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Syntaxin 6- And Microtubule- Mediated Intracellular Trafficking Contributes To Golgi And Nuclear Translocation Of Egfr

Yi Du

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**SYNTAXIN 6- AND MICROTUBULE- MEDIATED INTRACELLULAR TRAFFICKING
CONTRIBUTES TO THE GOLGI AND NUCLEAR TRANSLOCATION OF EGFR**

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**SYNTAXIN 6- AND MICROTUBULE- MEDIATED INTRACELLULAR
TRAFFICKING CONTRIBUTES TO THE GOLGI AND NUCLEAR
TRANSLOCATION OF EGFR**

A

DISSERTATION

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
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in Partial Fulfillment
of the Requirements
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DOCTOR OF PHILOSOPHY

By

Yi Du B.S.

Houston, Texas

May, 2012

Dedication

To my dearest wife

Lovely Kids,

Alex and Nicole

My parents and my sister

All friends

For their selfless and endless love.

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First of all, I would like to sincerely thank my supervisor, Dr. Mien-Chie Hung, who always guides, supports, and encourages me about my research and care about my career. I would also like to thank the members of my committee who devoted their time and efforts to enlighten me and teach me how to be a good scientist: Dr. Dihua Yu, Dr. Elsa Flores, Dr. Peng Huang, Dr. Ann-bin Shyu, Dr. Michael W. Van Dyke, Dr. Mong-Hong Lee, Dr. Michael Andreff, and Dr. Dennis Hugh.

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**Syntaxin 6- and microtubule- mediated intracellular trafficking contributes to
Golgi and nuclear translocation of EGFR**

Publication No. _____

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Receptor-mediated endocytosis is well known for its degradation and recycling trafficking. Recent evidence shows that these cell surface receptors translocate from cell surface to different cellular compartments, including the Golgi, mitochondria, endoplasmic reticulum (ER), and the nucleus to regulate physiological and pathological functions. Although some trafficking mechanisms have been resolved, the mechanism of intracellular trafficking from cell surface to the Golgi is not yet completed understood. Here we report a mechanism of Golgi translocation of EGFR in which EGF-induced EGFR travels to the Golgi via microtubule (MT)-dependent movement by interacting with dynein and fuses with the Golgi through syntaxin 6 (Syn6)-mediated membrane fusion. We also demonstrate that the Golgi translocation of EGFR is necessary for its consequent nuclear translocation and transcriptional activity. Interestingly, foreign protein such as bacterial cholera toxin, which is known to activate its pathological function through the Golgi/ER retrograde pathway, also utilizes the MT/Syn6 pathway. Thus, the MT, and syntaxin 6 mediated trafficking pathway from cell surface to the Golgi and ER defines a comprehensive retrograde trafficking route for both cellular and foreign molecules to travel from cell surface to the Golgi and the nucleus.

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Abbreviations

AP2	adaptor protein2
A β	amyloid β peptide
APP	β -amyloid precursor protein
ASGPR	asialoglycoprotein receptor
ATP	adenosine-5'-triphosphate
BSA	bovine serum albumin
BMP-7	bone morphogenetic protein 7
cAMP	cyclic adenosine monophosphate
Cbl	casitas b-lineage lymphoma
CCD	coiled-coil domain
CDK1	cyclin dependent kinase 1
ChIP	chromatin immunoprecipitation
CHX	cycloheximide
CME	clathrin mediated endocytosis
c-Met	cellular mesenchymal epithelial transition factor

COPI	coatomer protein I
COX-2	cyclooxygenase
CoxII	cytochrome oxidase subunit II
CREB	cAMP response element-binding
β -DG	β -dystroglycan
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DsRed	<i>Discosoma sp.</i> red fluorescent protein
DTT	dithiothreitol
EBV	epstein-barr virus
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
EGFR	epidermal growth factor receptor
EGFRvIII	epidermal growth factor receptor variant III
EHNA	erythro-9-[2-hydroxy-3-nonyl]-adenine
EM	electron microscopy

ErbB	erythroblastic leukemia viral oncogene homolog
ER	endoplasmic reticulum
FGFR	fibroblast growth factor receptor
FRET	fluorescence resonance energy transfer
GalNac T2	polypeptide N-acetylgalactosaminyltransferase 2
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
GR	glucocorticoid receptor
HER2	human epidermal growth factor receptor 2
HB-EGF	heparin-binding EGF-like growth factor
HBP	heparin-binding protein
ICD	intracellular domain
IGF1R	insulin-like growth factor 1 receptor
InsR	insulin receptor
IP	immunoprecipitation
MPR	mannose 6-phosphate receptor

MTOC	microtubule organizing centers
MUC1	heterodimeric transmembrane mucin
NDF	Neu differentiation factor
nEGFR	nuclear epidermal growth factor receptor
NK3R	tachykinin neurokinin 3 receptor
NLS	nuclear location signal
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDHK1	pyruvate dehydrogenase kinase 1
PDGFR	platelet derived growth factor receptor
PFA	paraformaldehyde
PIP2	phosphatidylinositol bisphosphate
PMSF	phenylmethanesulfonylfluoride
RFP	red fluorescent protein
RING	really interesting new gene
RIPA	radio immuno precipitation assay

RNA	ribonucleic acid
RSK1	ribosomal S6 kinase
RTKs	receptor tyrosine kinases
SH	Src Homology
shRNA	short hairpin RNA
siRNA	small interfering RNA
SN	stabilized Non-viral
SNARE	soluble NSF attachment protein receptor
STAT	signal transducers and activators of transcription protein
TGF- α	transforming growth factor alpha
TfR	transferrin receptor
tTG	transglutaminase II
VAMP	vesicle-associated membrane protein
VDR	vitamin D receptor
VEGFR	vascular endothelial growth factor receptor
Vps	vacuolar protein-sorting

Vti1a

vps10p tail interacting 1a

Chapter One

Introduction

1.1 Receptor tyrosine kinases and ErbB family

Receptor tyrosine kinases (RTKs) are a big family of cell surface transmembrane proteins which catalyze the transfer of the γ -phosphate of adenosine-5'-triphosphate (ATP) to hydroxyl groups of tyrosine on target proteins. RTKs play important physiological and pathological roles in response to their ligand's stimulation (Lemmon and Schlessinger, 2010; Yarden and Shilo, 2007). Ligand binding induces the dimerization of receptor and initiates the tyrosine kinases activity through the phosphorylation of tyrosine residues at their C-terminal domain (Sorkin and Goh, 2008). However, ligand is not always required for the tyrosine activity. In some cases, even in the absence of ligands, dimerization or oligomerization of receptors still occurs and turns on the tyrosine activity (Noordeen et al., 2006; Schlessinger et al., 2002; Weiss et al., 1997). Based on the structural and functional domain, about 20 subfamilies of RTKs are identified, such as erythroblastic leukemia viral oncogene homolog (ErbB) receptors, insulin receptor (InsR), platelet derived growth factor receptor (PDGFR), vascular endothelial growth factor receptor (VEGFR), and fibroblast growth factor receptor (FGFR) (Lemmon and Schlessinger, 2010).

ErbB proteins are a family which consists of ErbB1 (epidermal growth factor receptor (EGFR)), ErbB2 (human epidermal growth factor receptor 2 (HER2)), ErbB3, and ErbB4. Excessive ErbB signaling is associated with the development of a wide variety of human tumor (Hynes and Lane, 2005; Hynes and MacDonald, 2009). For example, aberrant expression or amplification of ErbB-1 and ErbB-2 are found in many human cancers, and their signaling may be critical in the development and

malignancy of these tumors. EGFR is the first ErbB family protein discovered as a receptor tyrosine kinase (Carpenter, 2000; Haigler et al., 1978). Epidermal growth factor (EGF), transforming growth factor alpha (TGF- α) or heparin-binding EGF-like growth factor (HB-EGF) stimulates the dimerization of EGFR which causes the autophosphorylation of several tyrosine (Y) residues at carboxy-terminal domain including Y992, Y1045, Y1068, Y1086, Y1148, and Y1173 (Figure 1-1) (Bishayee et al., 1999a, b; Hunter, 1984; Lombardo et al., 1995; Reynolds et al., 1981).

Figure 1-1

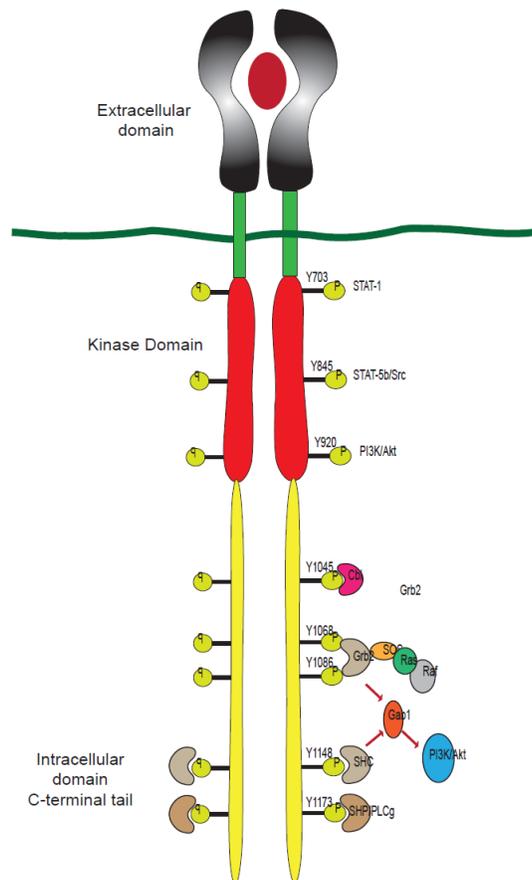


Figure 1-1: Tyrosine phosphorylation sites of EGFR. Downstream molecules bind to specific phosphorylated tyrosines at C-terminal domain which are involved in signaling transduction and intracellular trafficking.

These phosphorylated tyrosines provide docking sites for downstream signaling molecules such as Src homolog (SH) domain to transduce signal. Meanwhile, active EGFR also internalizes into the cytoplasm as endocytic vesicles for lysosome regulated degradation or recycles to the cell surface to keep signal transduction under certain conditions (Carpenter and Cohen, 1976; Sorkin and Goh, 2008).

1.2 Endosomal trafficking of EGFR

1.2.1 Traditional endocytosis, degradation, and recycling of EGFR

Receptor mediated endocytosis is considered as a major down-regulation of EGFR signaling (Figure 1-2) (Beguinot et al., 1984; Brown et al., 1983). Ligands induced dimerization has been thought as an initial step for signaling transduction via autophosphorylation of several tyrosine residues at C-terminal domain (Opresko et al., 1995). These phosphorylated tyrosines also turn on receptor mediated endocytosis, called clathrin mediated endocytosis (CME) (Figure 1-3), through series of protein-protein interaction (Sorkina et al., 1999). In CME, clathrin-coated pits are first constructed by recognition of the sorting signal (Y954xxθ) localized on EGFR by adaptor protein 2 (AP2) (Jones et al., 2002; Takei and Haucke, 2001). AP2 also has a binding domain to interact with phosphatidylinositol bisphosphate (PIP2) which concentrates on the plasma membrane (Robinson, 2004). Then clathrin is recruited to the plasma membrane via binding to a subunit of AP2 (Huang et al., 2001; Sorkin, 2004). When clusters of clathrin protein accumulate around EGFR, these protein-protein interactions force plasma membrane to endocytose. The clathrin-coated

vesicles then release receptor cargo from parental plasma membrane via internalization (Robinson, 2004).

Figure 1-2

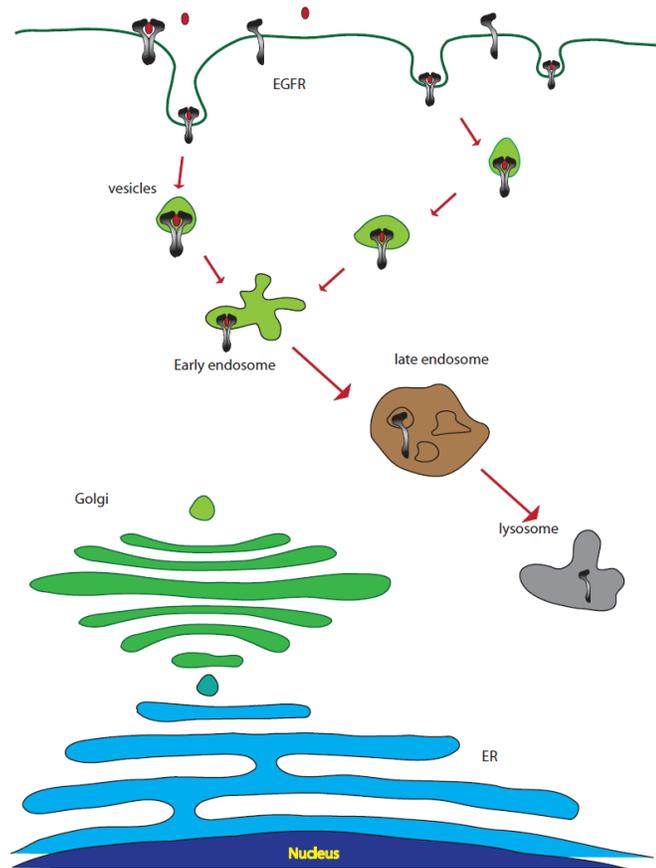


Figure 1-2: Endocytic trafficking of EGFR in response to the ligand's stimulation. Ligand induced dimerization of EGFR and internalization. Internalized EGFR transport from early endosome, late endosome to lysosome for degradation.

Figure 1-3

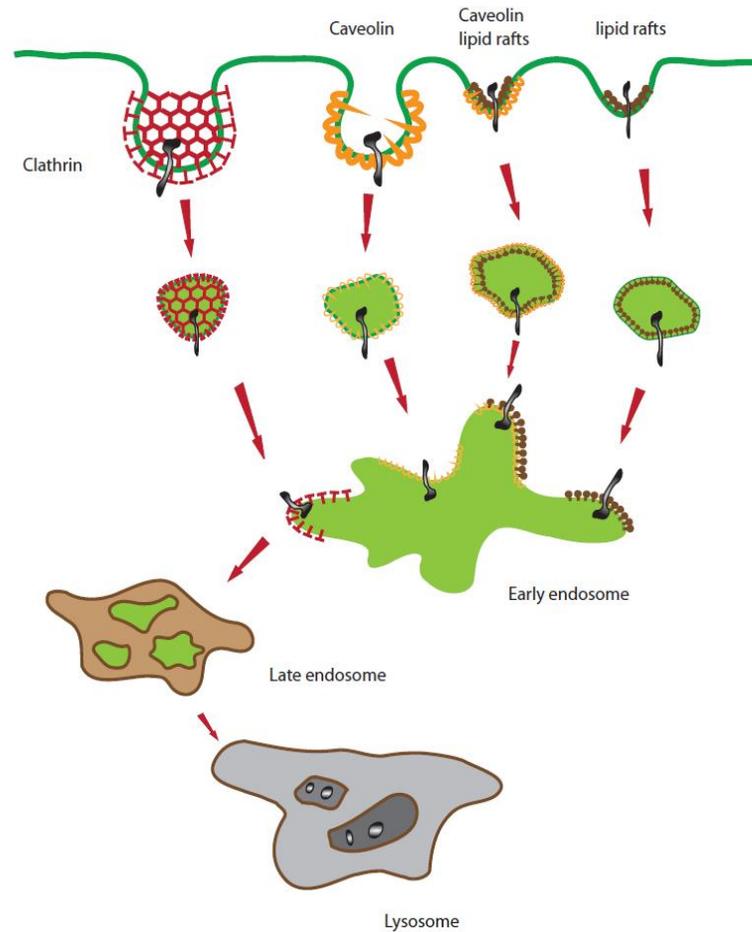


Figure 1-3: Comparison of endocytic trafficking mediated by clathrin, caveolin, and lipid rafts. Receptor-mediated endocytosis is mediated by clathrin from transport vesicles and early endosome to the lysosome in order to terminate signaling transduction. Caveolin-mediated caveolae is another endocytosis of cell surface receptor. Lipid rafts are also involved in the endocytosis and cooperated with caveolin under certain conditions.

EGF-induced degradation of EGFR is a well studied process to terminate EGFR signaling transduction (Wiley and Burke, 2001). After endocytosis, EGFR transports to the lysosome where it is degraded through the endosomal trafficking including early endosome and late endosome. The pH value of endosomal compartments have been shown to continuously decrease to pH5.0 for degradation process (Yamashiro and Maxfield, 1984; Yamashiro et al., 1984). In cells with moderate expression of EGFR, the turnover of $t_{1/2}$ is about 6 hours. However in cells with overexpression of EGFR such as A431, the turnover time $t_{1/2}$ is about 24 hours (Sorkin and Goh, 2008).

Ubiquitylation on lysine residues of EGFR during endocytic traffic is another biological event to down-regulate EGFR signaling pathway (Levkowitz et al., 1998). Ubiquitylation is a protein post-transcriptional modification with ubiquitin, a 76-amino acid molecule, to the ϵ -amino group of lysine in target proteins. There are two major ubiquitylations categorized by K48 linked and K63 linked ubiquitin. K48 linked ubiquitylation is thought to mediate proteasomal degradation, and K63 linked ubiquitylation is related to the signal transduction and vesicles trafficking (Hershko and Ciechanover, 1998; Hershko et al., 1983). Cbl (Casitas b-lineage lymphoma) is a critical E3 ligase that has been reported to form a complex with EGFR via phosphorylated tyrosine 1045 (Galisteo et al., 1995; Langdon, 1995). Cbl-mediated ubiquitylation of EGFR is also related to its lysosomal-proteasomal degradation (Galcheva-Gargova et al., 1995; Levkowitz et al., 1999).

Endocytosis recycling is an opposite biological process against endosomal degradation of EGFR to maintain its signaling transduction (Maxfield and McGraw,

2004). Once EGFR travels into the cytoplasm via the internalization in response to ligands' stimulation, EGFR recycles back to the cell surface to keep the signaling transduction (Masui et al., 1993; Sorkin et al., 1989). Neu differentiation factor (NDF/neuregulin) and TGF- α are ligands which have potential to cause EGFR recycling after endocytosis (Waterman et al., 1998).

1.2.2 Caveolin and lipid rafts regulate endocytosis of EGFR

Caveolin is another protein that regulates endocytosis of EGFR through ligand-independent pathway (Abulrob et al., 2004; Couet et al., 1997; Mineo et al., 1996) (Figure 1-3). Different from the ligand induced endocytosis which transports EGFR to the lysosomal to terminate signaling, caveolin-related endocytosis is thought to prolong signal transduction of EGFR (Khan et al., 2006; Kim and Bertics, 2002). Lipid rafts is an additional functional microdomain on the plasma membrane containing cholesterol, sphingolipid, and gangliosides (Simons and Toomre, 2000). In lipid raft-mediated endocytosis, lipid raft usually cooperates with caveolin to regulate endocytosis and signaling transduction. Lipid raft-mediated endocytosis is also involved in the EGFR signaling in response to virus infection (Eierhoff et al., 2010). Although lipid rafts usually inhibit ligand induced signaling activation, it enhances ligand-independent kinase activity of EGFR (Chen and Resh, 2002; Peres et al., 2003; Roepstorff et al., 2002; Zhuang et al., 2002).

1.3 Intracellular trafficking of cell surface molecules

Recent studies show that cell surface receptors translocate from cell surface to different cellular compartment, including the Golgi, mitochondria, endoplasmic reticulum (ER), and the nucleus. These results indicate that the function of RTKs on sub-cellular locations is more complicated than only down-regulate signal transduction or recycle to the plasma membrane (von Zastrow and Sorkin, 2007). Subcellular-localized cell surface proteins may play specific functions at different cellular compartments (Figure 1-4).

Figure 1-4

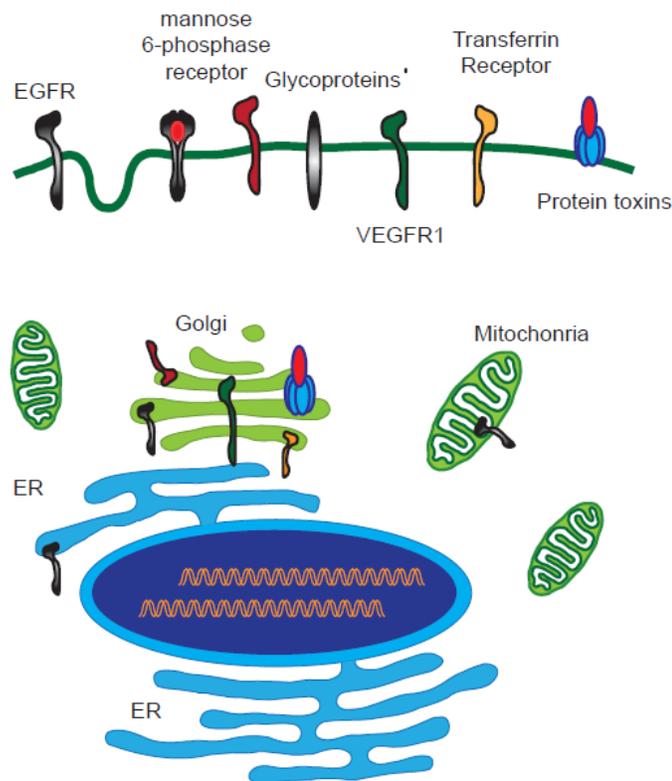


Figure 1-4: Cell surface molecules localized on non-traditional organelles. VEGFR, EGFR, MPR, TfR, and protein toxins have been reported to transport into the Golgi apparatus. FGFR1 and EGFR have been reported to localize on the mitochondria.

1.3.1 Golgi translocation of cell surface molecules

The Golgi apparatus is the first organelle where the cell surface molecules have been detected. Early in 1980's, non-specific plasma membrane markers such as dextrans and cationized ferritin were found to translocate from cell surface to the Golgi apparatus (Farquhar, 1985). This trafficking pathway was further supported by the observation of other cell surface proteins including asialoglycoprotein receptor (ASGPR) (Duncan and Kornfeld, 1988; Roth et al., 1985), transferrin receptor (TfR) (Snider and Rogers, 1985), and mannose 6-phosphate receptor (MPR) (Jin et al., 1989) translocated from plasma membrane to the Golgi apparatus. Further studies have identified the transport of more cell surface proteins, such as G-proteins and glycoprotein (Akgoz et al., 2004; Bos et al., 1995), to the Golgi apparatus.

Golgi-localized interferin receptor had been reported to regulate ligand uptake, glycoprotein repair, or homeostasis of membrane compartment to balance the loss by exocytosis (Snider and Rogers, 1985; Tauber et al., 1986; Tauber et al., 1983). Recently, the specific functions of more cell surface receptors localized at the Golgi apparatus were revealed. For example, Golgi localized VEGFR1 has been reported to balance the level of VEGFR1 and VEGFR2 on plasma membrane and to dictate endothelial signaling to influence vascular physiology (Mittar et al., 2009). Perinuclear accumulation of cellular mesenchymal epithelial transition factor (c-Met) was required for the downstream signaling (Kermorgant and Parker, 2008). However, the functions of Golgi-translocated EGFR, TfR, and G-protein coupled receptors are still unknown (Robertson et al., 1992; Saini et al., 2010; Wang et al., 2010a).

Compared to less known function of cellular cell surface receptors on the Golgi, foreign molecules such as bacterial protein toxins or viruses used Golgi translocation to regulate their pathological function in target cells such as toxin activity and viruses' Ribonucleic acid (RNA) replication and assembly (Boulant et al., 2008; Salanueva et al., 2003). For instance, the Golgi translocation of cholera toxin is a critical step to activate the cyclic adenosine monophosphate (cAMP), thus cause dehydration toxicity.

1.3.2 Translocation of cell surface molecules to mitochondria

Mitochondria are another cellular compartment on which cell surface receptors had been detected. For example, EGFR or EGFR variant III (EGFRvIII) transport to mitochondria to modulate the mitochondrial function via modification of cytochrome oxidase subunit II (CoxII) (Demory et al., 2009; Yue et al., 2008) or to cause resistance to drug treatment (Cao et al., 2011). Recent study indicated that FGFR1 localized on mitochondria to phosphorylate the metabolic enzyme pyruvate dehydrogenase kinase 1 (PDHK1) thus regulate mitochondrial activity in cancer cells (Hitosugi et al., 2011).

1.3.3 Nuclear translocation of cell surface proteins

Many cell surface receptors, such as EGFR family including EGFR, ErbB2, ErbB3, and ErbB4, VEGFR1, FGFR, c-Met, and insulin-like growth factor 1 receptor (IGF1R) (Feng et al., 1999; Kermorgant and Parker, 2008; Marti et al., 1991; Sehat et al., 2010; Stachowiak et al., 1996b; Wang and Hung, 2009) had been reported to

translocate into the nucleus and play important physiological and pathological roles (Figure 1-5). G protein-coupled receptors, such as tachykinin neurokinin 3 receptor (NK3R) (Jensen et al., 2008) and endothelin receptors (Boivin et al., 2003), are another type of cell surface receptors which have been detected in the nucleus.

Figure 1-5

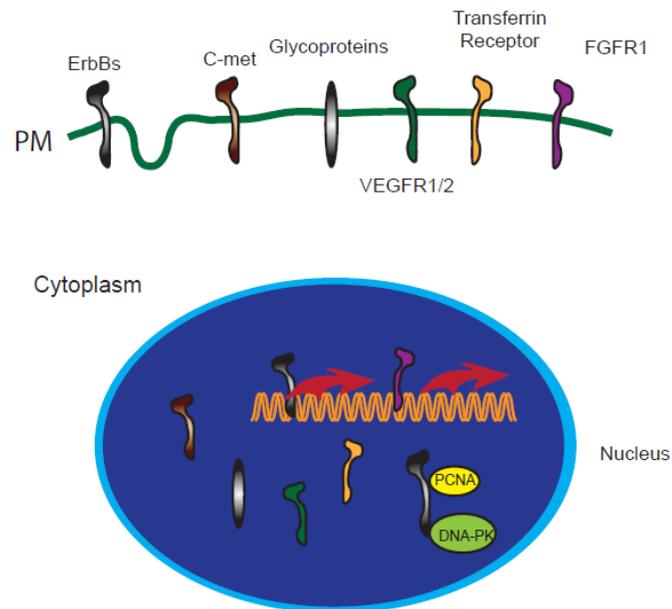


Figure 1-5: Cell surface receptors transport into the nucleus. RTKs including EGFR family, VEGFR1, FGFR and c-Met have been detected in the nucleus. G protein coupled receptors are reported to transport to the nucleus.

1.3.3.1 Nuclear translocation of EGFR family

All EGFR family including EGFR, ErbB2, ErbB3, and ErbB4 have been detected in the nucleus in different cell types and human cancers (Wang and Hung, 2009; Wang et al., 2010b). EGFR is a well-investigated RTK that is translocated into the nucleus from the cell surface in response to ligand stimulation or under certain stress conditions, such as ultraviolet or ionizing radiation (Dittmann et al., 2005; Lin et al., 2001; Xu et al., 2009). EGFR also exists in the nucleus in different human tissue and cancer cell types (Li et al., 2010; Marti et al., 1991; Psyrri et al., 2008; Raper et al., 1987; Xia et al., 2009). Multiple laboratories have reported the correlation between nuclear expression of EGFR (nEGFR) and poor prognosis for several different cancer types in humans (Hadzisejdic et al., 2010; Hoshino et al., 2007; Lo et al., 2005b; Psyrri et al., 2005; Xia et al., 2009). Also, functional studies have revealed that nEGFR functions as a transcriptional co-factor to regulate target gene expression in an EGF-dependent manner and promote cell proliferation (Huo et al., 2010; Lin et al., 2001; Lo and Hung, 2006). Transcriptional regulation of nEGFR is mediated by a DNA-binding domain-containing RNA helicase A to recognize a specific AT-rich sequence in the promoter regions of targeted genes. nEGFR can also interact with other co-regulators, such as signal transducers and activators of transcription protein 3 (STAT3), STAT5, heterodimeric transmembrane mucin (MUC1), and epstein-barr virus (EBV)-encoded latent membrane protein 1, to regulate gene expression (Bitler et al., 2010 ; Hung et al., 2008; Huo et al., 2010; Jaganathan et al., 2011; Lo et al., 2005a; Tao et al., 2005). Furthermore, nEGFR is involved in the regulation of deoxyribonucleic acid (DNA) replication and repair

(Chen and Nirodi, 2007; Das et al., 2007; Dittmann et al., 2008; Wang et al., 2006). More recently, nEGFR was found to contribute to the resistance to cetuximab, a monoclonal antibody against EGFR, and gefitinib, a tyrosine kinase inhibitor (Huang et al., 2011; Li et al., 2009). All of studies that shed light on the nuclear functions of EGFR may provide important clues about its potential clinical applications.

ErbB2 is another well studied receptor in the nucleus. Nuclear ErbB2 has been reported to function as a transcriptional regulator via binding to the promoter of cyclooxygenase-2 (COX-2) (Wang et al., 2004). Recent study has been shown that nuclear ErbB2 associates with β -actin and RNA polymerase I to enhance the rRNA transcription (Li et al., 2011). Not only the full length of ErbB2 can transport into the nucleus, but truncated form of ErbB-2 is also detected in the nucleus and contributes to the resistance of anti-HER2-targeting therapies (Scaltriti et al., 2007).

The clinical studies indicated that nuclear ErbB3 is correlated with prostate cancer disease progression (Cheng et al., 2007; Koumakpayi et al., 2006). A nuclear variant of ErbB3 has been shown to regulate myelination of Schwann cell (Adilakshmi et al., 2011).

ErbB4 is a well recognized cell surface receptor to transport into the nucleus as truncated form which associates with transcriptional factor STAT 5A at the β -casein promoter and thus regulates the activation of STAT 5A-stimulated gene (Williams et al., 2004). Furthermore, the fragment of ErbB-4 can function as a kinase to phosphorylate Mdm2 and to increase the ubiquitination of Mdm2. As results, ICD of ErbB4 enhances the protein levels of p53, p21, and transcriptional target of p53 (Arasada and Carpenter, 2005).

1.3.3.2 Nuclear translocation of VEGFR

VEGFR2 (Flk/KDR) is the receptor of VEGF-A. It has been detected in the nucleus and functions to regulate the activation of transcription factors (Feng et al., 1999) or to form a complex with transglutaminase II (tTG) and mediate the response to VEGF stimulation (Dardik and Inbal, 2006).

1.3.3.3 Nuclear translocation of FGFR-1

FGFR-1 is another well-studied tyrosine kinase receptor which can be accumulated in the nucleus (Bryant and Stow, 2005; Johnston et al., 1995; Stachowiak et al., 1996a, b) and functions as a transcriptional regulator. Nuclear FGFR1 induces the expression of c-Jun and serves as a common co-activator to activate cAMP response element-binding (CREB)-binding protein and regulate cell proliferation (Reilly and Maher, 2001). Nuclear FGFR1 is also involved in neuronal differentiation via mediation of cAMP and morphogenetic protein 7 (BMP-7) (Horbinski et al., 2002).

1.3.3.4 Nuclear translocation of other molecules

Other cell surface proteins, such as G proteins coupled receptors angiotensin I, II, endothelin, NK3R, and bradykinin, are detected in the nucleus (Chen et al., 2000; Lee et al., 2004). But their functions remain largely unknown. Nuclear translocation of NK3R occurs upon osmotic challenge (Jensen et al., 2008) and nuclear endothelin receptor is coupled with the signaling transduction machinery within the nuclear

membrane (Boivin et al., 2003). Although most nuclear G protein coupled receptors function to regulate signaling pathways which is similar to its function on the cell surface, some of them, such as nuclear PTH/PTHrP receptor, β -adrenergic receptor, and metabotropic glutamate receptor 5 (mGluR5), can directly regulate cell proliferation (Watson et al., 2000), transcriptional initiation (Boivin et al., 2006), gene expression (Jong et al., 2009; Savard et al., 2008; Vaniotis et al., 2011), and histone modification (Re et al., 2010). Recently, the extracellular matrix receptor β -dystroglycan (β -DG) has been detected in the nucleus. But the function of nuclear β -DG needs to be further investigated (Oppizzi et al., 2008).

More recently, membrane-anchored cell surface protein Heparin-binding EGF-like growth factor (HB-EGF) and its precursor proHB-EGF have been reported to transport to the inner nuclear membrane in response to stresses (Hieda et al., 2008; Kim et al., 2005). The studies of their nuclear translocation indicated that releasing from the cell surface membrane is not required for the nuclear translocation of cell surface proteins.

1.3.4 Mechanisms of intracellular trafficking

Investigation of mechanisms regulating intracellular trafficking of cell surface receptors is critical for further understanding of their functions at cellular compartment. However, the mechanism of how cellular proteins translocate to mitochondrial is not clear at all. The trafficking pathway of cellular proteins from cell surface to the Golgi apparatus is largely unknown although the studies of the Golgi translocation of bacterial protein toxins led to the identification of several regulators

mediating this trafficking pathway (Spooner et al., 2006). For example, the transport of shiga toxin from early endosomes to the Golgi apparatus is regulated by a series of soluble NSF attachment protein receptor (SNARE) and a small guanosine triphosphate (GTP) GTPase Rab6 isoform.

Because of the importance of cell surface receptors in the nucleus, uncovering the mechanisms of their nuclear translocation become very attractive. A protease-dependent mechanism shown in Figure 1-6 was proposed to address how these integrated receptors are released from the lipid bilayer.

Figure 1-6

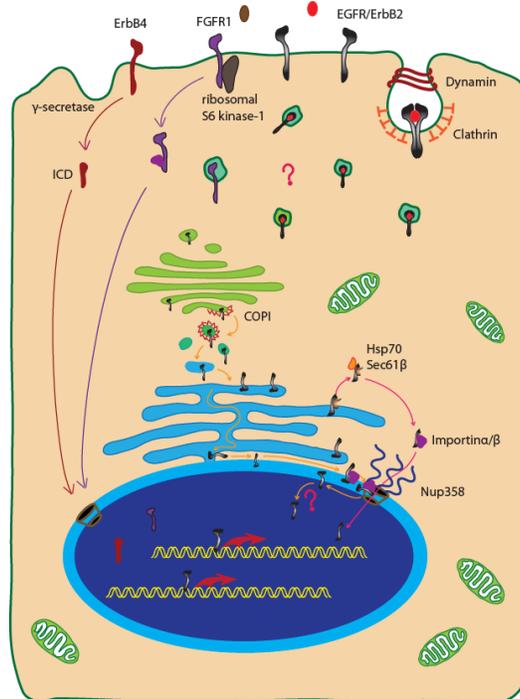


Figure 1-6: The mechanism of nuclear translocation of cell surface receptors. Truncated form of cell surface receptors is an accepted mechanism to explain how cell surface receptors transport into the nucleus. RSK-1 mediates releasing of FGFR1 from cell surface to the cytoplasm is another mechanism to explain nuclear translocation of FGFR1. Endocytosis is required for nuclear transport of ErbB2 and EGFR. Sec61 located either on ER membrane or inner nuclear membrane is involved in nuclear translocation of EGFR.

According to this mechanism, the translocation of transmembrane receptors into the nucleus is caused by the interaction of a nuclear import protein with the intracellular fragments of receptors. For example, in the nuclear translocation of ErbB4 (Ni et al., 2001), γ -secretase cleaves ErbB4 and releases the truncated form (intracellular domain (ICD)) of ErbB4 from membrane and then transport into the nucleus as a soluble protein. Similarly, β -secretase cleaves β -amyloid precursor protein (APP) and releases soluble fragment, an amyloid β peptide ($A\beta$) that is critical for Alzheimer's disease (Vassar et al., 1999). Proteolysis-mediated activation of Notch receptor is another sample (Maillard et al., 2005), in which the intracellular domain of Notch is released by ligands induced proteolytic cascade and then translocates to the nucleus to regulate the transcription of targeted genes.

Although some cell surface receptors translocate into the nucleus as soluble truncated form as mentioned above, most cell surface receptors detected in the nucleus are full length proteins. It has been reported that 90-kDa ribosomal S6 kinase (RSK1) (Hu et al., 2004) can directly release the full length of FGFR1 from plasma membrane and then into the nucleus. However, another report showed that endosomal trafficking is still required for the nuclear translocation of FGFR1 (Bryant et al., 2005).

The mechanism of nuclear translocation of EGFR and ErbB2 is well-studied. It has been reported that endocytosis is required for the nuclear translocation of EGFR and ErbB2 because blocking of endocytosis using a dominant negative mutation of dynamin or endocytosis inhibitors can decrease their nuclear translocation (Giri et al., 2005; Lo et al., 2006). The involvement of importin $\alpha 1/\beta 1$, a

critical molecule for the nucleus/cytoplasm shuttling, in the nuclear transport of EGFR and ErbB2 and the identification of nuclear location signal (NLS) in EGFR and ErbB2 (Hsu and Hung, 2007) indicated that the nuclear transport of EGFR or ErbB2 is regulated by nucleus/cytoplasm shuttling machinery. Translocon Sec61 localized either on the ER or inner nuclear membrane plays an important role in the release of EGFR or ErbB2 from cell surface or membrane compartment (Giri et al., 2005; Hsu and Hung, 2007; Liao and Carpenter, 2007; Wang et al., 2010a; Wang et al., 2010c). Recent studies demonstrated that coatamer protein I (COPI), which regulates the trafficking from the Golgi to ER, also functions for the nuclear trafficking of EGFR. This finding suggest that the machinery mediating the retrograde trafficking is also involved in the nuclear translocation of EGFR and membrane trafficking may be an important biological event to regulate the nuclear translocation of EGFR or other cell surface transmembrane receptors via membrane compartments.

1.4 Retrograde trafficking

After synthesis and post-transcription, proteins are delivered to the targeted cellular locations via exocytosis or secretory system including a series of membrane compartments (Burgoyne and Morgan, 2003) (Figure 1-7). In opposite, retrograde transport, an influx of proteins and lipids, is used to balance the outward flow of secretion. Retrograde trafficking is thought to from endosomal components including early endosomes and late endosomes or from the recycling endosomal compartments to the endoplasmic reticulum (ER) or the Golgi apparatus (Johannes and Popoff,

2008). Similar to endocytosis, adaptor proteins, such as AP-1 and coated proteins clathrin, are involved in the initiation of retrograde trafficking. Small GTPases such as Rab proteins are important for intracellular trafficking pathway. Different Rabs coordinates with other cofactors to tether and dock cargo proteins or vesicles containing transferred materials. It has been demonstrated that Rab6, Rab9, and Rab11 regulate retrograde trafficking from different endosomal compartments.

Figure 1-7

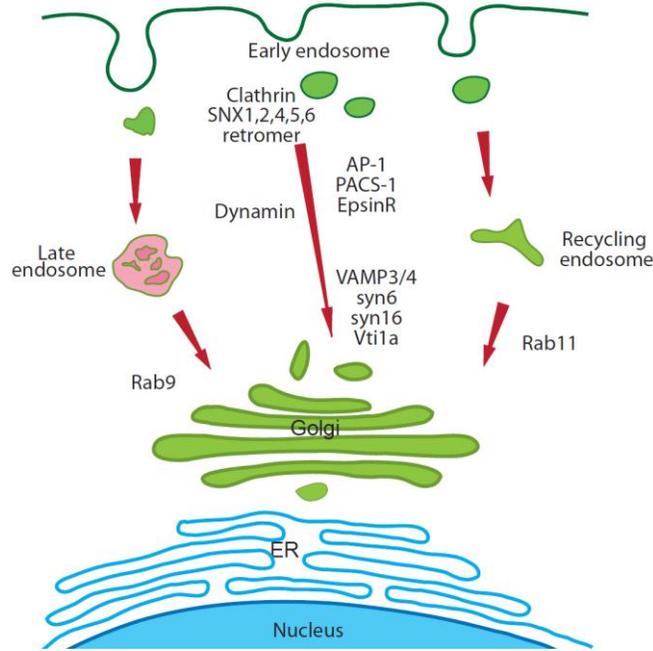


Figure 1-7: Retrograde trafficking pathway. Retrograde trafficking includes intracellular trafficking events from different endosome (early, late, recycling) to the Golgi apparatus. Adaptor proteins such as AP-1 and epsinR, membrane coat proteins such as clathrin, or the retromer complex are involve in the formation of intermediates containing cargo proteins. Tethering, docking, and fusion of retrograde transport intermediates with the TGN depend on a wide range of regulatory factors, such as golgin-97, golgin-245, GCC88, and GCC185. SNARE (soluble N-ethylmaleimide-sensitive fusion factor attachment receptor) complexes are required for membrane fusion.

1.5 SNARE proteins and membrane fusion

Membrane fusion is an important biological event occurring between cells, different intracellular compartments, intracellular compartments, and the plasma membrane (Bonifacino and Glick, 2004; Jahn and Scheller, 2006; Pfeffer, 2007). Studies of membrane fusion between vesicles and organelles have mainly focused on neural synaptic vesicles fusion (Hirokawa and Takemura, 2005) or endocytic pathways (Chernomordik and Kozlov, 2003; Soldati and Schliwa, 2006). The proteins that mediate membrane fusion include SNAREs, synaptotagmins, and viral fusion proteins (Martens and McMahon, 2008). SNARE family proteins are critical players for the intracellular events. In general, four SNARE proteins localized at different compartments are assembled to initiate the membrane fusion and to complete the organelles' transport and the delivery of cargo proteins from one compartment to another compartment (Jahn and Scheller, 2006).

There are two types of SNAREs proteins, vesicle SNAREs (v-SNAREs) and target SNAREs (t-SNAREs). v-SNAREs localize in vesicles or other forms of transport intermediates and t-SNAREs localize in the target compartment. The t-SNARE family members have two sub-classifications, heavy and light chains (Hong, 2005). Different SNARE proteins functions at different organelles to regulate the vesicular transport of cargo proteins. For example, v-SNARE proteins, vesicle-associated membrane protein (VAMP) 3, VAMP4, and VAMP5 localize on the plasma membrane. They can be internalized into cytoplasm in endosomes or vesicles (Tran et al., 2007; Zeng et al., 2003). T-SNARE proteins syntaxin 6, syntaxin 16, and vt1a localize on the trans-Golgi network or the endoplasmic reticulum and assemble

to initiate the fusion of small vesicles with organelles (Mallard et al., 2002; Zwilling et al., 2007).

The machinery of retrograde trafficking pathway from cell surface and endosomes to the trans-Golgi network has been well-studied (Bonifacino and Rojas, 2006). Although a comprehensive retrograde trafficking pathway has been uncovered and regulators and factors, such as adaptor proteins, small GTPase, coating protein, tethering factor, and SNARE proteins have been identified, only a few cellular proteins such as transferrin receptor and mannose 6-phosphate receptor and foreign protein such as protein toxins or viruses have been reported as a cargo protein regulated by retrograde trafficking pathway (Green and Kelly, 1990; Roth, 1987; Snider and Rogers, 1985). Clearly there is a big gap between retrograde trafficking pathway and other important cellular events, such as signaling transduction.

The focus of our study is to link retrograde trafficking pathway with non-canonical EGFR signaling and to investigate the mechanism of how EGFR translocate from cell surface to the Golgi and the nucleus and thus functions as a transcriptional regulator. Syntaxin 6 is a well-known SNARE protein that regulates endosomal trafficking via membrane fusion in the retrograde trafficking pathway (Figure1-7) (Jahn and Scheller, 2006; Johannes and Popoff, 2008; Martens and McMahon, 2008). Thus the role of syntaxin 6 in the trafficking of EGFR to the Golgi and nucleus is systematically investigated in our study.

1.6 Microtubule dependent intracellular trafficking

1.6.1 Microtubule cytoskeleton

Microtubules are a component of cytoskeleton. They serve as structural components within cells and are involved in many cellular processes including proliferative divisions and vesicular transport (Kelly, 1990). Microtubules are polymers of α - and β -tubulin dimers. The tubulin dimers polymerize end to end into protofilaments with α subunit and β subunit from different dimers. Protofilaments then bundle in hollow cylindrical filaments. Another important feature of microtubule structure is its polarity. In a microtubule, there is one (+) end with only β subunits exposed while the other (-) end has α subunits exposed. Usually, the (+) end is close to the cell surface and the (-) end is close to the nucleus (Nogales, 2000). In most cells, especially in the non-polarized cells, microtubules are nucleated and organized by the microtubule organizing centers (MTOC) (Cole and Lippincott-Schwartz, 1995; Rodriguez-Boulan et al., 2005), such as centrosomes, which usually locate near the nucleus and associate closely with the Golgi apparatus.

The critical role of microtubules during the transport of organelles and vesicles has been well studied and understood (Rogers and Gelfand, 2000). Microtubules provide the basis for the directional movement of organelles and vesicles. The specific motor proteins (Hirokawa and Takemura, 2005; Mallik and Gross, 2004), fusion proteins (Hong, 2005; Jahn and Scheller, 2006), and small GTPases (Grosshans et al., 2006; Schwartz et al., 2007) decide the direction and specificity. Microtubules also provide the roadway for the movement of the protein cargo. For example, intact microtubules have been shown to be necessary for the nuclear translocation of glucocorticoid receptor (GR) (Galigniana et al., 2004; Harrell et al., 2004). Overexpression of dynamitin which inhibits the function of dynein could

block the nuclear translocation of GR (Burkhardt et al., 1997; Melkonian et al., 2007). Moreover, microtubules function in the distribution of virus particles during the virus maturation (Boulant et al., 2008) and the transport of herpes simplex virus 1 into the nucleus (Sodeik et al., 1997). Another function of microtubules is to regulate the subcellular localization of mRNA (Messitt et al., 2008; Zimyanin et al., 2008). Microtubules are also an important component during the endocytic trafficking. Some studies have shown that intracellular transport of cargo proteins requires the cytoskeleton including microtubules and motor proteins (Allan and Schroer, 1999; Cole and Lippincott-Schwartz, 1995).

1.6.2 Microtubule motor proteins

Motor proteins are another kind of molecules regulating the microtubule-dependent movement (Figure 1-8). Dynein and kinesin are two major motor proteins utilized for the trafficking of cargo along the microtubules. The difference between these two motor proteins is the direction of the movement of cargo (Schliwa and Woehlke, 2003). Kinesin delivers cargo from inside of cells to the cell surface and dynein transports cargo from cell surface to the center of cells (Caviston and Holzbaaur, 2006; Hirokawa and Takemura, 2005).

Dynein is composed of heavy chains, light chains, and intermediate chains (Porter and Johnson, 1989). It carries the cargo proteins and moves along microtubules utilizing different functional domains such as the cargo binding domain, ATPase domain, and the microtubules binding domain. Dynein provides the energy for the minus-end movement of cargoes or vesicles along microtubules (Ross et al.,

2008). Dynactin is another complex which helps dynein to complete its function (Schroer, 2004).

Figure 1-8

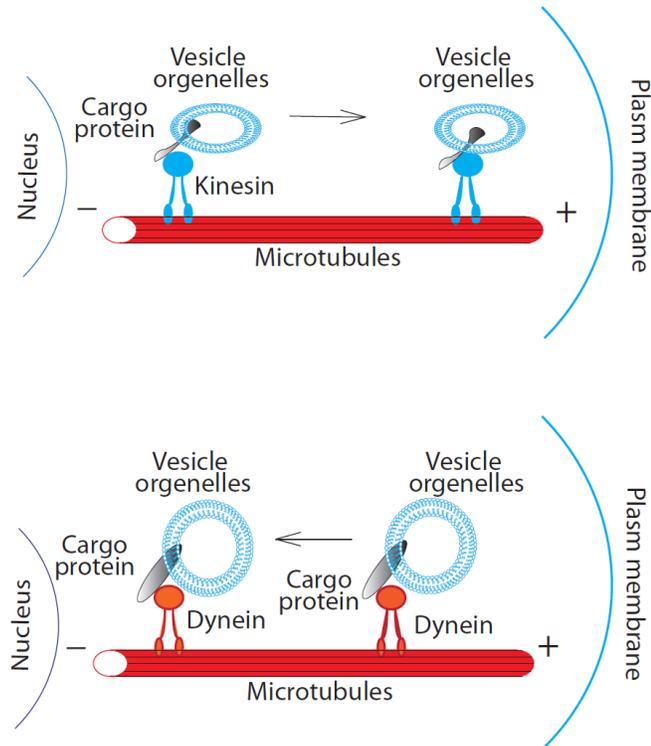


Figure 1-8: Motor proteins involved in intracellular transport of cargo proteins. Microtubule motor proteins dynein and kinesin regulate the direction of the movement of cargo along the microtubules. Myosin functions as a motor for trafficking along actin filaments.

There are two groups of dynein: cytoplasmic dynein and axonemal dynein. Cytoplasmic dynein is necessary for organelles transport and centrosome assembly. Axonemal dynein functions in the sliding of the microtubule in the axonemes of cilia and flagella. Cytoplasmic dynein is a motor protein which provides the power for the movement along microtubules.

As described above, intracellular transport of cargo proteins requires the cytoskeleton including microtubules (MTs) and motor proteins (Allan and Schroer,

1999; Cole and Lippincott-Schwartz, 1995). More recent studies demonstrated that microtubule-dependent intracellular trafficking is used for EGFR degradation (Deribe et al., 2009; Gao et al., 2010). Moreover, although a plenty of data have shown that cytoskeleton provides the real pathway for the movement of vesicles, endosomes, and organelles in the cytoplasm (Allan and Schroer, 1999; Bananis et al., 2000; Murray et al., 2000; Rodriguez-Boulan et al., 2005), there are limited studies addressing the relationship between nuclear trafficking of endocytic cell surface receptors and cytoskeletons. Therefore, in the present study, we investigated the roles of microtubules and motor proteins in the Golgi and nuclear translocation of EGFR.

Chapter Two

Materials and Methods

2.1 Chemicals and antibodies

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO). The following antibodies were used in this study: anti-EGFR (Santa Cruz Biotechnology, Inc. Santa Cruz, CA and Neomarkers, Fremont, CA); anti-dynein IC (Santa Cruz Biotechnology, Inc. Santa Cruz); anti-tubulin (Abcam, Cambridge, MA; Sigma-Aldrich Co. St. Louis, MO); anti-syntaxin6 (BD Bioscience, San Jose, CA); anti-lamin B (Calbiochem, San Diego, CA); anti-calregulin (Santa Cruz Biotechnology, Inc. Santa Cruz); anti-vacuolar protein-sorting (vps) vps10p tail interacting 1a (Vti1a) (BD Bioscience, San Jose, CA); anti-actin, anti-myc and anti-HA (Roche, Indianapolis, IN). All fluorescence-labeled secondary antibodies were obtained from Invitrogen (Carlsbad, CA).

2.2 Cell culture and treatment

All cells lines were maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 medium supplemented with 10% fetal bovine serum and antibiotics. Cells were serum-starved overnight or 12 hr before EGF stimulation. For experiments using inhibitors, serum starved cells were treated with inhibitors first and then stimulated with EGF.

2.3 Plasmid constructs, small interfering RNA oligonucleotides, and short hairpin RNA

The syntaxin 6 full-length plasmid was purchased from the Origene. The coiled domain of syntaxin6 was subcloned into the pcDNA6His-MycA (Invitrogen,

Carlsbad, CA) and pDsRedC1 (Clontech, Mountain View, CA) for fluorescence staining. The GalNAc-T2-GFP plasmid was a gift from Dr. B. Storrie (University of Arkansas for Medical Sciences). The plasmid expressing cyclin dependent kinase 1 (CDK1) (#1888), cyclin B (#10911), and RFP-tubulin (#21041) were obtained from Addgene. pEGFP-EGFR was constructed by subcloning full length EGFR into the pEGFP-N1 (Clontech, Mountain View, CA) with HindIII and KpnI.

siRNA oligonucleotides targeting dynein IC (siRNA ID: SASI_Hs01_00129737 and SASI_Hs01_00129739), syntaxin 6 (siRNA ID: SASI_Hs01_00129146 and SASI_Hs01_00129147), non-specific siRNA control (Hurtado et al., 2008) (containing the sequences 5'-AUCACAUCUGUCAAAUUAUU-3', 5'-GAACGUGGCUCUCAAGUUU-3', 5'-AAAGGAAAUCGACACUGAUU-3' and 5'-GCCCUGGGAUUUAUGAUGAUU-3'), and short hairpin RNA (shRNA) targeting dynein IC (TRCN0000116797 and TRCN0000116799) were purchased from Sigma-Aldrich (St. Louis, MO). For small interfering RNA (siRNA) transfection, the siRNAs were transfected into cells using the cationic liposome SN (Stabilized Non-viral) or lipofectamine 2000 (Invitrogen, Carlsbad, CA) as previously described (Yan et al., 2004). Briefly, cells were grown overnight and incubated with plasmid/liposome complexes in Opti-MEM medium for 4 hr, followed by replacement of complete medium and incubation at 37°C for 24 to 48 h. pLKO based shRNA was co-transfected with packaging vector and envelope vector into A293T cells for virus production. After 48 hr transfection, media containing lentivirus were harvested by centrifugation. Media were further filtered by 0.45 µm filter and used to infect target cells.

2.4 Nuclear fractionation

Treated cells were collected, washed with ice-cold phosphate buffered saline (PBS), swelled, and solubilized in lysis buffer (10 mM Tris-HCl, pH 8.0, 10 mM KCl, 0.5% NP-40, 2mM MgCl₂, 0.5 mM dithiothreitol (DTT), 1 mM phenylmethanesulfonylfluoride or phenylmethylsulfonyl fluoride (PMSF), and 0.15 u/ml aprotinin) for 20 min on ice. After cells were homogenized with a Dounce homogenizer (20 strokes) on ice, the nuclei were pelleted by centrifugation at 600 x g for 5 min, and then the supernatant was collected as cytoplasmic fraction. The nuclei pellet was washed with lysis buffer 3 times to remove any cytoplasmic contamination. Finally, the nuclei pellet was solubilized in the Radio Immuno Precipitation Assay (RIPA) buffer (10 mM Tris•HCl pH 8.0, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 0.5 mM DTT, 1 mM PMSF, and 0.15 u/ml aprotinin), and sonicated to disrupt all nuclei, followed by centrifugation at 16,000 ×g for 20 min at 4°C. The supernatant was collected as nuclear lysate.

2.5 Purification of the Golgi apparatus

The Golgi apparatus were purified using the OptiPrep density gradient medium by following the manufacturer's guidelines with a slight modification (Sigma-Aldrich, St. Louis, MO). Briefly, cultured cells were harvested and resuspended in a homogenization buffer (10 mM Hepes, pH 8.0, 250 mM sucrose, 140 mM NaCl, and protease inhibitor cocktail). Cells were homogenized using 20 strokes with a Dounce homogenizer in the same buffer and then centrifuged at 800× g for 5 min at 4°C. The supernatant was collected and then loaded onto continuous

iodixanol gradients from 0% to 30% and centrifuged at 48,000× g for 16 hr at 4°C. The gradients were unloaded in 0.6 ml fractions. Markers for the Golgi apparatus, early endosome, and ER in each fraction were analyzed.

2.6 Immunoprecipitation and immunoblotting

For immunoprecipitation (IP) experiments, cytoplasmic fractions of HeLa cells were treated as described above and precleared with 1 µg of mouse or rabbit IgG and 20 µl of protein G-agarose (Roche, Indianapolis, IN) for 1 hr at 4°C. Precleared lysates were then incubated with 1 µg of primary antibodies or mouse IgG at 4°C overnight with gentle agitation. Following the addition of protein G-agarose, incubation was continued for an additional 30 min at 4°C. Protein G-agarose pellets were collected and washed for multiple cycles at 4°C. The washed immunoprecipitates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis as previously described (Giri et al., 2005).

2.7 Chromatin IP (ChIP)

Chromatin immunoprecipitation (ChIP) was performed by following the manufacturer's guidelines. In brief, treated cells were fixed with 1% high quality of formaldehyde for 10 minutes at room temperature. Then cells were lysated with lysis buffer and then sonicated to shear the genome DNA to manageable fragments. Lysis were precleared with protein G agarose and then incubated with indicated antibody or

IgG control. DNA was isolated with the EZ-ChIP kit and subjected to polymerase chain reaction (PCR) to analyze relative level of target genes.

2.8 Confocal microscopy analysis

For fixed cells, all experiments were performed as previously described (Giri et al., 2005). Briefly, cells grown on chamber slides (Labtek, Scotts Valley, CA) were treated as described above. After washing with ice-cold PBS, cells were fixed, permeabilized, and incubated with primary antibodies and fluorescence-labeled secondary antibodies. Immunostained cells were examined using an Olympus FluoView FV300 confocal microscope (Olympus America, Melville, LA) or Zeiss LSM 710 laser-scanning microscope (Carl Zeiss, Inc. Thornwood, NY) with a 63X/1.4 objective. For live cell imaging, HeLa cells were grown in 35-mm glass-bottomed dishes (MatTek Corporation, Ashland, MA). Imaging was performed using the Zeiss LSM 710 microscope with a 37°C incubation chamber using a 40X/1.2 NA objective. A laser (488 and 561 nm) was used to obtain the images. EGFR was labeled with EGFP, tubulin was labeled with mRFP, and syntaxin 6 was labeled with DsRed. After 48 hr of transfection, cells were serum-starved overnight. After EGF stimulation, cells were monitored with 30 min time lapse and 15 seconds interval (EGFR and microtubules); 40 minutes time lapse and 20 second interval (EGFR and syntaxin 6). The ZEN and AxioVison software programs (Carl Zeiss MicroImaging, Inc., Thornwood, NY) and ImageJ software program (National Institutes of Health, Bethesda, MD) were used for data analysis.

2.9 Fluorescence resonance energy transfer (FRET)

HeLa cells were transfected with pEGFP-EGFR (donor) for 48 hr. Cells were exposed to serum-free medium overnight, treated with EGF (50ng/ml) for 30min following fixation overnight at 4°C with 4% paraformaldehyde (PFA), and then washed three times with PBS. Cells were incubated with 0.05% Triton X-100 for 15 min and washed with PBS three times. Cells were blocked with 3% bovine serum albumin (BSA) in PBS and incubated with primary mouse anti- α -tubulin antibody and secondary mouse Alexa Fluor-555 antibody (acceptor). The slides were examined with Zeiss LSM710 confocal microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY). For FRET data acquisition, three channels were set-up: Donor (GFP), Acceptor (Alexa Fluor 555), and the FRET channel. The donor channel has a 488nm excitation and 495-525nm emission collection and the acceptor channel has a 561nm excitation and 575-635nm emission collection. In contrast, the FRET channel has a 488nm donor-excitation and 575-635nm acceptor emission collection in order to image acceptor emission resulted from energy transferred from the donor. Then optimal laser power levels and PMT settings were determined for the double-labeled sample to avoid photobleaching and saturation in all three imaging channels. After optimization, nine images were acquired for background and spectral bleed-through correction and subsequent FRET analysis from single-label donor sample, single-label acceptor sample, and double-label sample containing donor and acceptor fluorophore. For analysis of FRET efficiency quantitatively, Youvan method was chose to calculate the raw FRET images based on the following formula and a color-coded FRET image was then created.

$$F_c = (\text{fret}_{\text{gv}} - \text{bg}_{\text{fret}}) - \text{cf}_{\text{don}} * (\text{don}_{\text{gv}} - \text{bg}_{\text{don}}) - \text{cf}_{\text{acc}} * (\text{acc}_{\text{gv}} - \text{bg}_{\text{acc}})$$

F_c: FRET as calculated by the Youvan method

gv: intensity as gray value

bg: background intensity

cf: correction factor

fret: raw fret-channel image

don: donor channel image

acc: acceptor channel image

2.10 Immunoelectron microscopy (Immuno-EM)

Cells were fixed with 2% paraformaldehyde containing 0.1% glutaraldehyde for 1 hr, permeabilized with 0.5% Triton X-100 for 15 min, and then incubated with 5% bovine serum albumin for 15 min. After overnight incubation at 4°C with primary antibodies, cells were then washed with PBS and further incubated with the gold particle-labeled secondary antibody (Electron microscopy science, Hatfield, PA) overnight at 4°C for immunogold labeling. After post-fixation with 2% glutaraldehyde, cells were washed and stained with 1% Millipore-filtered uranyl acetate. The samples were dehydrated in increasing concentrations of ethanol, infiltrated, and embedded in Spurr's low viscosity medium. The samples were polymerized in a 70°C oven for 2 days. The glass cover slips were removed by dipping the blocks in liquid nitrogen. Ultrathin sections were cut with a Leica Ultracut microtome (Leica, Deerfield, IL), stained with uranyl acetate and lead citrate in a Leica EM Stainer, and examined using a JEM 1010 transmission electron

microscope (JEOL, USA, Inc., Peabody, MA) at an accelerating voltage of 80 kV. Digital images were obtained using an AMT Imaging System (Advanced Microscopy Techniques Corp, Danvers, MA).

2.11 RT-PCR and quantitative real-time PCR

First-strand cDNA was obtained from 1 µg of total RNA isolated with the Trizol Reagent (15596-026, Invitrogen, Carlsbad, CA) using the SuperScript III First-Strand Synthesis System (18080-051, Invitrogen) and oligo(dT)₂₀ primers. For RT-PCR, primers (5'-GTCATGGGAGAAAACAACAC-3' and 5'-CTTTGCCTCCTTCTGCATGG-3') were used to amplify the EGFR gene fragment. The PCR was performed in a total volume of 20 µl Taq reaction buffer containing 6 nmol dNTPs, 20 pmol of each primer, 1 µl Dimethyl sulfoxide (DMSO), and 0.2 µl Taq polymerase. The PCR cycling conditions were as follows: one cycle at 94°C for 5 min; 30 cycles of 94°C for 45 s, 57°C for 45 s, and 72°C for 1 min; and a final extension cycle at 72°C for 10 min. For quantitative RT-PCR, the cDNAs were amplified in iQ SYBR Green Supermix (170-8880, Bio-Rad, Hercules, CA). The relative amount of mRNA was determined by performing RT-PCR in triplicate using specific primers with the following sequences: GAPDH forward, 5'-GGTGGTCTCCTCTGACTTCAACA-3'; and GAPDH reverse, 5'-GTTGCTGTAGCCAAATTCGTTGT-3'. ACTB forward: 5'-GTTGTCGACGACGAGCG-3', ACTB reverse 5'-GCACAGAGCCTCGCCTT-3'

2.12 Luciferase reporter assay

HeLa cells plated in 12-well culture plates were transfected with pCCD1-Luc and the control Recilla luciferase reporter construct pRL-TK (Promega) as previously described (Lo et al., 2005a). Twenty-four hours following transfection, cells were serum starved overnight, stimulated with 100 ng/ml EGF for indicated time, harvested, and subjected to luciferase assay using the dual luciferase assay kit and the TD20/20 luminometer (Promega, Madison, WI). Following normalization with the Recilla luciferase activity (transfection efficiency control), mean luciferase activities and standard deviations were derived from three independent experiments.

Chapter Three

Syntaxin 6-mediated Golgi trafficking regulates transcriptional activity of nuclear EGFR

3.1 EGF induces translocation of EGFR to the Golgi apparatus

3.1.1 EGF stimulation enhances the protein level of EGFR in the Golgi-enriched fraction

It has been shown that retrograde trafficking from Golgi to ER is required for the nuclear translocation of EGFR (Wang et al., 2010a). However, it is not yet clear how EGFR moves from cell surface to the Golgi apparatus. To address this issue, we first asked whether EGFR transport to the Golgi could be stimulated by its ligand, EGF. We separated the Golgi apparatus using iodixanol density gradient ultracentrifugation and examined the presence of EGFR in the Golgi-enriched fraction. As shown in the top panel of Figure 3-1, left panel, using two Golgi apparatus markers, syntaxin 6 and Vt11b, we found that fraction 9 was the Golgi-enriched fraction from normal culture condition. EGFR expression level in the Golgi-enriched fraction was significantly higher in cells treated with EGF than that in cells without EGF treatment (Figure 3-1, right panel).

Figure 3-1

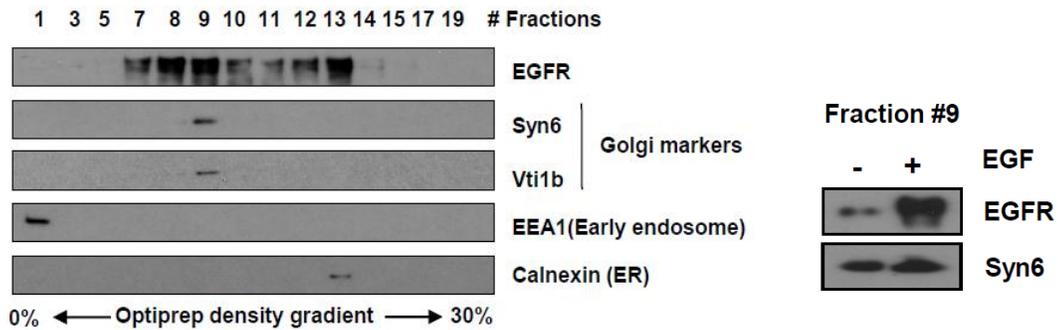


Figure 3-1: EGF induces translocation of EGFR to the Golgi apparatus. (Top) The 0-30% OptiPrep continuous density gradient was constructed using the Gradient Station (BIOCOMP, Fredericton, NB). Cell lysate in normal culture was loaded onto a density gradient medium and subjected to ultracentrifugation, and fractions were separated using the Gradient Station. The early endosome, the Golgi, and ER markers were used to analyze fractions. **(right)** HeLa cells were treated with or without EGF (50 ng/ml) for 20 min after starvation overnight. The EGFR level in the Golgi-enriched fraction (fraction 9) was analyzed using immunoblotting.

3.1.2 EGF induces the colocalization of EGFR with the Golgi marker

Polypeptide N-acetylgalactosaminyl transferase 2 (GalNac T2) (Storrie et al., 1998) is another marker of the Golgi apparatus. Using confocal microscopy analysis, we detected the localization of EGFR on the GalNac T2 positive compartment under a time-course treatment of EGF indicating that EGFR was on the Golgi apparatus. The colocalization peaked at 30 min and then gradually decreased at 60 min post EGF stimulation (Figure 3-2). We quantified the colocalization of EGFR with GalNac T2 by counting the numbers of yellow spots that resulted from the merge of the green signal of EGFR with the red signal of GalNac T2 (right panel of Figure 3-2). These results indicated that EGFR colocalized with the Golgi apparatus, which is in consistent with biochemical results shown in Figure 3-1.

Figure 3-2

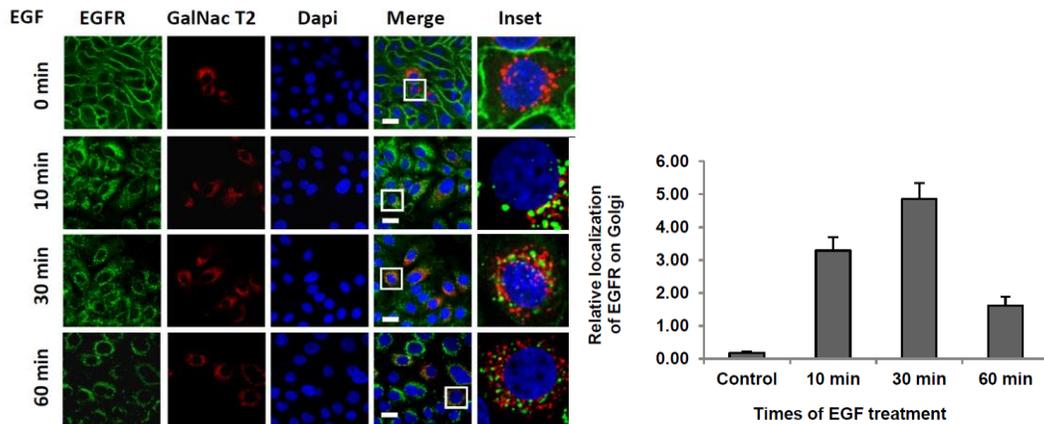


Figure 3-2: EGF stimulates the colocalization of EGFR with the Golgi marker GalNac T2. HeLa cells were transfected with GalNac T2 expression plasmid. Cells maintained in serum-free media overnight were treated with EGF (50 ng/ml) for the indicated time. The colocalization of EGFR with GalNac T2 was examined with confocal microscopy. All nuclei were confirmed by DAPI staining. Green, red, and blue channels: EGFR, GalNac T2, and nuclei, respectively. Scale bars, 20 μ m. Quantitation of cells with nEGFR are shown in the right panel.

3.1.3 Inhibition of protein synthesis does not affect EGF-induced Golgi translocation

To rule out the possibility that EGF stimulation induces EGFR synthesis in the ER and its posttranslational modification at the Golgi apparatus, we treated HeLa cells with protein synthesis inhibitor, cycloheximide (CHX), for 6 hr and still observed the colocalization of EGFR with GalNac T2 upon EGF stimulation (Figure 3-3), indicating that EGF can induce translocation of EGFR to the Golgi apparatus in the absence of protein synthesis. This notion was further supported by our observation that the EGFR protein and mRNA levels did not change significantly upon treatment with EGF for 30 min (Figures 3-4).

Figure 3-3

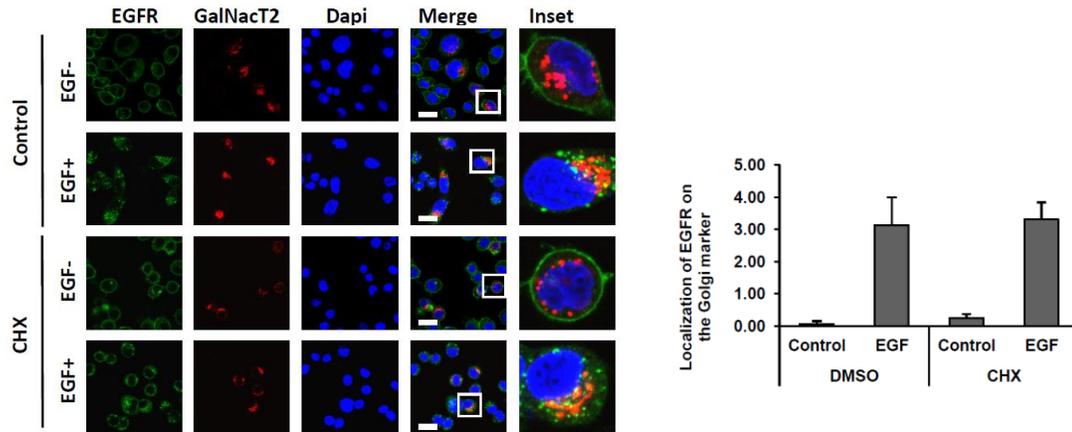


Figure 3-3: Inhibition of protein synthesis does not affect EGF-induced nuclear translocation of EGFR. HeLa cells were transfected with a plasmid encoding EGFP-GalNac T2, and cells were exposed to serum-free media overnight following treatment with EGF (50 ng/ml) for 20 min with and without CHX treatment. The colocalization of EGFR with GalNac T2 was examined with confocal microscopy. All nuclei were confirmed by DAPI staining. Green, red, and blue channels: EGFR, GalNac T2, and nuclei, respectively. Scale bars, 20 μ m. The boxed areas are shown in detail in the insets. Quantitation of positive cells with nEGFR is shown in the lower panel. DMSO, dimethyl sulfoxide; CHX, cycloheximide, a protein synthesis inhibitor.

Figure 3-4

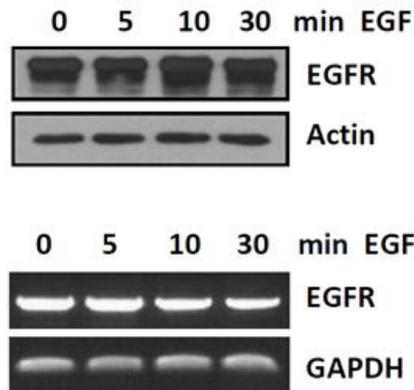


Figure 3-4: EGF stimulation does not change the protein and mRNA level of EGFR. HeLa cells were treated with EGF for different time. Total lysates were examined by immunoblotting analysis with indicated antibodies. Total mRNA isolated from cells was reversed transcribed to cDNA. mRNA level of EGFR and GAPDH were examined by RT-PCR.

3.1.4 EGF induces dynamic translocation of EGFR to the Golgi

Furthermore, we examined the dynamic process of EGFR and Golgi colocalization using a time-lapse confocal microscopy assay. We observed the localization of enhanced green fluorescent protein (EGFP)-labeled EGFR on the cell surface at time 0 of EGF stimulation after overnight serum starvation. After EGF stimulation, we observed a gradual move of EGFR into the cytoplasm in which some EGFR began to colocalize with *Discosoma sp.* red fluorescent protein (DsRed)-syntaxin 6. In representative images from time-lapse confocal microscopy shown in Figure 3-5, the green spots (EGFR) in inset 1 at the 6'20" time point moved closer to the red spots (syntaxin 6; arrows) and some merged into yellow spots in insets 3-5 at time points 7'00" to 7'40".

Taken together, our data showed that EGF stimulation enhances the dynamic translocation of EGFR to the Golgi apparatus.

Figure 3-5

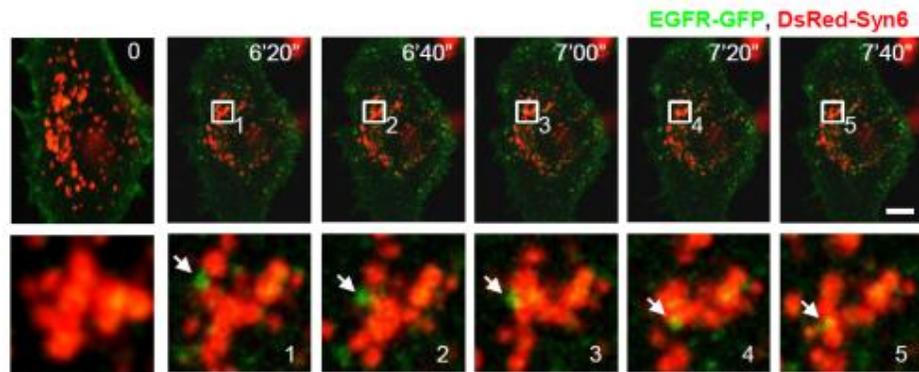


Figure 3-5: EGF induces the dynamic translocation of EGFR to the Golgi apparatus. Fluorescence-labeled EGFP-EGFR (green) and DsRed-syntaxin 6 (red) were transfected into HeLa cells. The movement of EGFR after EGF stimulation was monitored using a live cell time-lapse confocal microscopy. Images were collected at 20-sec intervals and representative snapshot images at indicated time were shown. Arrows indicate the representative colocalization of EGFR with syntaxin 6. Scale bar, 5 μ m.

3.2 Syntaxin 6 is required for the Golgi translocation of EGFR

3.2.1 EGF induces association of EGFR with syntaxin 6

Since syntaxin 6 is a key molecule to regulate membrane fusion between endosomal vesicle and Golgi apparatus, we next asked whether syntaxin 6 associates with EGFR to regulate the Golgi translocation of EGFR.

We first tested whether EGF can induce the colocalization of EGFR with syntaxin 6 using confocal microscopy. As shown in Figure 3-6, EGFR colocalized with syntaxin 6 upon EGF stimulation indicating the association between EGFR with syntaxin 6 in response to EGF stimulation.

Figure 3-6

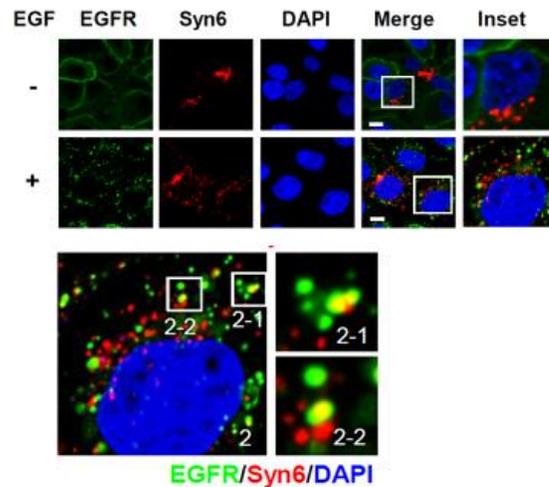


Figure 3-6: EGF induces the colocalization of EGFR with the Golgi marker, syntaxin 6. HeLa cells were transfected with pDsRed-Syn6 expression plasmid. Cells were exposed to serum-free media overnight following treatment with EGF (50 ng/ml) for 20 min. The colocalization of EGFR with syntaxin 6 was examined with confocal microscopy. All nuclei of cells were confirmed by DAPI staining. Green, red, and blue channels: EGFR, Syn6, and nuclei, respectively. The boxed areas are shown in detail in insets. Insets 2-1 and 2-2 are representative images showing the colocalization of EGFR with syntaxin 6. Scale bars: 10 μ m.

We then examined whether EGFR interacts with syntaxin 6 using immunoprecipitation (IP) assay. Our data showed EGFR was coimmunoprecipitated with syntaxin 6 as examined with IP using anti-syntaxin 6 antibody followed by immunoblotting (IB) using anti-EGFR antibody (Figure 3-7, left panel). This interaction was confirmed with reciprocal IP using anti-EGFR antibody followed by IB using anti-syntaxin 6 antibody (Figure 3-7, right panel). Taken together, our results suggested that syntaxin 6 might have a role in Golgi transport of EGFR.

Figure 3-7

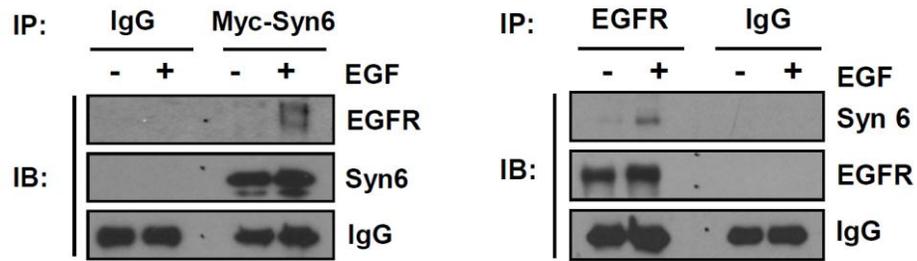


Figure 3-7: EGF induces the association between EGFR and syntaxin 6. HeLa cells were serum-starved overnight and stimulated with EGF (50 ng/ml) for 20 min. Cell lysates were immunoprecipitated with the indicated antibodies and then subjected to immunoblot (IB) analysis as indicated.

3.2.2 Downregulation of syntaxin 6 reduces the localization of EGFR at the Golgi apparatus

To explore the function of syntaxin 6 in regulating the Golgi translocation of EGFR, we used two small interfering RNAs (siRNAs) to knockdown the expression of syntaxin 6 and examined its effect on the Golgi translocation of EGFR. As shown in Figure 3-8 and Figure 3-9, knockdown of syntaxin 6 decreased the protein level of EGFR in the Golgi-enriched fraction and EGF-induced colocalization of EGFR with the Golgi marker, GalNac T2. When we used a dominant negative mutant of syntaxin 6, coiled-coil domain (CCD) (Kabayama et al., 2008), to inhibit the function of syntaxin 6, we observed similar results, in which the EGF-induced colocalization of EGFR with GalNac T2 was blocked (Figure 3-10). Quantitative results are shown in the lower panels of Figures 3-9 and Figure 3-10. These results indicate that syntaxin 6 is required for the Golgi translocation of EGFR and imply that membrane fusion at

the Golgi apparatus is involved in the transport of EGFR from cell surface to the Golgi apparatus.

Figure 3-8

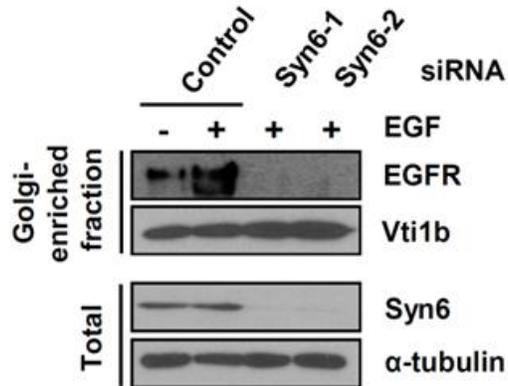


Figure 3-8: Knockdown of syntaxin 6 reduced EGFR protein level in the Golgi enriched fraction. HeLa Cells were transfected with siRNA of syntaxin 6 or control siRNA for 72 hr. After that, cells were serum starved overnight and then treated with EGF (50 ng/ml) for 20 min. The EGFR level in the Golgi-enriched fraction (fraction 9) was analyzed using immunoblotting.

Figure 3-9

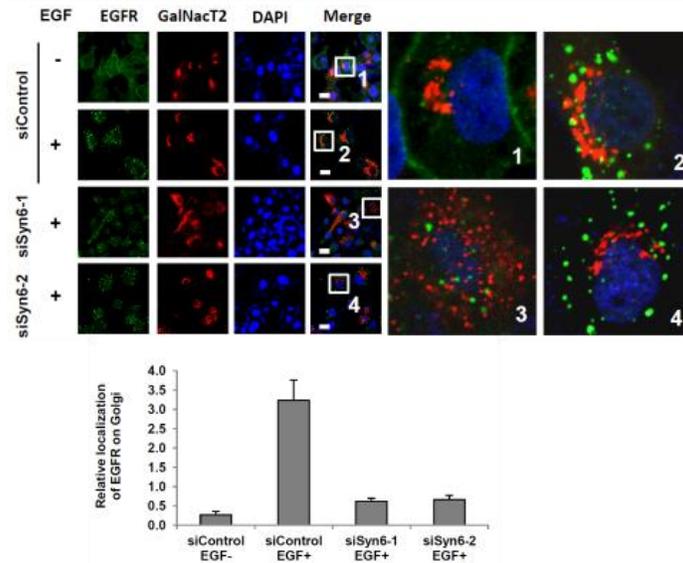


Figure 3-9: Knockdown of syntaxin 6 expression reduces the colocalization of EGFR with the Golgi marker GalNac T2. HeLa Cells were transfected with siRNA of syntaxin 6 or control siRNA for 24 hr, and then transfected with GalNac T2 expression plasmid for 48 hr. After that, cells were serum starved overnight and then treated with EGF (50 ng/ml) for 20 min. Then the colocalization of EGFR with GalNac T2 was examined with confocal microscopy assay. All nuclei were confirmed by DAPI staining. Green, red, and blue channels: EGFR, GalNac T2, and nuclei, respectively. Scale bars: 20 μ m. The boxed areas are shown in detail in the insets. Quantitative results are shown in the lower panel.

Figure 3-10

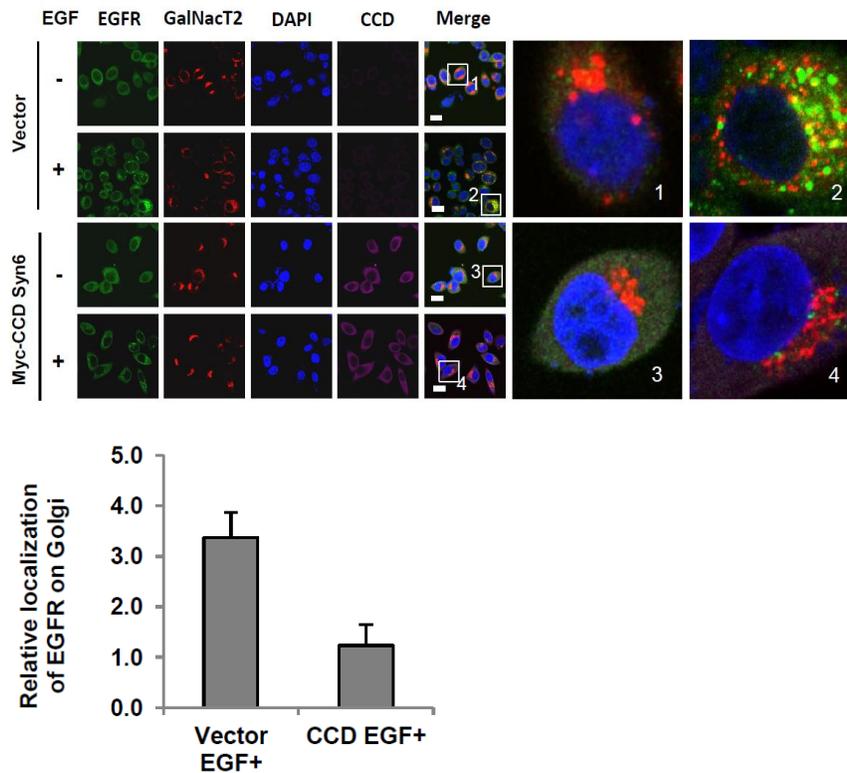


Figure 3-10: Dominant negative mutation (CCD) of syntaxin 6 decreases the colocalization of EGFR with the Golgi marker GalNac T2. Cells were transfected with CCD domain of syntaxin 6 or control vector. After 48 hr transfection, cells were serum starved overnight and then treated with EGF (50 ng/ml) for 20 min. Then the colocalization of EGFR with GalNac T2 was examined with confocal microscopy. All nuclei were confirmed by DAPI staining. Green, red, and blue channels: EGFR, GalNac T2, and nuclei, respectively. Scale bars: 20 μ m. The boxed areas are shown in detail in the insets. Quantitative results are shown in the lower panel.

3.3 Syntaxin 6 is required for nuclear translocation of EGFR

3.3.1 Depletion of syntaxin 6 by siRNAs decreases nuclear translocation of EGFR

COPI-regulated retrograde transport from the Golgi apparatus to the ER has been reported to mediate the nuclear translocation of EGFR (Wang et al., 2010a). Our

results indicated that syntaxin 6-, microtubule-, and dynein-mediated intracellular trafficking is required for EGF-induced Golgi translocation of EGFR. Therefore, we asked whether syntaxin 6 is required for the downstream trafficking pathway of EGFR to the nucleus.

We knocked down expression of syntaxin 6 by siRNAs and analyzed EGFR expression in nuclear fractions. As shown in Figure 3-11, upon EGF stimulation, EGFR was detected in the nuclear fraction of cells with syntaxin 6 expression. However, when syntaxin 6 expression was knocked down, the level of nEGFR significantly decreased. Consistently, confocal microscopy analysis showed EGF-induced localization of EGFR in the nucleus (Figures 3-12, insets 1 and 2; green signal of EGFR merged with the blue signal of nucleus) was reduced in cells with knockdown of syntaxin 6 (cells that do not have red color surrounding the nucleus) compared to cells with syntaxin expression (Figure 3-12, insets 3 and 4; cells in red color surrounding the nucleus). We quantitated the percentage of nEGFR-positive cells in 100 cells under different conditions (lower panel of Figure 3-12) and found the number of nEGFR-positive cells decreased as the results of syntaxin 6 knockdown.

Figure 3-11

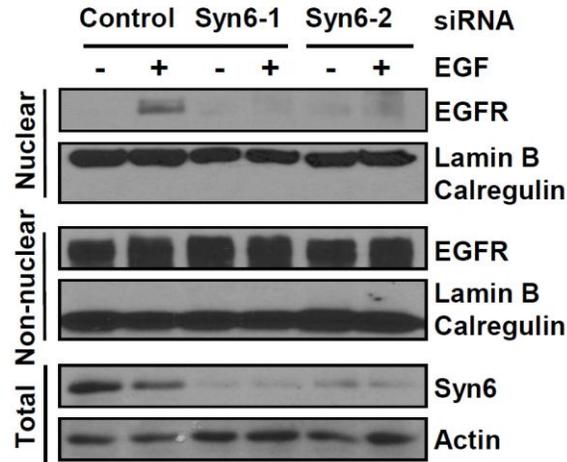


Figure 3-11: Knockdown of syntaxin 6 expression reduces nuclear EGFR level. Cells were transfected with siRNAs of syntaxin 6 or control siRNA. Cells were maintained in serum free media overnight and then treated with EGF (50 ng/ml) for 30 min. Non-nuclear and nuclear fractions were separated using cellular fractionation and then subjected to immunoblot analysis using the indicated antibodies.

Figure 3-12

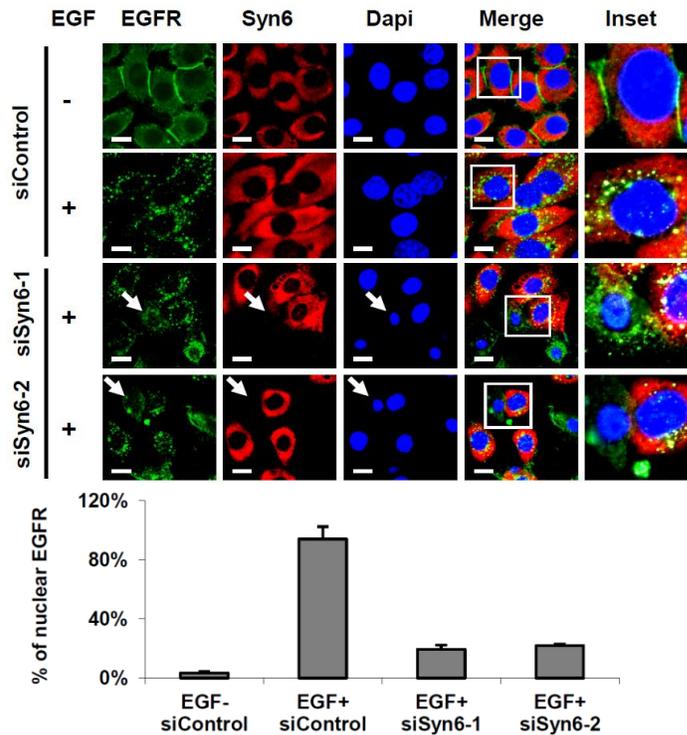


Figure 3-12: Knockdown of syntaxin 6 expression blocks nuclear translocation of EGFR. HeLa cells were transfected with siRNA of syntaxin 6 or a control siRNA. Cells were maintained in serum-free media overnight and then treated with EGF (50 ng/ml) for 30 min. The colocalization of EGFR with syntaxin 6 was examined with confocal microscopy. All nuclei were confirmed by DAPI staining. Green channel, EGFR; red channel, syntaxin 6; blue channel, nuclei. The details of cells indicated by arrows were shown in insets. Quantitation of positive cells with nEGFR is shown in the lower panel. Scale bars, 20 μ m.

3.3.2 Dominant negative mutation of syntaxin 6 reduces nuclear translocation of EGFR

Similarly, when a dominant negative mutant of syntaxin 6, coiled-coil domain (CCD) (Kabayama et al., 2008), was used to inhibit the function of syntaxin 6, the protein level of nEGFR (Figure 3-13) and the localization of EGFR in the nucleus (Figure 3-14, insets 2 and 4; green signal of EGFR merged with the blue signal of

nucleus) were decreased. Thus, we concluded that syntaxin 6-regulated Golgi translocation of EGFR is critical for EGF-induced nuclear translocation of EGFR.

In summary, reduced nuclear translocation of EGFR by knockdown of syntaxin 6 and inhibition of its function using CCD domain suggested that syntaxin 6 is required for the nuclear translocation of EGFR.

Figure 3-13

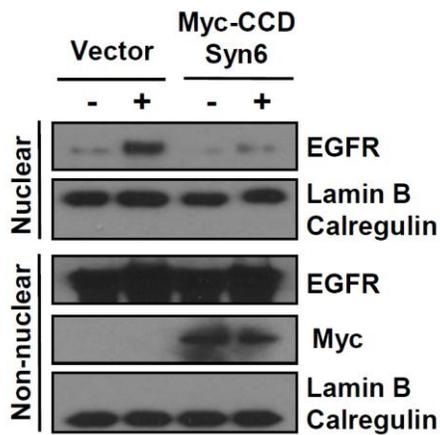


Figure 3-13: The CCD of syntaxin 6 decreases nuclear EGFR expression. HeLa cells were transfected with a control vector or syntaxin 6 CCD. The cells were maintained in serum-free media overnight and then stimulated with EGF (50 ng/ml) for 30 min. Nuclear and nonnuclear fractions were subjected to immunoblot analysis with the indicated antibodies.

Figure 3-14

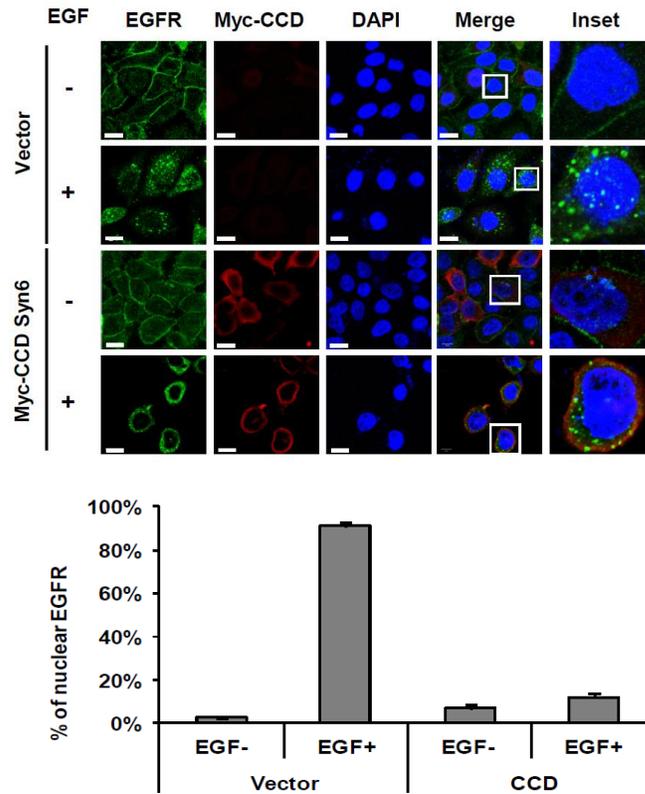


Figure 3-14: CCD domain decreases the nuclear translocation of EGFR. HeLa cells were transfected with a control vector or syntaxin CCD. The cells were maintained in serum-free media overnight and then stimulated with EGF (50 ng/ml) for 30 min. The colocalization of EGFR with CCD domain of syntaxin 6 was examined with confocal microscopy. All nuclei were confirmed by DAPI staining. Green channel, EGFR; red channel, CCD. The boxed areas are shown in detail in insets. Scale bars, 20 μ m.

3.4 Syntaxin 6 is required for the transcriptional activity of nuclear EGFR

3.4.1 Downregulation of syntaxin 6 using siRNAs reduces DNA binding ability of nuclear EGFR to the promoter of cyclin D1

EGFR has been reported to associate with cyclin D1 promoter to activate its transcription in the nucleus (Huo et al., 2010; Lin et al., 2001). If syntaxin 6 is

important for the Golgi translocation and the downstream nuclear translocation of EGFR, the inhibition of syntaxin 6 should affect the function of nuclear EGFR.

To confirm that syntaxin 6 is required for the function of nuclear EGFR, we evaluated the effect of knockdown of syntaxin 6 on the DNA binding ability of nuclear EGFR to the promoter of cyclin D1. As shown in Figure 3-15, when syntaxin 6 was knocked down by siRNAs, the DNA binding ability of nuclear EGFR was decreased as indicated by ChIP assay.

Figure 3-15

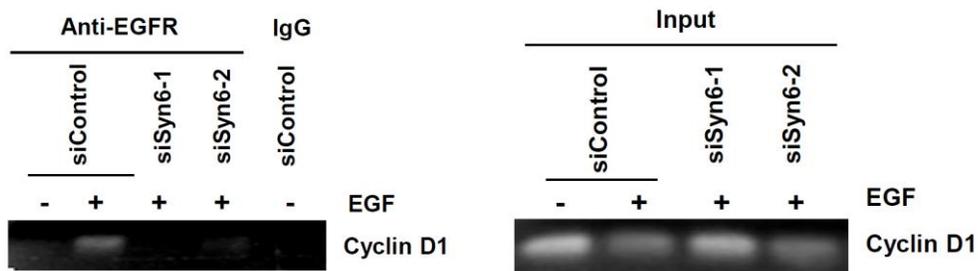


Figure 3-15: Knockdown of syntaxin 6 reduces the binding of EGFR to the promoter of cyclin D1. Cells were transfected with siRNAs of syntaxin 6. After 72 hr transfection, cells were serum starved overnight then stimulated with EGF for 30min. cells were then performed with chromatin-IP assay. For IgG control, lysate of cells without EGF stimulation was used. Lower panel: Input of upper panel.

3.4.2 Downregulation of syntaxin 6 using siRNAs decreases EGF-induced mRNA level of cyclin D1

To further confirm that syntaxin 6 regulated Golgi translocation of EGFR is important for the function of nEGFR, we performed Luciferase assay using the reporter plasmids containing cyclin D1 promoter. As shown in Figure 3-16, knockdown of syntaxin 6 decreased EGF induced luciferase activity which indicated

that knockdown of syntaxin 6 decreased the binding of nEGFR to the cyclin D1 promoter. Consistently, quantitative RT-PCR showed that when syntaxin 6 was knocked down, the EGF-induced mRNA level of cyclin D1 was lower than that in cells with control siRNA upon EGF stimulation (Figure 3-17).

Figure 3-16

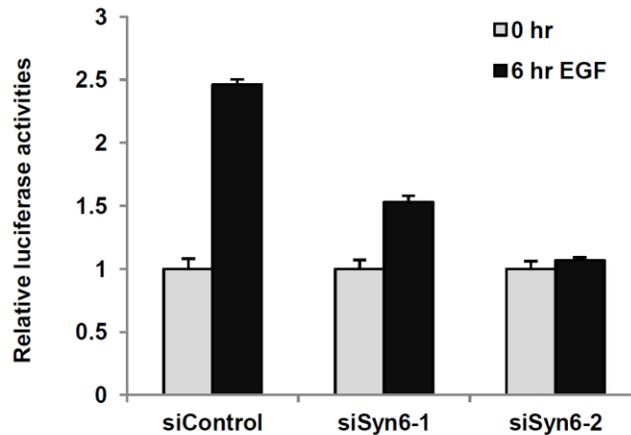


Figure 3-16: Knockdown of syntaxin 6 inhibits EGF-induced luciferase activity of cyclin D1 promoter. HeLa cells transfected with control siRNA or siRNAs of syntaxin 6 were transfected with reporter plasmids containing cyclin D1 promoter. Then after 24 hr transfection, cells were serum starved overnight and treated with EGF for indicated time. Total cell lysates were used for luciferase assay. Error bars were derived from three independent experiments.

Figure 3-17

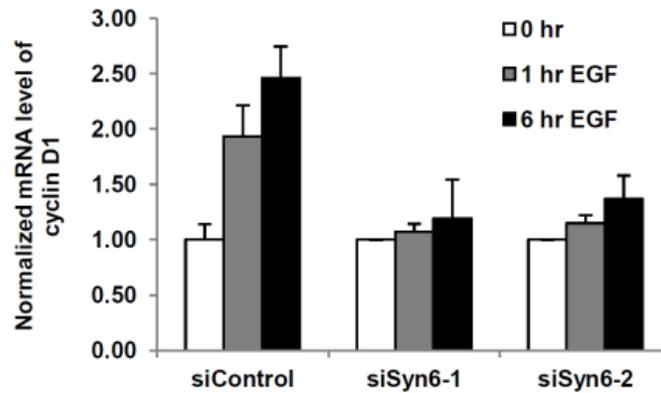


Figure 3-17: Depletion of syntaxin 6 reduces mRNA expression of cyclin D1. Cells were transfected with shRNA of syntaxin 6. After 72 hr transfection, cells were serum starved overnight and then stimulated with EGF for indicated time. Quantitative-PCR was used to analyze the mRNA level of cyclin D1. Error bars were derived from three independent experiments.

Taken together, these results indicated that syntaxin 6 is critical for the transcriptional activity of nEGFR and provided another layer of support for the importance of syntaxin 6-mediated Golgi translocation in EGF-induced nuclear translocation of EGFR.

Chapter Four
Microtubule cytoskeleton regulates the Golgi
and nuclear translocation of EGFR

4.1 Microtubules and dynein are required for EGFR translocation from the cell surface to the Golgi and nucleus

4.1.1 Disruption of microtubules blocks the Golgi translocation of EGFR

Since microtubules and their motor proteins play critical roles in intracellular trafficking of most organelles in cytoplasm including the Golgi apparatus (Caviston and Holzbaaur, 2006; Rogers and Gelfand, 2000; Soldati and Schliwa, 2006), we asked whether EGFR-embedded endocytic vesicles move along the microtubules to reach the Golgi apparatus.

To this end, we first tested whether trafficking of EGFR from the cell surface to the Golgi apparatus requires microtubule formation. Indeed, treatment of cells with microtubule inhibitors, nocodazole and paclitaxel, decreased EGF-induced EGFR translocation to the Golgi apparatus as is evident from the decreased EGFR protein level in Golgi-enriched fraction 9 (Figure 4-1, lanes 3 and 4 vs. lane 2). This was further supported by confocal microscopy analysis. As shown in Figure 4-2, EGF-induced colocalization of EGFR with the Golgi apparatus, which is indicated by the merged EGFR (green) and GalNac T2 (red) signals shown in yellow (Figure 4-2, inset 2 vs. inset 1), was disrupted in cells pretreated with microtubule inhibitors, nocodazole and paclitaxel (Figure 4-2, insets 3 and 4 vs. inset 2; quantitative results were shown in the lower panel of Figure 4-2). Moreover, using time-lapse confocal microscope assay, we observed the blocking of dynamic trafficking of EGFR to the Golgi apparatus by microtubule inhibitor nocodazole (Movie 2 vs. 3).

Figure 4-1

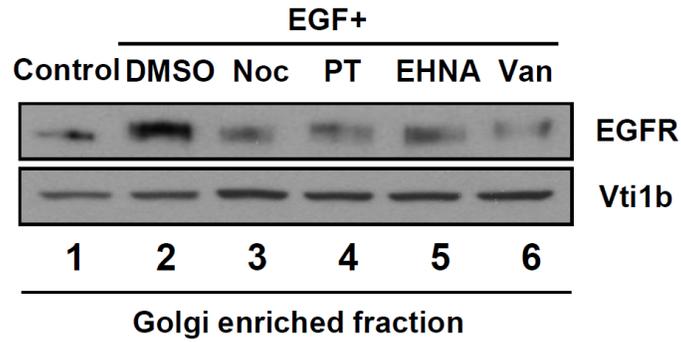


Figure 4-1: Inhibitors of microtubules and dynein decrease EGFR protein level in the Golgi-enriched fraction. Serum-starved HeLa cells were treated with microtubules inhibitors (nocodazole or paclitaxel) or dynein inhibitors (EHNA and vanadate) and then stimulated with EGF (50ng/ml). The expression of EGFR in Golgi-enriched fraction was analyzed with immunoblotting. Noc, nocodazole; PT, paclitaxel; Van, vanadate; Vti1b, marker of Golgi.

Figure 4-2

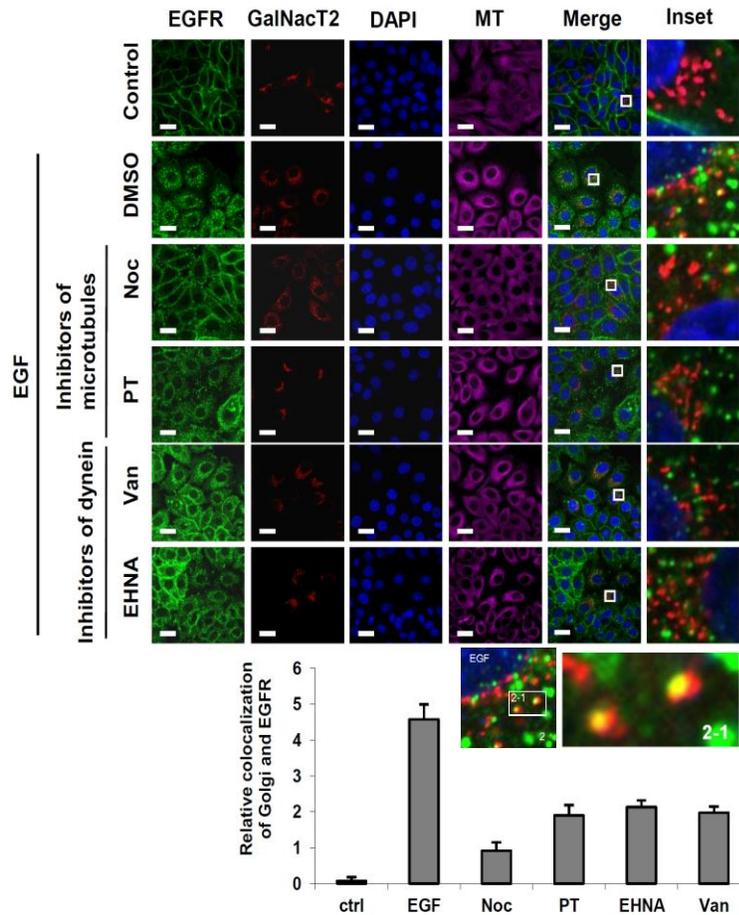


Figure 4-2: Inhibitors of microtubules and dynein block the colocalization of EGFR with the Golgi marker GalNacT2. HeLa cells were transfected with GalNac T2 expression plasmid. After 24 hr transfection, cells were treated with microtubules inhibitors (nocodazole or paclitaxel) or dynein inhibitors (EHNA and vanadate). Cells were then serum-starved overnight and stimulated with EGF (50ng/ml). The colocalization of EGFR with GalNac T2 was examined with confocal microscopy. All nuclei were confirmed by DAPI staining. Green, red, and blue channels: EGFR, GalNac T2, and nuclei, respectively. Scale bars, 20 μ m. The boxed areas are shown in detail in the insets. Representative colocalization of EGFR with GalNac T2 is shown in inset 2-1. Quantitated colocalization of EGFR and Golgi marker is shown in the lower panel. Noc., nocodazole; PT., paclitaxel; Van., vanadate.

4.1.2 CDK1 decreases Golgi translocation of EGFR

CDK1 has been reported to depolymerize microtubules through direct phosphorylation of β -tubulin or indirect phosphorylation of microtubule associated protein. To further confirm the function and importance of microtubules in Golgi translocation of EGFR, we disrupted microtubules' formation by overexpression of CDK1 and its activator, cyclin B, to depolymerize microtubules through a natural occurring process. We found that overexpression of CDK1 decreased the colocalization of EGFR with the Golgi marker, GalNac T2, in response to the EGF stimulation (Figure 4-3).

Figure 4-3

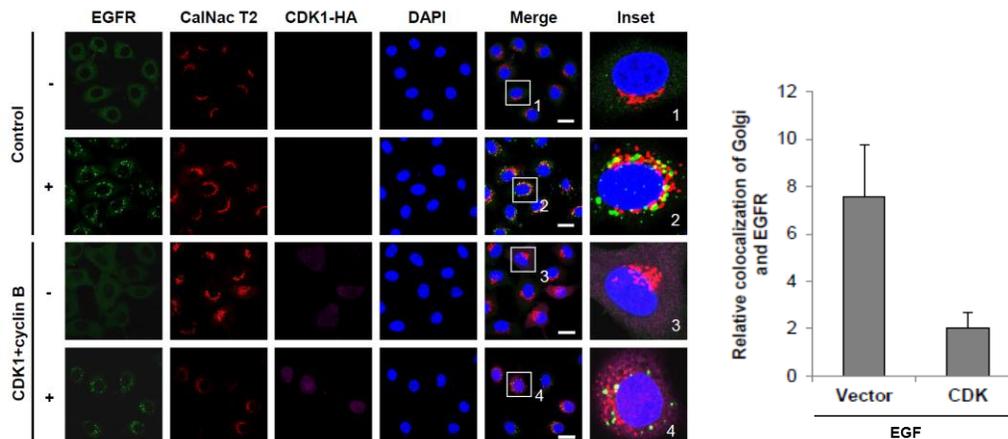


Figure 4-3: CDK1 decreases the colocalization of EGFR with the Golgi marker GalNac T2. HeLa cells were transfected with GalNac T2 expression plasmid. After 24 hr transfection, cells were transfected with plasmids coding CDK1 and cyclin B. Cells were then serum-starved overnight and stimulated with EGF (50ng/ml). The colocalization of EGFR with GalNac T2 was examined with confocal microscopy. All nuclei were confirmed by DAPI staining. Green, red, magenta, and blue channels: EGFR, GalNac T2, CDK1, and nuclei, respectively. The boxed areas are shown in detail in the insets. Quantitated colocalization of EGFR and Golgi marker is shown in the right panel.

Taken together, reduced Golgi translocation of EGFR by depletion of microtubules with inhibitors and depolymerization of microtubules by CDK1 suggested that microtubule formation is required for EGF-induced EGFR trafficking from the cell surface to the Golgi apparatus.

4.1.3 Dynein is required for the Golgi translocation of EGFR

Microtubule-dependent nucleated movement requires dynein, which contains ATPase functional domain to utilize ATP to generate energy for movement. Therefore, we asked whether dynein also plays a role in EGF-induced EGFR trafficking to the Golgi apparatus. When cells were treated with dynein inhibitors, EHNA (erythro-9-[2-hydroxy-3-nonyl]-adenine) and vanadate, EGF-induced translocation of EGFR to the Golgi was attenuated as indicated by decreased EGFR protein level in the Golgi-enriched fraction (Figure 4-1, lanes 5 and 6 vs. lane 2) as well as colocalization of EGFR with the Golgi marker GalNac T2 (Figure 4-2, insets 5 and 6 vs. inset 2).

In addition, we knocked down the expression of dynein by short hairpin RNAs (shRNAs) and found that depletion of dynein decreased EGF-induced EGFR protein level on the Golgi-enriched fraction (Figure 4-4).

Therefore, reduced Golgi translocation of EGFR by inhibition of dynein with inhibitors and depletion of dynein with shRNAs suggested that dynein is required for the translocation of EGFR from the cell surface to the Golgi.

Figure 4-4

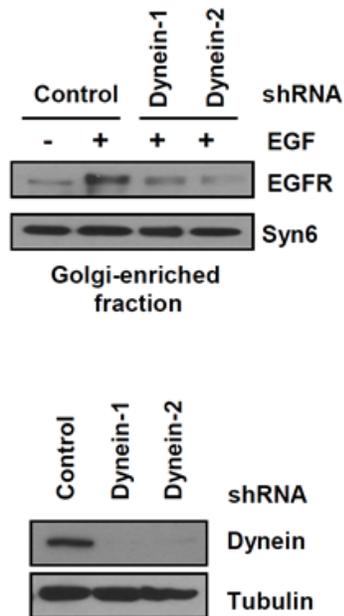


Figure 4-4: Knockdown of dynein expression decreases EGFR protein level in Golgi-enriched fraction. Serum-starved HeLa cells were transfected with dynein shRNAs. After 72 hr transfection, cells were serum starved overnight and then stimulated with EGF (50ng/ml). The Golgi-enriched fraction was purified and subjected to immunoblot analysis with indicated antibodies.

4.1.4 Inhibitors of microtubules block the colocalization of EGFR with syntaxin6

Syntaxin 6 is a key molecule to regulate membrane fusion between endosomal vesicle and Golgi apparatus. Our results have demonstrated that EGFR associates with syntaxin 6 and syntaxin 6 is required for the Golgi translocation of EGFR (Chapter 3). We further asked whether microtubules also play a role in the association of EGFR with syntaxin 6. Using time-lapse confocal microscope assay, we observed the colocalization of EGFR with syntaxin 6 occurred at about 20 min upon EGF treatment (Figure 4-5 and Movies 2 vs. 3). However, when we pre-treated cells with nocodazole to disrupt microtubules, the colocalization of EGFR with syntaxin 6 was

blocked (Figure 4-5, lower panel) suggesting that microtubules play an important role in the association of EGFR with syntaxin 6.

In summary, our data showed that functional microtubules and dynein are critical for the translocation of EGFR from the cell surface to the Golgi apparatus and suggested that EGFR likely travels along the microtubules to reach Golgi and interacts with syntaxin 6, which then facilitates membrane fusion between the Golgi and the endocytic vesicle that carries EGFR.

Figure 4-5

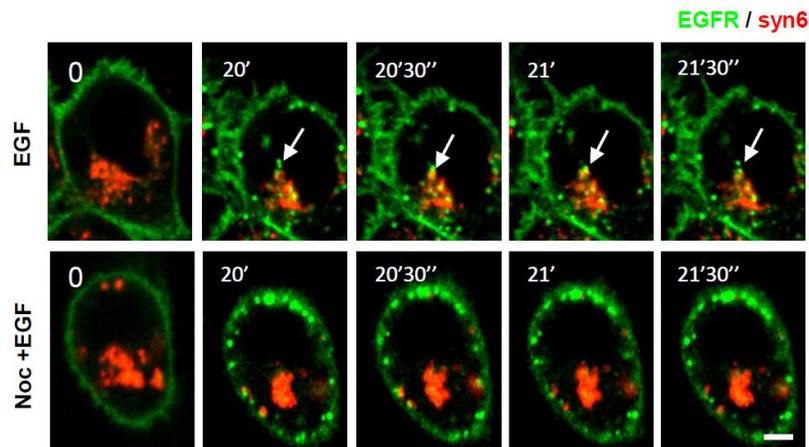


Figure 4-5: Colocalization of EGFR with syntaxin 6 is blocked by microtubules inhibitor nocodazole. Fluorescence-labeled EGFP-EGFR (green) and DsRed-syntaxin 6 (red) expression plasmids were transfected into HeLa cells. The movement of EGFR after EGF stimulation was monitored using a live cell time-lapse confocal microscopy. Images were collected at 30-sec intervals and representative snapshot images at indicated time were shown. Arrows indicate the representative colocalization of EGFR with syntaxin 6. Scale bar, 5 μ m. Noc, nocodazole.

4.2 EGF induces EGFR/microtubule and EGFR/dynein association

Our results demonstrated the Golgi translocation of EGFR upon EGF stimulation (Chapter Three). Microtubules and their motor protein dynein are required for this process. In this Chapter, we explored how EGF induces the Golgi translocation of EGFR via microtubules and dynein by a combination of different approach.

4.2.1 EGF induces association of EGFR with microtubules

We tested whether EGFR associates with microtubules using immunoprecipitation (IP) assay. As shown in Figure 4-6 (left panel), upon EGF stimulation, coimmunoprecipitation of EGFR with tubulin was significantly increased as examined with IP using anti- α -tubulin antibody followed by immunoblotting (IB) using anti-EGFR antibody. This association was confirmed with reciprocal IP using anti-EGFR antibody followed by IB using anti- α -tubulin antibody (Figure 4-6, right panel).

Figure 4-6

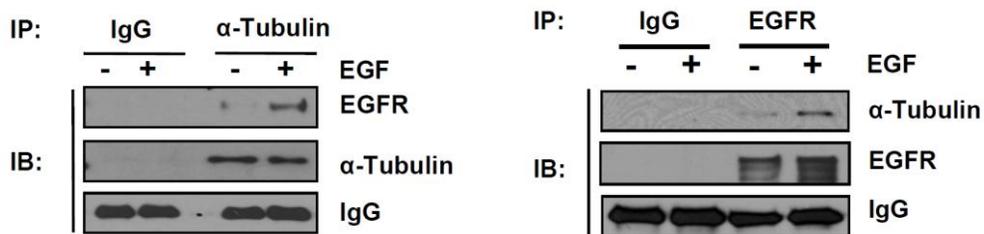


Figure 4-6: EGF induces association of EGFR with α -tubulin. HeLa cells were serum-starved overnight and stimulated with EGF (50 ng/ml) for 20 min. Cell lysates were immunoprecipitated with the indicated antibodies and then subjected to immunoblot (IB) analysis as indicated

4.2.2 EGF induces the colocalization of EGFR with microtubules

We next examined whether EGFR travels along the microtubules using confocal microscopy analysis. As shown in Figure 4-7, EGFR localized on the cell surface at 0 min of EGF stimulation. Then EGFR gradually transported into cytoplasm when treated with EGF for 5 minutes and then accumulated around the nucleus. Meanwhile, the green signal of EGFR on the cell surface merged with the red signal of microtubules in the cytoplasm and produced yellow spots indicating the colocalization of EGFR with microtubules.

The colocalization of EGFR with microtubules was further supported by immunoelectron microscopy (Immuno-EM) analysis. As shown in Figure 4-8, without EGF stimulation, EGFR was detected on the cell surface (inset 3); however, upon addition of EGF, EGFR (black spots in insets 1 and 2) localized around microtubules (triangles in insets 1 and 2).

Therefore, both confocal microscopy and Immuno-EM analyses demonstrated that EGFR transport along microtubules upon EGF stimulation.

Figure 4-7

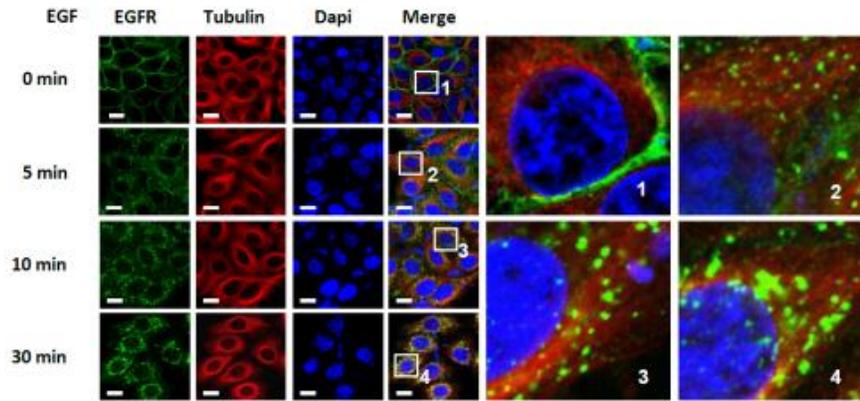


Figure 4-7: EGF induces the colocalization of EGFR with microtubules. HeLa cells maintained in serum-free media overnight were treated with EGF (20 ng/ml) for different time. EGFR and microtubules were labeled with primary antibodies and fluorescence secondary antibodies. The colocalization of EGFR with tubulin was examined with confocal microscopy. All nuclei of cells were confirmed by DAPI staining. Green, red, and blue channels represent EGFR, tubulin, and nuclei, respectively. The boxed areas are shown in detail in insets. Scale bars, 20 μ m.

Figure 4-8

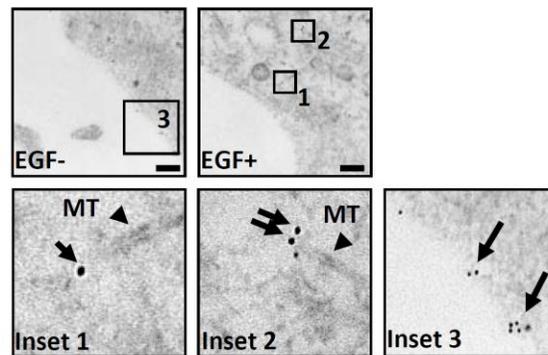


Figure 4-8: EGF induces localization of EGFR near microtubules. HeLa cells were treated with or without EGF for 30 min and subjected to immuno-EM as described in methods. MT, microtubules. Scale bars, 200 nm. Arrows indicate EGFR and triangles indicate microtubules.

4.2.3 EGF enhances the FRET efficiency from EGFR to microtubules

To further confirm that EGF induces EGFR to travel along the microtubules, we studied the association of EGFR with microtubules upon EGF stimulation using fluorescence resonance energy transfer (FRET), in which EGFP fused-EGFR served as a donor and Alexa Flour 555 stained α -tubulin served as an acceptor. The collected raw data were calculated as described in Chapter two and the Fc image was generated using the Youvan method (Douglas C. Youvan and William J. Coleman, 1997) and the Zeiss FRET software program (Carl Zeiss, Thornwood, NY). As shown in Figure 4-9, we detected FRET from the acceptor (Fc Image; arrows) only in cells expressing EGFR (Donor-EGFR) (compare images 1, 2 and 3 without EGF stimulation or compare images 4, 5 and 6 with EGF stimulation). However, upon EGF stimulation, FRET efficiency from the acceptor (microtubules) excited by the donor (EGFR) was increased as the microtubule-like image (image 3 vs. 6). Quantitation of the FRET efficiency is shown in the right panel (Figure 4-9). These results indicated that EGF induced the localization of EGFR around microtubules and further confirmed that EGFR travels along microtubules upon EGF stimulation.

Figure 4-9

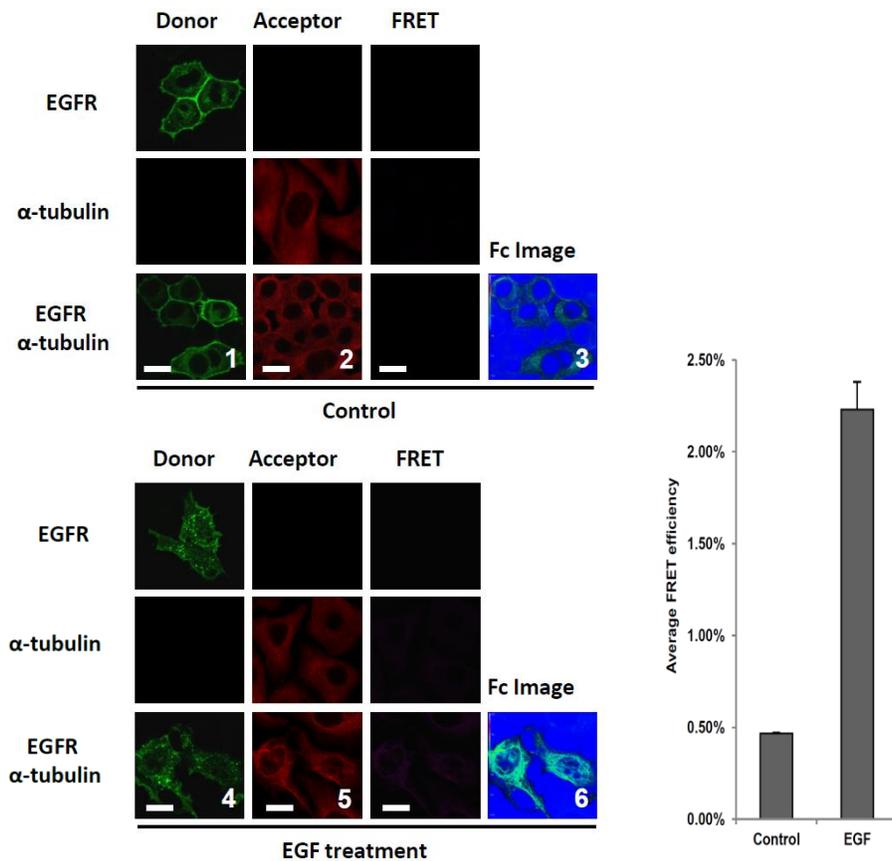


Figure 4-9: EGF enhances the FRET efficiency of microtubules from EGFR. HeLa cells were transfected with pEGFP-EGFR (donor; green) alone or labeled with a primary anti- α -tubulin antibody and Alexa Fluor 555 (acceptor; red) antibody alone. A third condition was a combination of EGFP-EGFR and Alexa Fluor 555 with α -tubulin. A Fc image was obtained using the Zeiss FRET software program. Scale bars, 20 μ m. Quantitation of the FRET efficiency is shown in the right panel.

4.2.4 EGF-induced perinuclear accumulation of EGFR requires the formation of microtubules

IP, confocal microscopy, EM, and FRET analyses demonstrated that EGFR transport along microtubules upon EGF stimulation. Finally, co-expressed EGFP-EGFR and mRFP-tubulin were used to visualize the movement of EGFR in cells using live cell imaging system. Under EGF stimulation, most EGFR moved forward and back in the certain area in parallel to microtubule. This movement was disrupted by microtubule inhibitor nocodazole (Figure 4-10). The amount of EGFR spots in the perinuclear area at 15 and 30 minutes after EGF treatment was also decreased by nocodazole (the lower panel of Figure 4-10) treatment. Although the movement of single endocytic EGFR from cell surface to the nucleus along microtubule was not observed, our data showed that the travel of EGFR from cell surface to the perinuclear area requires the formation of microtubules.

4.2.5 Nocodazole reduces the ability of EGFR to move parallel to microtubule

Using Zeiss AxioVision tracking application, we tracked the movement of more than 20 spots of EGFR. In Figure 4-11, we measured the maximum distance of movement of each spot. Y-axis stands for the movement parallel to microtubules and X-axis stands for the movement vertical to microtubules. Compared with EGF treatment, nocodazole treatment significantly reduced the EGF-induced movement of EGFR parallel to microtubules.

Figure 4-10

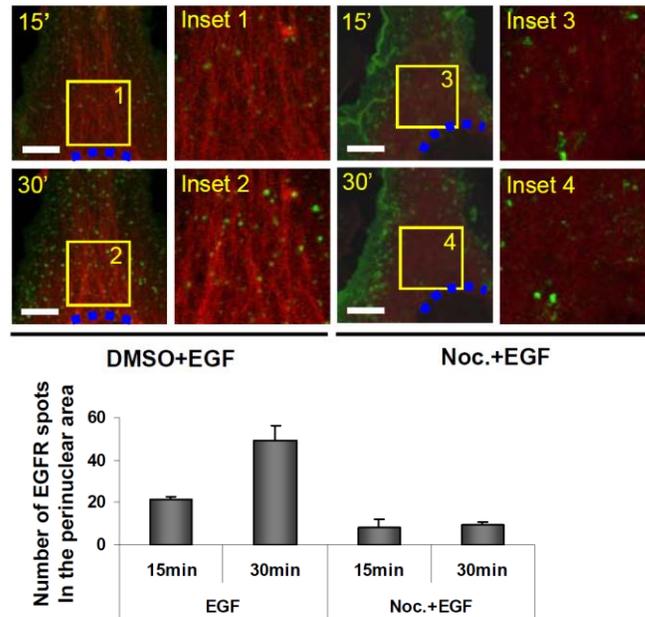


Figure 4-10: EGF induced perinuclear accumulation of EGFR requires the formation of microtubule. HeLa cells transfected with EGFP-EGFR and mRFP- α -tubulin were maintained in serum-free media overnight. EGF stimulated cells with or without nocodazole pre-treatment were subjected to time-lapse confocal microscopy. Images at 15 and 30 min under each condition were used to quantify particles in the perinuclear region. Noc, nocodazole.

Figure 4-11

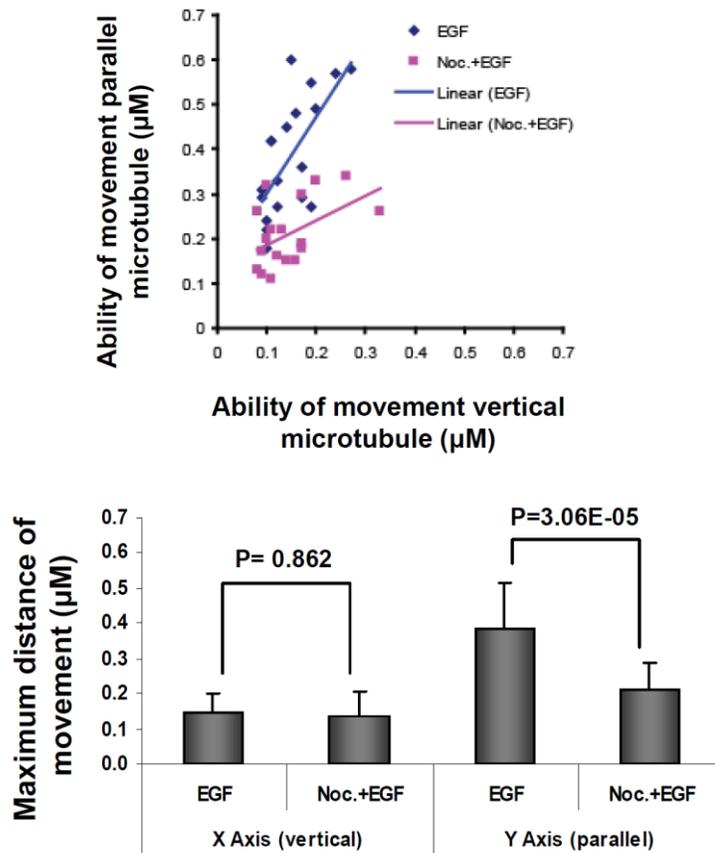


Figure 4-11: Nocodazole reduces the ability of EGFR to move parallel to microtubule. HeLa cells were transfected with EGFP-EGFR and mRFP-tubule. After collection of data using time-lapse confocal microscopy of living cells pretreated with or without nocodazole plus EGF stimulation, EGF induced particles were tracked by the AxioVision Tracking program. Spots in the plot indicated maximum movement of these particles in two directions, parallel to microtubule or vertical to microtubule. Blue spots indicated the particles under EGF stimulation and pink spots indicated the particles under pretreatment of Nocodazole plus EGF stimulation.

4.2.6 EGF induces association of EGFR with dynein

Dynein is a major motor proteins utilized for the trafficking of cargo along the microtubules. After demonstrating the movement of EGFR along the microtubules by a combination of different approach, we asked whether dynein is involved in the movement of EGFR. We first examined the association of EGFR with dynein by IP

(Figure 4-12) and confocal microscopy (Figure 4-13) analyses. Both approaches showed the association of EGFR with dynein upon EGF stimulation.

Figure 4-12

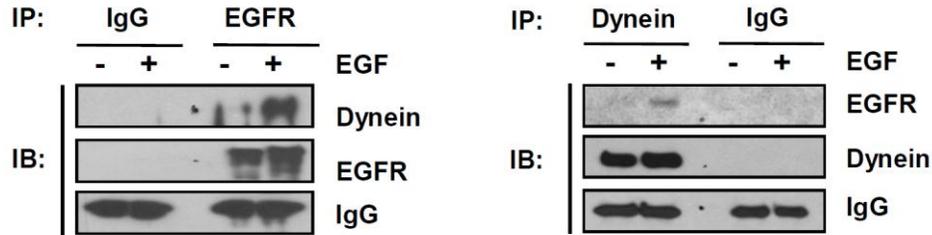


Figure 4-12: EGF induces association of EGFR with dynein. Serum-starved HeLa cells were stimulated with EGF (50ng/ml) for 20 min. Cell lysates were immunoprecipitated with the indicated antibodies and subjected to immunoblot (IB) analysis as indicated.

Figure 4-13

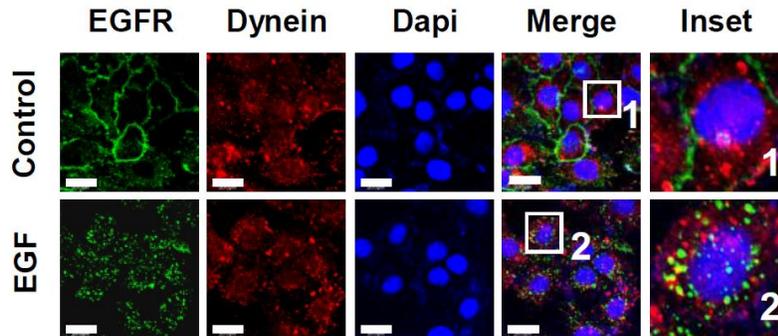


Figure 4-13: EGF induces the colocalization of EGFR with dynein. HeLa cells maintained in serum-free media overnight were treated with EGF (20 ng/ml). EGFR and dynein were labeled with primary antibodies and fluorescence secondary antibodies. The colocalization of EGFR with dynein was examined with confocal microscopy. All nuclei of cells were confirmed by DAPI staining. Green, red, and blue channels represent EGFR, dynein, and nuclei, respectively. The boxed areas are shown in detail in insets. Scale bars, 20µm. Ctrl, control.

4.2.7 Knockdown of dynein expression decreases association of EGFR with tubulin

We then asked whether dynein is required for the association of EGFR with tubulin. To that end, we used shRNAs targeting dynein to knock down dynein expression and found that in the absence of dynein, EGFR no longer interacted with α -tubulin (Figure 4-14) indicating that dynein is required for microtubule-dependent movement of EGFR.

In summary, our results indicated that EGF induces trafficking of EGFR along the microtubules. The requirement of dynein for EGFR/ α -tubulin interaction suggested that dynein may link EGFR to microtubules and facilitate microtubule-dependent movement as the power provider for intracellular trafficking of EGFR. These results resemble the previous studies in which transport of vesicles containing an N-methyl-D-aspartate or γ -aminobutyric acid receptor along microtubules were shown to be regulated by motor proteins or other co-regulators (Heisler et al., 2011; Setou et al., 2000). Thus, it is conceivable that analogous to the previous model, the endocytic vesicle containing EGFR interacts with dynein and tubulin and travels along the microtubules (the right panel of Figure 4-14).

Figure 4-14

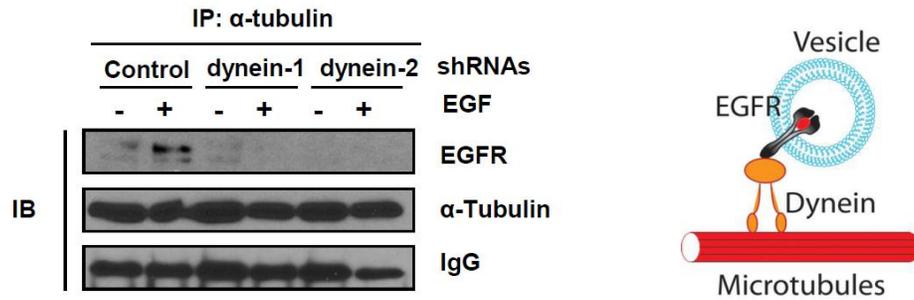


Figure 4-14: Knockdown of dynein expression decreases the association of EGFR with tubulin. Serum-starved HeLa cells with knockdown of dynein expression were stimulated with EGF (50 ng/ml) for 20 min. Cell lysates were immunoprecipitated with anti- α -tubulin antibody and subjected to immunoblot as indicated. Right panel: model of EGFR transport along microtubules regulated by dynein.

4.3 Microtubules mediate nuclear trafficking of EGFR

4.3.1 Disruption of microtubules using inhibitors blocks EGFR transport to the nucleus

So far, our data indicated that EGF-induced endocytic EGFR travels to the Golgi apparatus via microtubule-dependent movement and fuses with the Golgi through syntaxin 6-mediated membrane fusion. Our group have demonstrated that EGFR utilizes the COPI-regulated retrograde trafficking pathway (Wang et al., 2010a) to move from the Golgi to ER, inner nuclear membrane, and then nuclear plasma (Wang et al., 2010c). Based on these observations, we hypothesized that microtubule- and dynein-mediated movement of EGFR is also required for its downstream nuclear translocation.

To test this possibility, we disrupted the formation of microtubules by pretreating cells with microtubule inhibitors nocodazole or paclitaxel. Indeed, we

found that EGF-induced nuclear level of EGFR (Figure 4-15) and colocalization of EGFR with the nucleus (Figure 4-16) significantly decreased. Quantitation of the confocal microscopy images is shown in the lower panel of Figure 4-16.

Figure 4-15

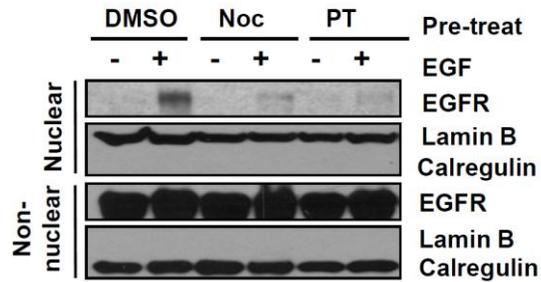


Figure 4-15: Microtubule inhibitors nocodazole and paclitaxel decrease nEGFR expression. HeLa cells maintained in serum-free media overnight were treated with nocodazole (Noc) or paclitaxel (PT) and then stimulated with EGF (50 ng/ml). Nonnuclear and nuclear fractions were separated using cellular fractionation and then subjected to immunoblot assay with indicated antibodies.

Figure 4-16

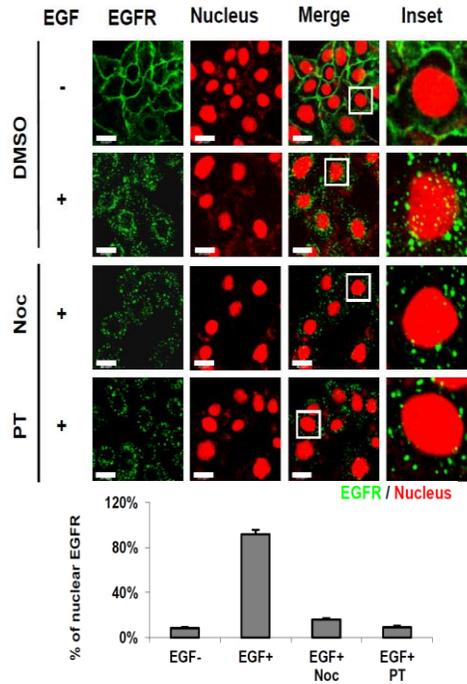


Figure 4-16: Microtubule inhibitors block nuclear localization of EGFR. HeLa cells were treated under the same conditions as in Figure 8-1. The localization of EGFR in nucleus was examined with confocal microscopy. All nuclei were confirmed by TO-PRO-3 staining. Green and red channels: EGFR and nuclei, respectively. Quantitation of positive cells with nEGFR is shown in the lower panel. Scale bars, 20 μ m. Noc, nocodazole; PT, paclitaxel.

4.3.2 Depolymerization of microtubules by CDK1 decreases nuclear translocation of EGFR

In addition to disrupt microtubule formation using chemical inhibitors, we further used a naturally occurred process to depolymerize microtubules by overexpressing CDK1 and its activator, cyclin B (Fourest-Lieuvin et al., 2006), and tested the effects of depolymerized microtubules on nuclear translocation of EGFR. As shown in Figure 4-17, compared with vector control, overexpression of CDK1 decreased the expression of EGFR in nucleus. Similar results were obtained using confocal microscopy assay (Figure 4-18, inset 4 vs. inset 2). Quantitation of cells with

nuclear EGFR in confocal microscopic images is shown in the lower panel of Figure 4-18.

Figure 4-17

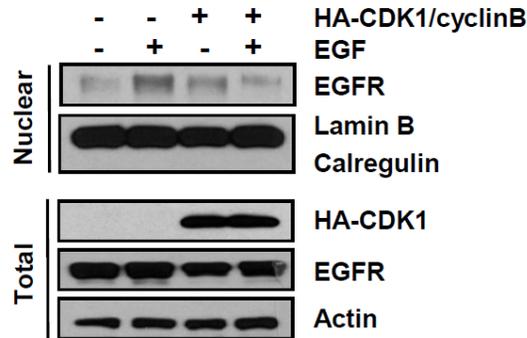


Figure 4-17: CDK1 decreases EGFR expression in nucleus. HeLa cells were transfected with a control vector or HA-CDK1 and cyclin B expression vectors for 48 hr. After that, cells were maintained in serum-free media overnight and then stimulated with EGF. Nuclear fraction was separated using cellular fractionation. Nuclear fraction and total lysates were subjected to immunoblotting with the indicated antibodies.

Figure 4-18

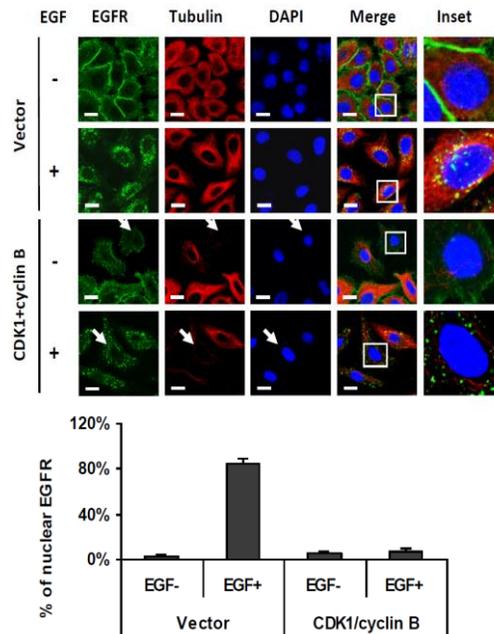


Figure 4-18: CDK1 decreases EGF induced nuclear localization of EGFR. HeLa cells were transfected with a control vector or HA-CDK1 and cyclin B expression vectors for 48 hr. Cells were serum-starved overnight and then stimulated with EGF. The colocalization of EGFR with tubulin was examined with confocal microscopy. All nuclei were confirmed by DAPI staining. Green, red, and blue channels: EGFR, tubulin, and nuclei, respectively. The boxed areas are shown in detail in insets. Quantitation of positive cells with nEGFR is shown in the lower panel. Scale bars, 20 μ m.

4.3.3 Nocodazole reduces nuclear translocation of EGFR in live cells

Finally, we used time-lapse confocal microscopy to examine the effect of microtubule inhibitor nocodazole on the dynamic nuclear translocation of EGFR. EGFR was labeled with GFP and the inner nuclear membrane structure protein Lamin B representing the boundary of nucleus was labeled with RFP. As shown in the upper panel of Figure 4-19 (also in Movies S6 vs. S7), strong trafficking activity of EGFR was observed as indicated by the movement of the green spots (EGFR) from cell surface into cytosol. A few green spots representing nEGFR were detected in nucleus

at the 23-min and 23.5-min after EGF stimulation (Figure 4-19, upper panels). However, in the presence of nocodazole, without microtubules to serve as a trafficking route, the endocytic EGFR stacked on the cell surface membrane, the active trafficking of EGFR disappeared, and no EGFR signal could be detected in the nucleus (Figure 4-19, lower panels). These results suggested that microtubules are required for the nuclear translocation of EGFR and this is likely through the regulation of Golgi translocation.

In summary, reduced nuclear translocation of EGFR by disrupting the formation of microtubules and depolymerizing microtubules suggested that microtubules coordinate the nuclear translocation of EGFR.

Figure 4-19

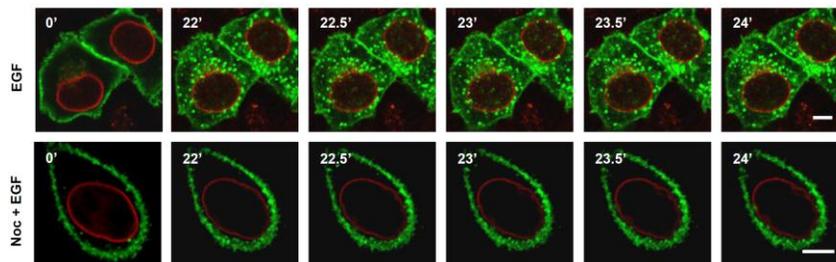


Figure 4-19: Microtubule inhibitor nocodazole blocks the dynamic nuclear translocation of EGFR. Fluorescence-labeled EGFP-EGFR (green channel) and RFP-lamin B (red channel) were transfected into HeLa cells. The movement of EGFR after EGF stimulation was monitored using a live cell time-lapse confocal microscopy. Images were obtained at 30-sec intervals and representative snapshot images at indicated time points were shown. Scale bars, 10 μ m. Noc, nocodazole.

4.4 Dynein coordinates the nuclear translocation of EGFR

4.4.1 Disruption dynein using inhibitors blocks the transport of EGFR to the nucleus

We further asked whether dynein also regulates nuclear trafficking of EGFR. To answer this question, we treated cells with dynein inhibitors, EHNA and vanadate, and found that disruption of dynein ATPase activity decreased EGF-induced nuclear translocation of EGFR according to the Western blot (Figure 4-20) and confocal microscopy analyses (Figure 4-21). Quantitation of cells with nEGFR from the confocal microscopy images is shown in the lower panel of Figure 4-21.

Figure 4-20

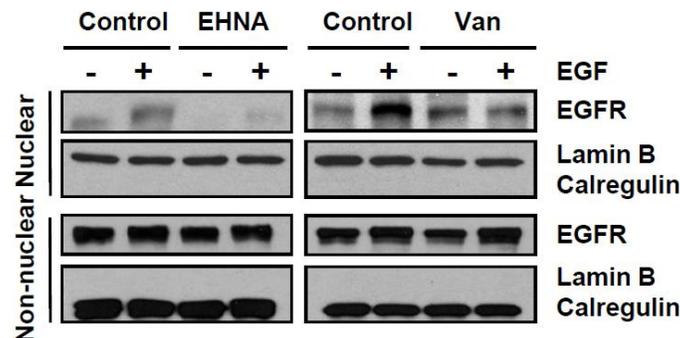


Figure 4-20: Inhibitors of dynein, EHNA and vanadate, decrease the protein level of EGFR in the nucleus. Serum-starved HeLa cells were treated with EHNA or vanadate and then stimulated with EGF (50 ng/ml). Nuclear and nonnuclear fractions were separated using cellular fractionation and subjected to immunoblot analysis with the indicated antibodies.

Figure 4-21

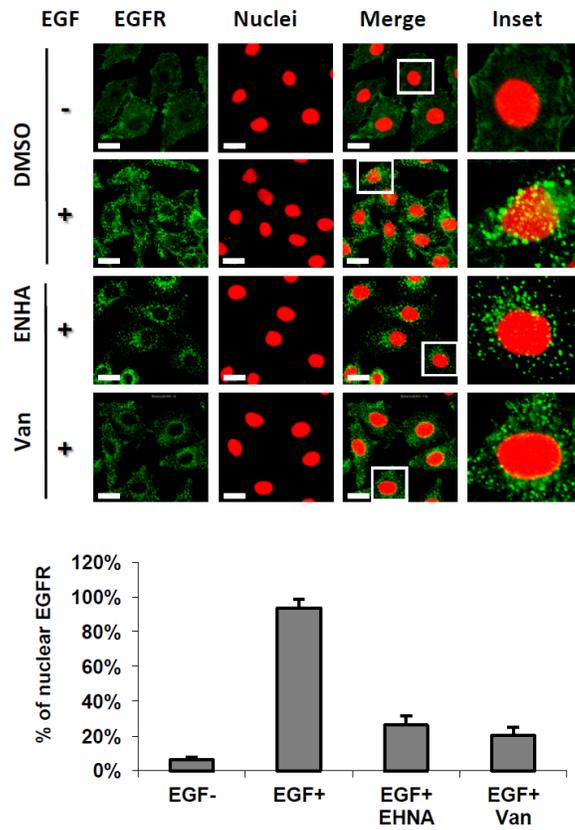


Figure 4-21: Inhibitors of dynein, EHNA and vanadate, decrease the localization of EGFR in the nucleus. HeLa cells were treated as described in Figure 9-1. The localization of EGFR in nucleus was examined with confocal microscopy. All nuclei were confirmed by TO-PRO-3 staining. Green and red channels: EGFR and nuclei, respectively. Quantitation of positive cells with nEGFR is shown in the lower panel. Scale bars, 20 μ m. Van, vanadate.

4.4.2 Depletion of dynein by siRNAs reduces nuclear translocation of EGFR

To rule out the nonspecific effects of these inhibitors, we used siRNAs to knock down the expression of dynein (Figure 4-22, lower panel) and examined the effect of dynein knockdown on the nuclear translocation of EGFR. As shown in the upper panel of Figure 4-22, depletion of dynein expression decreased EGF-induced

nuclear translocation of EGFR. Confocal microscopy assay also showed that knockdown of dynein expression decreased EGF-induced nuclear translocation of EGFR (Figure 4-23). Thus, our data showed that microtubule motor protein dynein is required for EGF-induced nuclear translocation of EGFR.

Figure 4-22

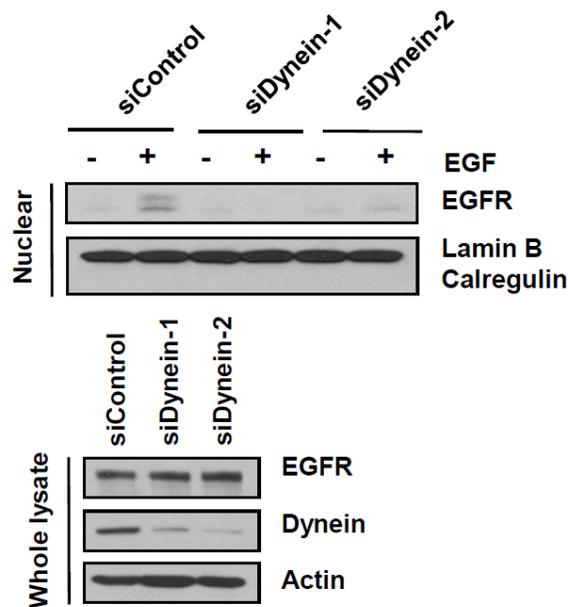


Figure 4-22: Knockdown of dynein decreases the expression of EGFR in nucleus. HeLa cells were transfected with two dynein siRNAs for 48 hr, serum-starved overnight, and then treated with 50 ng/ml EGF for 30 min. Non-nuclear and nuclear fractions were separated using cellular fractionation and subjected to immunoblot analysis with the indicated antibodies. Knockdown of dynein expression is analyzed with immunoblot (lower panel).

Figure 4-23

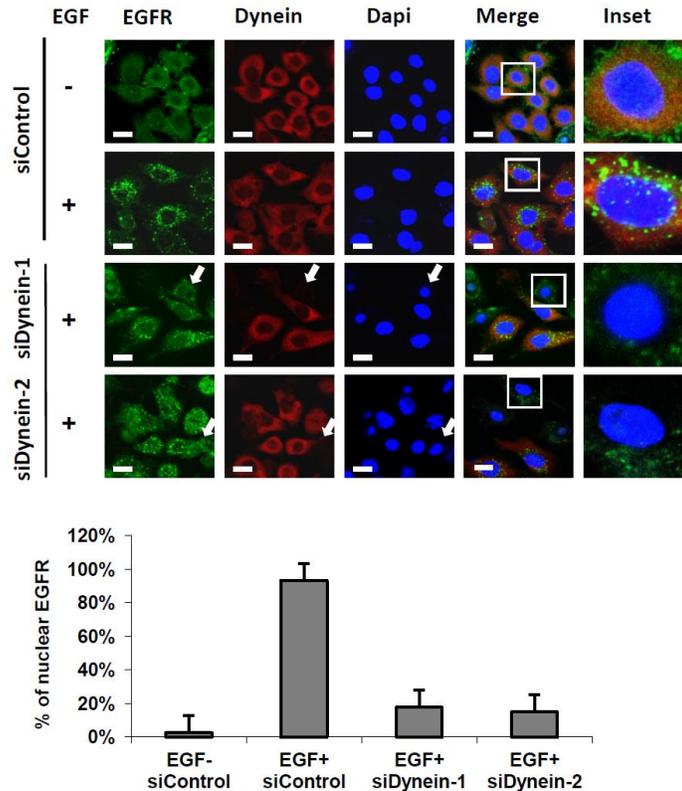


Figure 4-23: Knockdown of dynein inhibits nuclear location of EGFR. HeLa cells were transfected with dynein siRNA and then treated as described in Figure 9-3. The colocalization of EGFR with dynein was examined with confocal microscopy. All nuclei were confirmed by DAPI staining. Green, red, and blue channels: EGFR, dynein, and nuclei, respectively. Quantitation of positive cells with nEGFR is shown in the lower panel. The boxed areas are shown in detail in the insets. Scale bars, 20 μ m.

4.5 Disruption of microtubules and dynein reduces DNA binding ability of nuclear EGFR to the promoter of cyclin D1

If microtubules and dynein are important for the Golgi translocation and the downstream nuclear translocation of EGFR, the inhibition of microtubules and dynein activities should affect the function of nEGFR.

Chapter Five

**Syntaxin 6 and microtubule cytoskeleton
regulate the Golgi translocation of cholera toxin,
c-Met, and ErbB2**

5.1 Syntaxin 6 and microtubule cytoskeleton regulate the Golgi translocation of cholera toxin

5.1.1 Disruption of microtubule and dynein decrease Golgi translocation of cholera toxin

The data we presented above revealed a novel trafficking pathway regulating the Golgi translocation of cellular protein, EGFR, via retrograde transport from the cell surface. Foreign protein like cholera toxin has been reported to translocate to the Golgi apparatus through the retrograde trafficking to activate its toxicity; however, the mechanism is not well understood. Therefore, we asked whether microtubulin/dynein/syntaxin 6 axis identified in our study is also used by cholera toxin.

We first confirmed the localization of cholera toxin at the Golgi apparatus using confocal microscopy analysis (Figure 5-1, yellow spots in inset 2). Quantitation of cells with cholera toxin localized at Golgi is shown in the lower panel of Figure 5-1. When microtubules and dynein were disrupted with inhibitors, EHNA and nocodazole, colocalization of cholera toxin with the marker of Golgi apparatus was significantly decreased (Figure 5-1, insets 3 and 4).

We further isolated the Golgi-enriched fraction and examined the effects of disruption of microtubules and dynein on the localization of cholera toxin at the Golgi apparatus. As shown in Figure 5-2, the level of cholera toxin in Golgi-enriched fraction decreased when cells were pretreated with inhibitors of microtubules and dynein.

Figure 5-1

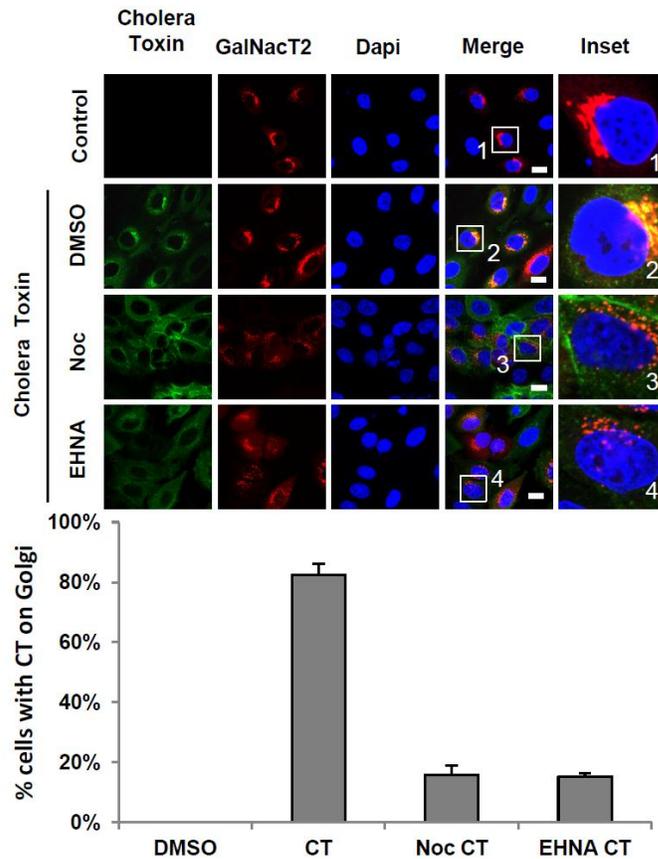


Figure 5-1: Inhibitors of microtubules and dynein reduce accumulation of cholera toxin at the Golgi apparatus. HeLa cells transfected with GalNac T2 expression plasmid for 48 hr were pretreated with control DMSO or indicated inhibitors for 30 min and then treated with RFP fused cholera toxin for 1 hr. The colocalization of cholera with GalNac T2 was examined with confocal microscopy. All nuclei were confirmed by DAPI staining. Green, red, and blue channels: cholera toxin (CT), GalNac T2, and nuclei, respectively. Scale bars, 20 μ m. Noc, nocodazole.

Figure 5-2

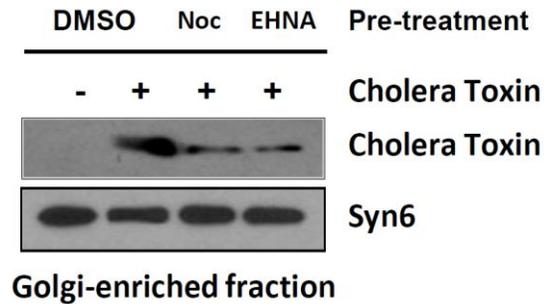


Figure 5-2: Inhibitors of microtubules and dynein reduce cholera toxin at the Golgi enriched fraction. HeLa cells transfected with GalNac T2 expression plasmid for 48 hr were pretreated with control DMSO or indicated inhibitors for 30 min and then treated with cholera toxin for 1 hr. The presence of cholera toxin at Golgi enriched fraction was examined with Western blot assay. Noc, nocodazole.

5.1.2 Depletion of syntaxin 6 by siRNAs blocks Golgi translocation of cholera toxin

Next we knocked down the expression of syntaxin 6 and examined its effect on the Golgi trafficking of cholera toxin. As shown in Figure 5-3, cholera toxin colocalized with the Golgi marker, GalNac T2, in cells transfected with control siRNA (Figure 5-3, yellow color in inset 1). When syntaxin 6 was knocked down, colocalization of cholera toxin with GalNac T2 was significantly reduced (Figure 5-3, insets 2 and 3). Quantitation of cells with cholera toxin at the Golgi apparatus is shown in the right panel of Figure 5-3. We also observed similar results when we used the dominant negative mutant (CCD domain) of syntaxin 6 to inhibit the function of syntaxin 6. Compared with vector control, the CCD domain of syntaxin 6 significantly decreased the colocalization of cholera toxin with GalNac T2 (Figure 5-4, insets 1 and 2), indicating that syntaxin 6 is required for the Golgi accumulation of cholera toxin.

Collectively, these results further demonstrated that not only do syntaxin 6 and microtubules regulate intracellular trafficking of EGFR to Golgi and contribute to downstream nuclear transport but also mediate Golgi translocation of foreign molecule such as the cholera toxin. This pathway which regulates the Golgi translocation through microtubule-dependent movement and syntaxin 6-mediated membrane fusion likely serves as a general model for intracellular trafficking of cellular or foreign molecules.

Figure 5-3

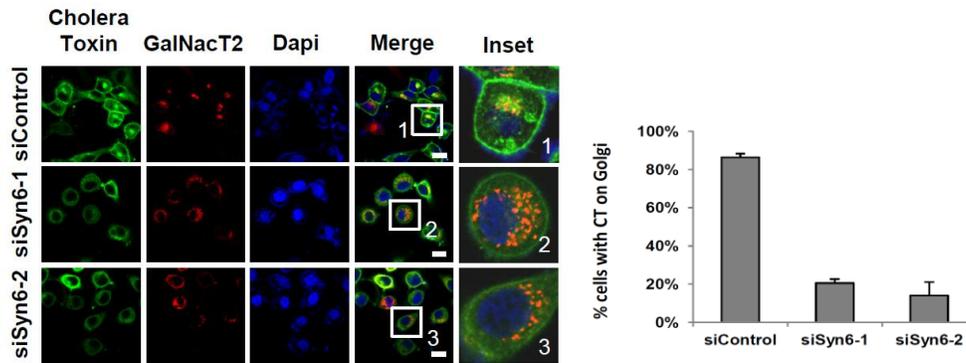


Figure 5-3: Depletion of syntaxin 6 by siRNAs reduces the localization of cholera toxin at the Golgi apparatus. HeLa cells were transfected with siRNAs of syntaxin 6 for 24 hr then transfected with GalNac T2 expression plasmid for 48 hr. Transfected cells were then treated with RFP fused cholera toxin for 1 hr. The colocalization of cholera toxin with GalNac T2 was examined with confocal microscopy. All nuclei were confirmed by DAPI staining. Green, red, and blue channels: cholera toxin (CT), GalNac T2, and nuclei, respectively. Scale bars, 20 μ m.

Figure 5-4

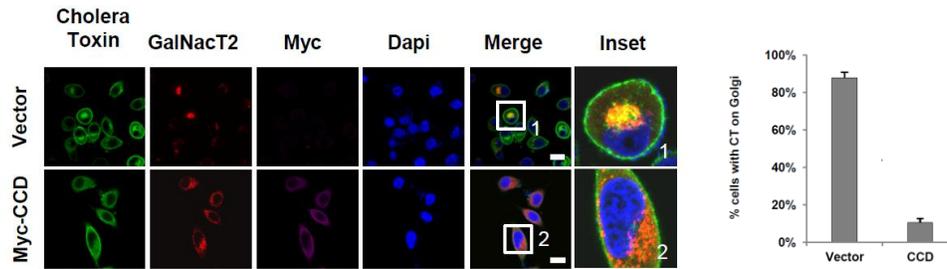


Figure 5-4: Downregulation of syntaxin 6 by CCD domain reduces the localization of cholera toxin at the Golgi apparatus. HeLa cells were co-transfected with CCD domain of syntaxin 6 and GalNac T2 expression plasmid for 48 hr. Transfected cells were then treated with RFP fused cholera toxin for 1 hr. The colocalization of cholera toxin with GalNac T2 was examined with confocal microscopy. All nuclei were confirmed by DAPI staining. Green, red, and blue channels: cholera toxin (CT), GalNac T2, and nuclei, respectively. Scale bars, 20 μ m.

5.2 Depletion of syntaxin 6 and disruption of microtubule-dependent movement decrease Golgi translocation of c-Met

C-Met has been reported to transport to the Golgi apparatus in response to HGF stimulation. However its mechanism is not clear. Therefore, we tested whether c-Met utilizes the same trafficking pathway identified in our study for its Golgi translocation.

We first knocked down the expression of syntaxin 6 in HeLa cells using siRNAs of syntaxin 6 and tested its effect on the localization of c-Met at the Golgi apparatus. As shown in Figure 5-5, we detected the colocalization of c-Met with the Golgi marker, GalNac T2, upon HGF stimulation. When syntaxin 6 expression was knocked down by siRNA, the colocalization of c-Met with GalNac T2 was decreased

which implied that syntaxin 6 regulated membrane fusion is involved in the Golgi translocation of c-Met.

We then examined the function of microtubules and its motor protein dynein during the Golgi translocation of c-Met. We disrupted the function of microtubules and dynein with inhibitors, nocodazole and EHNA, and found this treatment reduced the colocalization of c-Met with the Golgi apparatus upon HGF stimulation (Figure 5-6). These results indicated that microtubules and dynein are required for the Golgi translocation of c-Met.

Figure 5-5

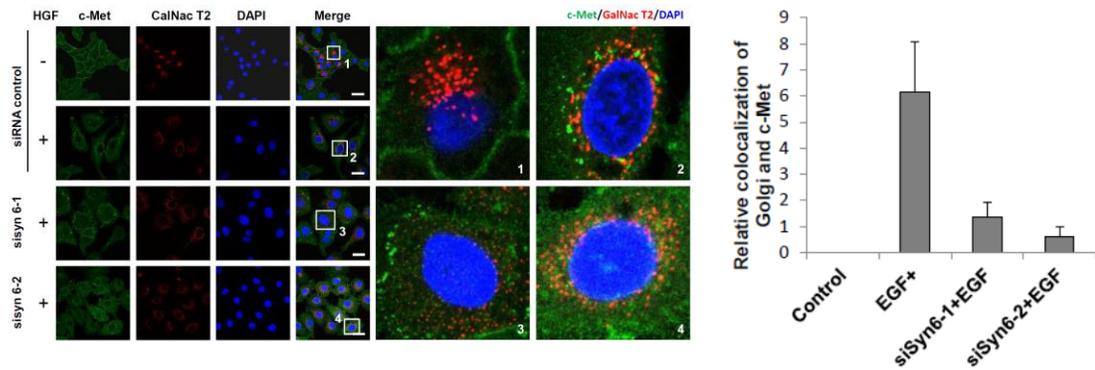


Figure 5-5: Knockdown of syntaxin 6 reduces the Golgi localization of c-Met. Cells were transfected with siRNAs of syntaxin 6 or control siRNA. After 24 hr transfection, HeLa cells were transfected with GalNac T2 expression plasmid for 24 hr. Cells were then serum-starved overnight and then treated with HGF (100 ng/ml) for 60 min. The colocalization of c-Met with the Golgi marker GalNac T2 was examined using confocal microscopy assay. All nuclei were confirmed by DAPI staining. Green, red, and blue channels: EGFR, GalNac T2, and nuclei, respectively. Scale bars, 20 μ m. The boxed areas are shown in detail in the insets.

Figure 5-6

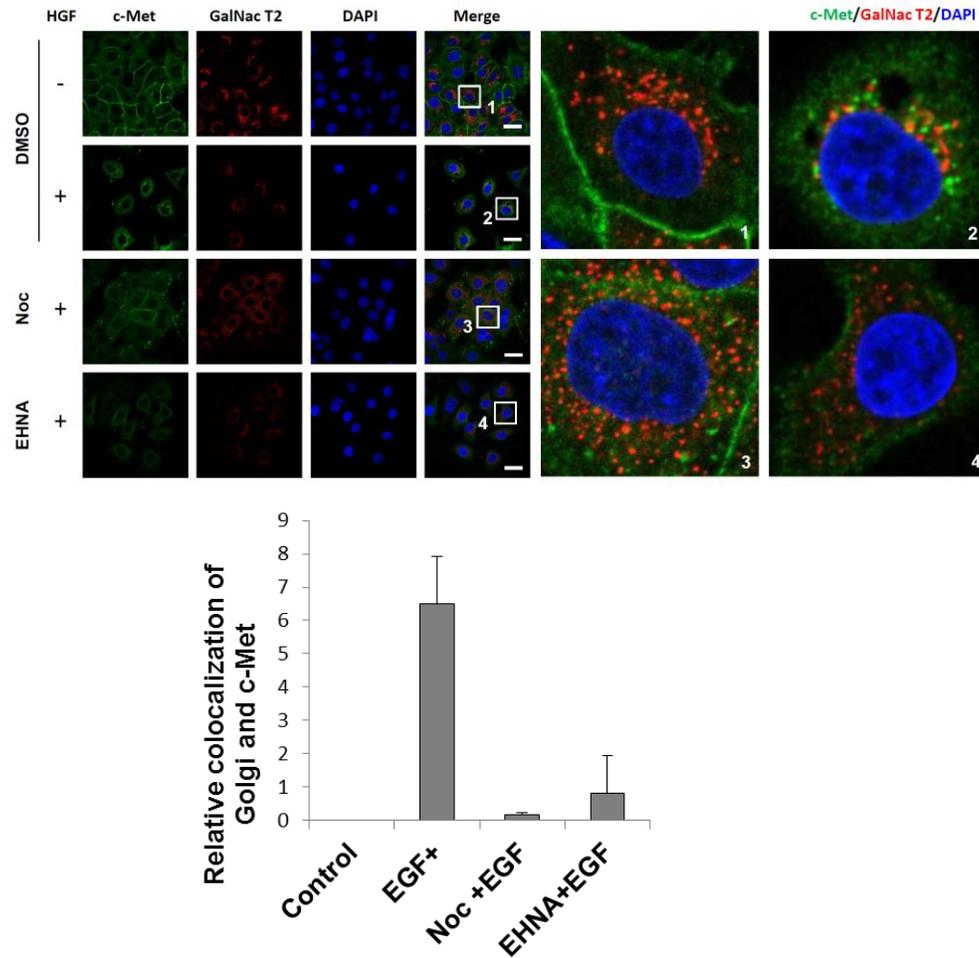


Figure 5-6: Inhibitors of microtubules and dynein block the colocalization of c-Met with the Golgi apparatus. HeLa cells transfected with GalNac T2 were treated with control DMSO or indicated inhibitors for 30 min. The colocalization of c-Met with the Golgi marker GalNac T2 was examined using confocal microscopy assay. All nuclei were confirmed by DAPI staining. Green, red, and blue channels: c-Met, GalNac T2, and nuclei, respectively. Scale bars, 20 μ m. The boxed areas are shown in detail in the insets.

5.3 Disruption of microtubule and dynein decreases nuclear translocation of ErbB2

We also depleted dynein and syntaxin 6 with specific siRNAs and then analyzed the nuclear translocation of ErbB2 using Western blotting. Similar to the

results of EGFR, nuclear translocation of ErbB2 was decreased when microtubules (Figure 5-7), dynein (Figures 5-7 and 5-8), and syntaxin 6 (Figure 5-9) were disrupted, demonstrating that nuclear translocation of ErbB2 occurs through regulated trafficking from the cell surface to the Golgi apparatus. This process is also mediated by syntaxin 6, microtubules, and dynein.

In summary, trafficking of cell surface receptors, c-Met and ErbB2, utilize similar pathway to transport either to the Golgi apparatus (c-Met) or the nucleus (ErbB2).

Figure 5-7

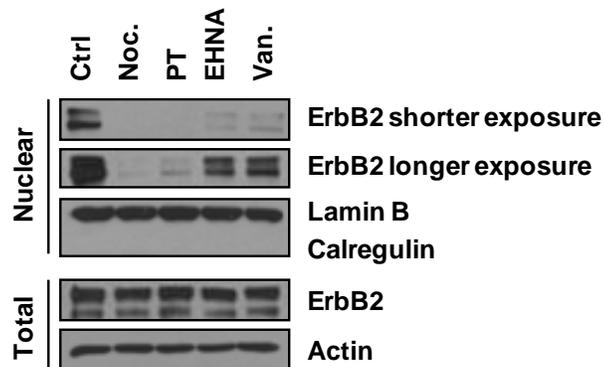


Figure 5-7: Inhibition of microtubules and dynein decreases nuclear translocation of ErbB2. SKBr3 cells were treated with microtubule or dynein inhibitors for 1 hr. Nuclear fraction was separated using cellular fractionation and then subjected to immunoblotting with the indicated antibodies. Noc., nocodazole; PT., paclitaxel; Van., vanadate.

Figure 5-8

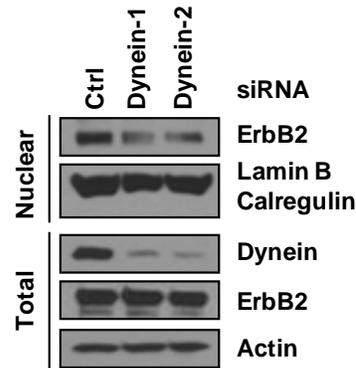


Figure 5-8: Knockdown of dynein decreases the nuclear translocation of ErbB2. SKBr3 cells were transfected with dynein siRNAs or control siRNA. Nuclear fractions were separated using cellular fractionation. The presence of ErbB2 in nuclear fractions and total lysis were analyzed using immunoblotting assay with indicated antibodies. Lower panel: knockdown of dynein.

Figure 5-9

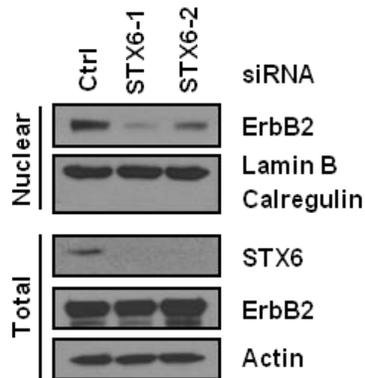


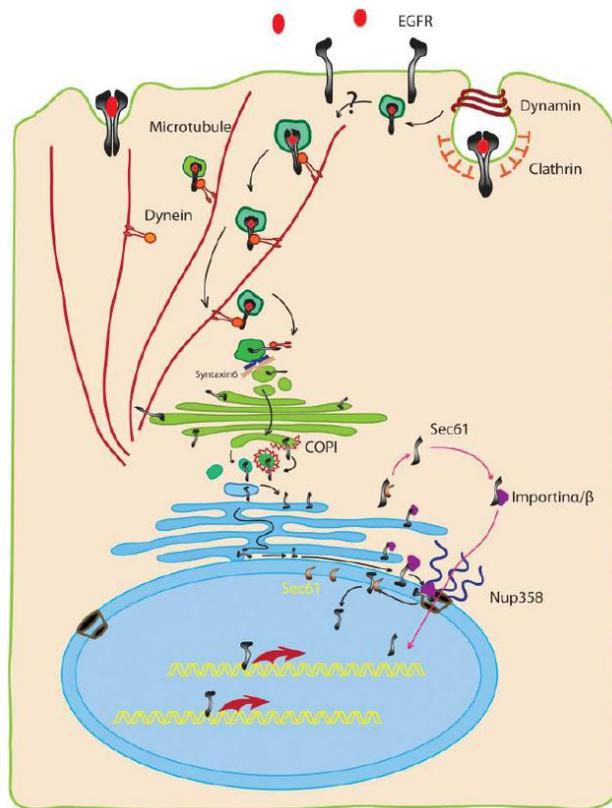
Figure 5-9: Knockdown of syntaxin 6 decreases the nuclear translocation of ErbB2. SKBr3 cells were transfected with syntaxin 6 siRNAs or control siRNA. Nuclear fractions were separated using cellular fractionation. The presence of ErbB2 in nuclear fractions and total lysis were analyzed using immunoblotting assay with indicated antibodies. Lower panel: knockdown of syntaxin 6.

Chapter Six
**Summary, discussion, significance, and future
directions**

6.1 Summary

A schematic representation of EGFR trafficking to the Golgi and nucleus proposed in our study was shown in Figure 6-1. This model is supported by the results described here.

Figure 6-1: Study model.



First, we showed that EGF induced EGFR to transport into the Golgi apparatus supported by the colocalization of EGFR with different Golgi markers using confocal microscopy assay. This notion is further supported by biochemical method in which the EGFR level in the Golgi-enriched fraction was enhanced by

EGF stimulation. Inhibition of protein synthesis does not change the colocalization pattern of EGFR with the Golgi marker upon EGF stimulation indicating that EGFR on the Golgi induced by EGF comes from the cell surface. Most importantly, the dynamic translocation of EGFR to the Golgi was observed by a living cell imaging system.

Second, we found syntaxin 6, microtubules, and dynein are required for the Golgi transport of EGFR. It is supported by the blocking of EGFR translocation from cell surface into the Golgi apparatus due to downregulation of syntaxin 6 and inhibition of microtubule and motor protein dynein. We also showed EGF induces the association of EGFR with syntaxin 6, tubulin, and motor protein dynein. Our data suggest that dynein may link EGFR to microtubules and facilitate microtubule-dependent movement as the power provider for intracellular trafficking of EGFR. Based on these results, we proposed that EGFR likely travels along the microtubules to reach Golgi and interacts with syntaxin 6, which then facilitates membrane fusion between the Golgi and the endocytic vesicle that carries EGFR.

Third, we demonstrated that syntaxin 6, microtubule, and dynein are required for the nuclear trafficking of EGFR via mediation of trafficking from the cell surface to the Golgi apparatus. This is supported by the decrease of EGFR nuclear translocation due to downregulation of syntaxin 6 either by siRNAs knockdown or dominant negative mutant of syntaxin 6 and disruption of microtubules and dynein by inhibitors and siRNAs.

More importantly, we demonstrated that syntaxin 6 and microtubule-dependent movement of EGFR are critical for the transcriptional activity of nEGFR.

This is supported by the decreased association of nuclear EGFR with the promoter of cyclin D1 and the decreased transcriptional activity of nEGFR due to knockdown of syntaxin 6 and inhibition of microtubules and dynein. These results provide another layer of support for the importance of microtubule- and syntaxin 6-mediated Golgi translocation in EGF-induced nuclear translocation of EGFR.

In addition, we showed that syntaxin 6 and microtubules not only regulate intracellular trafficking also mediate the Golgi translocation of foreign molecules, such as the cholera toxin, and other cell surface molecules, such as c-Met, as well as the nuclear translocation of ErbB2. This is supported by the decreased Golgi translocation of cholera toxin and c-Met and nuclear translocation of ErbB2 by syntaxin 6 knockdown and inhibition of microtubules and dynein.

In summary, this pathway which regulates the Golgi translocation through microtubule-dependent movement and syntaxin 6-mediated membrane fusion likely serves as a general model for intracellular trafficking of cellular or foreign molecules.

6.2 Discussion

Cellular distribution of cell surface receptors is considered to be more complicated than degradation and recycling. After receptor-regulated endocytosis, RTKs continue to activate downstream signals (Lemmon and Schlessinger, 2010; Sadowski et al., 2009). Recent studies have reported that RTKs, such as EGFR, FGFR1, and VEGFR1, transport to the Golgi, mitochondrial, and nucleus, (Demory et al., 2009; Hitosugi et al., 2011; Mittar et al., 2009; Wang et al., 2010b), implying that

cell surface receptors may have non-canonical function at different cellular compartments.

Since the discovery of nuclear translocation of EGFR more than 20 years ago (Kamio et al., 1990; Raper et al., 1987), many studies have identified important biological functions of nuclear EGFR, including cell proliferation, DNA synthesis, DNA repair, radiation response, and drug resistance (Dittmann et al., 2010; Huang et al., 2011; Li et al., 2009; Li et al., 2010; Liccardi et al., 2011; Lo and Hung, 2006; Wang and Hung, 2009). However, the field of nuclear RTKs has progressed slowly partly due to a lack of clear trafficking mechanism of cell surface receptors to the nucleus. Although the partial mechanism of nuclear translocation of EGFR has been demonstrated, including endocytosis (Bryant et al., 2005; Giri et al., 2005; Lo et al., 2006), nuclear localization signals (NLS), importin α 1/ β 1 (Giri et al., 2005; Hsu and Hung, 2007; Lin et al., 2001; Lo et al., 2006; Offterdinger et al., 2002; Reilly and Maher, 2001; Srinivasan et al., 2000), COPI-mediated retrograde trafficking from the Golgi to the ER (Wang et al., 2010a), translocon, and Sec61-regulated release of EGFR from the ER membrane (Liao and Carpenter, 2007; Wang et al., 2010c), experimental data demonstrating the Golgi translocation of EGFR is still absent. Our present findings fill the gap and indicate how EGFR is transported to the Golgi/ER after endocytosis and then into the nucleus.

Our results suggest that SNARE protein, syntaxin 6, regulates EGFR translocation to the Golgi and intracellular trafficking of EGFR is not a random process. Rather, EGFR moves along MTs powered by the motor protein, dynein. In addition, we also showed that cholera toxin uses similar trafficking pathway for its

pathological function. Several unique observations led to these conclusions: 1) Cellular protein like EGFR is transported to the Golgi in response to EGF stimulation and this process requires syntaxin 6, indicating that membrane fusion may be a critical trafficking event for the Golgi translocation. EGFR remains membrane-bound during the Golgi trafficking. 2) MTs and dynein are both required for the translocation of EGFR to the Golgi. MTs have been reported to function in endosomal trafficking to the lysosome and membrane trafficking between the Golgi and ER (Caviston and Holzbaur, 2006). Our study further shows that the intracellular trafficking of cell surface receptors from endosome to the Golgi also requires MTs, indicating that MTs may serve for most intracellular trafficking; and 3) MT and dynein inhibitors blocked nuclear translocation of EGFR and decreased transcriptional activity of nEGFR, indicating that the Golgi translocation of EGFR is a critical trafficking step for downstream trafficking of EGFR, nuclear translocation. Together with the previous studies (Liao and Carpenter, 2007; Wang et al., 2010a; Wang et al., 2010c), our study reveals a model showing schematic representation of the Golgi translocation of EGFR. EGFR, carried by endocytic vesicles along the MT cytoskeleton, is transported to the Golgi by syntaxin 6-mediated membrane fusion. In conjunction with the previous studies showing both transport of EGFR from the Golgi to the ER via COPI-regulated retrograde trafficking and translocon Sec61-mediated trafficking of EGFR released from ER or the inner nuclear membrane, our study provides a clear mechanism outlining the Golgi and nuclear translocation of EGFR.

This newly identified mechanism not only contributes to nuclear translocation of cell surface receptors but also reveals a potential trafficking route of foreign

proteins like the cholera toxin. Protein toxins enter the cell through retrograde trafficking pathway to the Golgi and ER and then released into the cytoplasm by Sec61-regulated ER-associated degradation (ERAD). However, instead of being degraded, they are activated to increase cAMP activation, which in turn causes the dehydration of target cells. Although retrograde transport is partially involved, the complete mechanism is unknown. The proposed mechanism provides a comprehensive trafficking pathway showing that MTs, dynein, and syntaxin 6 regulate the trafficking of cholera toxin into the Golgi and ER. Current treatment for cholera is primarily oral rehydration salts. Although antibiotic can also be used to kill the bacteria, it takes time for the effect to take place and is not effective in stopping dehydration as the effect of the toxin lingers on even after all the bacteria are killed. If the mechanism we have provided can be used to decrease the toxin activity in target cells, disruption of Golgi trafficking of cholera toxin may be a potential treatment by quickly reducing dehydration in target cells.

6.3 Significance

Combined with previous studies, our works provide a systemic model to explain how cell surface receptors containing the transmembrane domain translocate into the nucleus. Our study is unique and significant for the following reasons.

First, it provides a new concept that membrane fusion mediated by SNARE protein may be involved in the nuclear translocation of RTKs. Previous studies of nuclear transport of cell surface receptors focused on how these molecules are released from membrane components (Massie and Mills, 2006). However, in our

model, we opened another perspective to the mechanism of nuclear transport of RTKs and even other cell surface receptors. By this general mechanism, these membrane proteins are anchored on the membrane following retrograde trafficking pathway and membrane flux to reach the nucleus. Our findings fill the gap of how the EGFR is transported to the ER after endocytosis.

Second, it is the first time to reveal the correlation between cytoskeleton and nuclear translocation of EGFR by demonstrating that microtubule cytoskeleton provides a directional movement pathway for the nuclear translocation of EGFR.

Third, we showed that other cell surface receptor, such as ErbB2, also uses the same mechanism to translocate into the nucleus (Figures 12-3, 12-4, and 12-5). It suggests that the model proposed in our study is a general mechanism through which RTKs and other cell surface receptors translocate from cell surface into the nucleus to carry out their nuclear functions.

Finally, our study opens a new avenue to understand the trafficking mechanism for not only EGFR but also other cell surface molecules which have similar trafficking events. Since non-canonical trafficking of cell surface receptors are usually correlates with tumorigenesis, understanding their trafficking mechanisms may provide potential clinical implication for current therapy of cancer patients.

6.4 Future Directions

While the Golgi trafficking model depicted in our study is attractive, a few questions still need to be further investigated. For example, it is not known whether EGFRs have specific functions at the Golgi apparatus in addition to docking at the

Golgi for further intracellular trafficking. It has been reported that other SNARE proteins, e.g., vSNARE, may cooperate with syntaxin 6 to regulate membrane fusion (Jahn and Scheller, 2006). For example, VAMP3 and VAMP4 have been reported to cooperate with syntaxin 6 to regulate membrane fusion between vesicles and the Golgi (Zeng et al., 2003; Zwilling et al., 2007). Therefore, it is worth investigating whether vSNARE is required for the Golgi translocation of EGFR. Another group of molecules, small GTPase Rabs, are involved in retrograde trafficking pathway (Johannes and Popoff, 2008). It would also be interesting to investigate which Rab protein regulates the transport of EGFR to the Golgi after endocytosis. It is worthwhile to mention that EGF induces EGFR degradation through receptor-mediated endocytosis and endosomal trafficking to the lysosomes. How EGFR bypasses this degradation pathway or whether different populations of EGFR have different fates, for example either into lysosome or other cellular compartments such as mitochondria (Boerner et al., 2004) or the nucleus, remains to be investigated. More importantly, we will address whether blocking of nuclear trafficking of EGFR affects the sensitivities of cancer cells to tyrosine kinase inhibitors or radiotherapy.

In summary, the following questions need to be addressed in our future work.

1. How is the different cellular compartment trafficking of EGFR decided upon EGF stimulation? What molecules control the fate of intracellular trafficking of EGFR?

EGF induced the endosomal trafficking of EGFR for degradation in the lysosome in order to terminate EGFR signal transduction (Waterman et al., 1998). However, in

our study, we found that EGF also induced the Golgi and nuclear translocation. Thus we will investigate the switch which controls the different trafficking events of EGFR upon EGF stimulation.

2. What is the specific function of EGFR on the Golgi apparatus?

In our study, EGFR translocated into the Golgi and linked with the ER translocation for the final destination of nuclear location. Intracellular trafficking of proteins is usually related to some specific functions. For example, the lysosomal trafficking of EGFR is for degradation (Yarden and Shilo, 2007). Nuclear trafficking of EGFR is related to the transcriptional regulation, DNA repair, and DNA replication (Wang et al., 2010b). Recent study showed that EGFR transport to the mitochondria to regulate mitochondrial function through phosphorylation of COXII (Demory et al., 2009) or is correlated with cell survival (Yue et al., 2008).

However, the functions of Golgi translocated EGFR and even other cell surface proteins are not clear so far. When the Golgi translocation of transferrin receptor was observed 30 years ago, its function was proposed to repair the damaged glycolysation of receptor (Snider and Rogers, 1985). However, there was no experimental evidence to support this hypothesis. Recently some results showed that Golgi localized c-Met is still active (Kermorgant and Parker, 2008) indicating that Golgi localized cellular protein may have some specific functions and the Golgi apparatus is not just the place for glycolysation of newly synthesized proteins. Using unbiased assay, Dr. Lynda Chin's team reported a critical function of one Golgi associated protein in tumorigenesis (Scott and Chin, 2010). Their studies demonstrated that Golgi may not

only be the place to modify proteins, but also has physiological and pathological function through regulation of signaling cascades. Our data demonstrated that EGF-induced Golgi translocation of EGFR is a critical trafficking step for the nuclear translocation of EGFR and contributes to the transcriptional activity of nuclear EGFR. In our future work we will investigate whether Golgi-localized EGFR has other specific functions.

3. What are the functions of other SNARE proteins paired with syntaxin 6 in regulating the Golgi and nuclear translocation of EGFR?

Target SNARE usually pairs with vesicle SNARE and other two target SNAREs to play a full function in membrane fusion (Johannes and Popoff, 2008). Thus the syntaxin 6 as a target SNARE may work with other SNARE protein to regulate the Golgi translocation of EGFR. The identification of other SNARE proteins may provide fully understanding for the membrane fusion event which regulates the Golgi translocation of EGFR.

4. Are there any other type of proteins regulating the Golgi and nuclear translocation of EGFR? Which Rab protein plays a role during these traffickings?

It is known that other molecules including adaptor proteins, membrane coated proteins, tethering factors, and docking factors are required for the retrograde trafficking pathway (Johannes and Popoff, 2008). These proteins cooperate with each other to mediate membrane fusion events. Further identifying other regulators

required for the Golgi translocation of EGFR will provide more potential clinical applications for diseases caused by nuclear translocation of EGFR.

5. What is the mechanism of ligand-independent nuclear trafficking of EGFR?

Our studies identified the intracellular trafficking pathway of EGFR upon EGF stimulation from cell surface to the Golgi apparatus and demonstrated that this pathway is critical for downstream nuclear transport and its transcriptional function. However, as we know that nuclear localization of EGFR had been detected under different stress exposure including oxygen stress, DNA damage reagents, and radiation treatment (Dittmann et al., 2010; Li et al., 2010). Whether intracellular trafficking pathway identified in our study is also used for ligand-independent trafficking of EGFR is an interesting question. We will also investigate whether there are other mechanisms to regulate ligand-independent nuclear transport of EGFR.

6. Whether blocking nuclear trafficking of EGFR can increase the sensitivity of cancer cells to TKIs, Cetuximab, or radiotherapy?

Recent study demonstrated that nuclear EGFR contributes to the resistance of cancer cells to tyrosine kinase inhibitor through turning on the expression of BCRP gene (Huang et al., 2011), which may play a major role in multi-drug resistance. In addition, it has been reported that acquired-resistance of lung cancer cell lines to Cetuximab is caused by the nuclear EGFR (Brand et al., 2011). Furthermore, radiation treatment is reported to be another stress to induce nuclear translocation of EGFR to enhancing DNA repair (Dittmann et al., 2010). These results suggest that blocking nuclear translocation of EGFR may result in synergistic effect when

combined with chemotherapies or radiation therapies. Therefore, we will investigate the effects of blocking EGFR nuclear trafficking on the sensitivities of cancer cells to TKIs, Cetuximab, or radiotherapy in our future work.

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