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NUCLEAR TRANSLOCATION OF MET VIA INTERNET MECHANISM

MEI-KUANG CHEN

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By

Mei-Kuang Chen M.S.

APPROVED

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Supervisory Professor: Mien-Chie Hung Ph.D.

_____

Paul J. Chiao Ph.D.

_____

Min Gyu Lee Ph.D.

_____

Zhen Fan M.D.

_____

Jeffrey T. Chang Ph.D.

APPROVED

_____

Dean, The University of Texas

Graduate School of Biomedical Sciences at Houston

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A

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Mei-Kuang, Chen M.S.

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Dedication

To

my dearest parents,

Family

And fiancé

Acknowledgements

Here, I want to express my appreciation to all my friends studying or working in GSBS and MD Anderson Cancer Center. With their friendship, I survive and adapt myself in Houston without great obstacles. Their friendship help me go through all frustration and also delight my days.

Finally, I want to thank my family for endless love and support. My parents and all family members provide me a confidence and all the love I need to go through everything in my life. And I also thank my fiancé and his family for their support so that I can focus on my study.

NUCLEAR TRANSLOCATION OF MET VIA INTERNET MECHANISM

Abstract

Mei-Kuang Chen, M.S.

Advisory Professor: Mien-Chie Hung, Ph.D.

MET is one of the receptor tyrosine kinases (RTKs) that are overexpressed in malignant cancer types, including breast cancer. While RTKs are traditionally known for their roles in signaling transduction from the cell surface, recent studies have provided evidence demonstrating that most of RTKs can translocate into nucleus to regulate cellular processes in response to both ligand and stress stimulation. Oxidative stress is a common stress in cancer cells due to alteration of metabolism, and constitutive oxidative stress related to reactive oxygen species (ROS) has been observed in breast cancer cells. Here, we show that hepatocyte growth factor (HGF) as well as hydrogen peroxide (H_2O_2) can induce nuclear translocation of full-length MET holoreceptor via a membrane-bound vesicle transport mechanism in breast cancer cells. Our findings provide a putative mechanism by which breast cancer cells adapt to oxidative stress.

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Chapter 1 Introduction

1.1 Receptor Tyrosine Kinase and Cell Signaling

Receptor tyrosine kinases (RTKs) are cell surface transmembrane receptors for growth

factors, cytokines and hormones.　The first RTK was discovered in 1960s, and so far there

are 58 known RTKs in human and are categorized into 20 subfamilies (Figure. 1) (Gschwind

et al., 2004; Lemmon and Schlessinger, 2010; Robinson et al., 2000).

Receptor Tyrosine Kinase Subfamilies
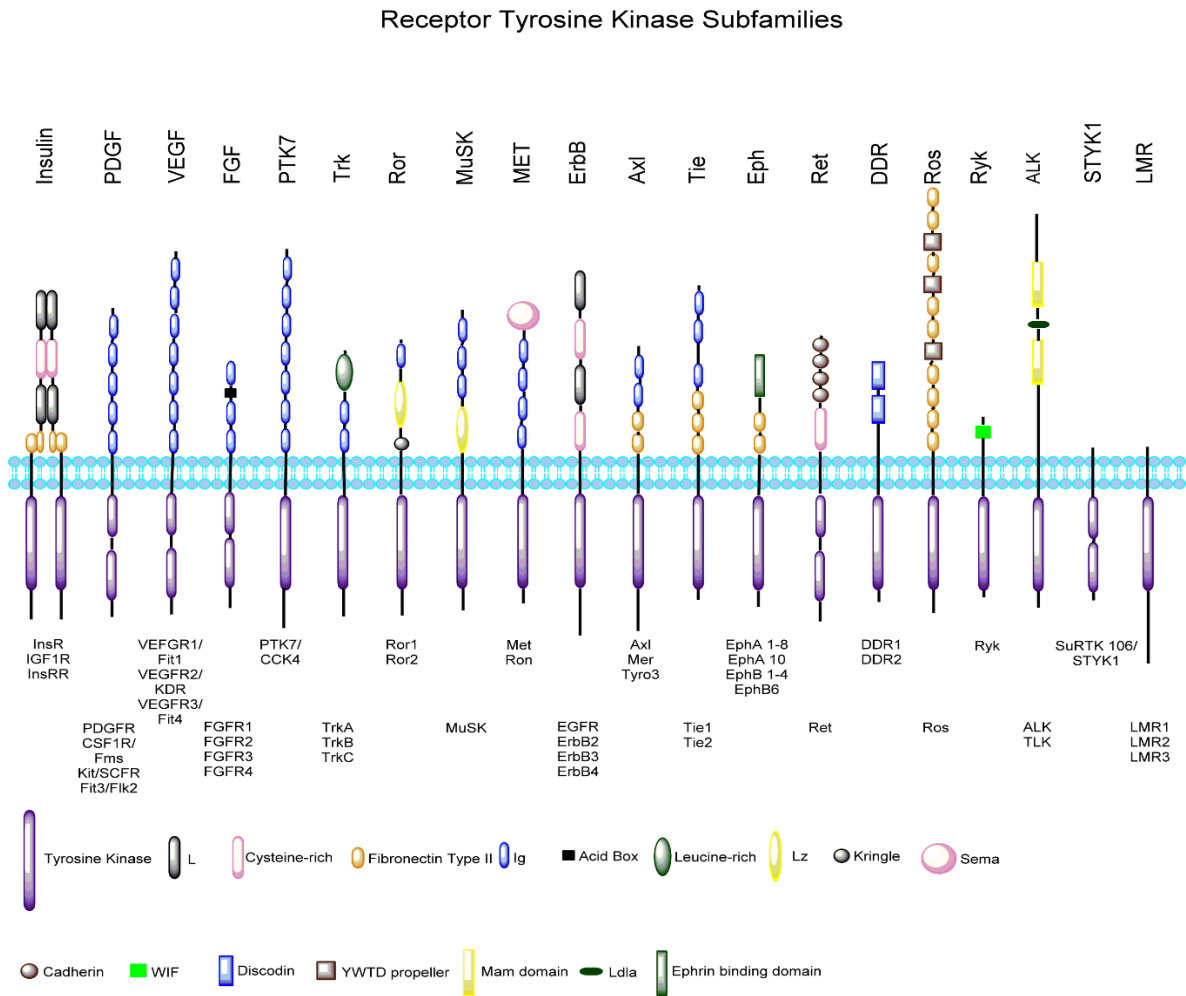
Figure 1. Receptor Tyrosine Kinases Subfamilies.　The human receptor tyrosine
kinases contain 20 subfamilies as listed on the top of the figure.

1.1.1 Ligand-induced RTK activation

These RTKs have various ligand-binding domains in extracellular region, a single transmembrane domain, cytoplasmic domain and carboxy-terminal domain (C-term) next to the juxtamembrane regions (Lemmon and Schlessinger, 2010). Generally, most of the activation of RTKs begins with ligand induced receptor oligomerization (Ullrich and Schlessinger, 1990). After stimulation, the receptors were *trans* autophosphorylated (Favelyukis et al., 2001; Furdui et al., 2006; Honegger et al., 1989) and can dramatically increase its own kinase activity (Cobb et al., 1989; Furdui et al., 2006). The main function of RTKs is catalyzing the transfer of γ-phosphate from adenosine-5'-triphosphate (ATP) to the target proteins on hydroxyl group of its tyrosine (Y) residues. The downstream adaptor proteins commonly have a plasma membrane targeting site at N-terminal as well as Src homology 2 (SH2) or phosphotyrosine-binding domain (PTB) domain that recognizes phosphotyrosine on RTKs (Krauss, 2014; Lemmon and Schlessinger, 2010). With the activated C-terminal domain, the RTK recruits adaptor proteins close to plasma membrane and induces signaling cascade through phosphorylating and activating the downstream proteins (Figure 2). The type of downstream proteins recruited determines the signaling pathway activated by RTKs (Riedel et al., 1989). Some RTKs are considered oncogenic and

also considered as therapeutic target because their high mutation rate in cancer. These mutated RTK signaling promotes essential progresses in tumorigenesis such as cell proliferation, migration and survival (Feldser and Kern, 2001).
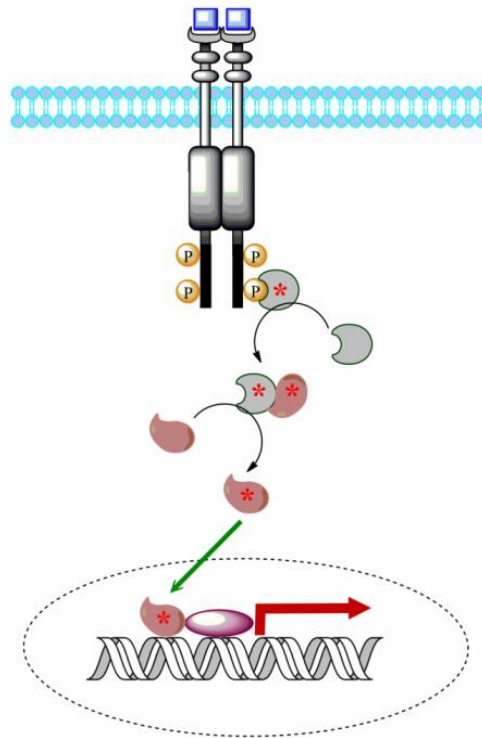
Figure 2. Canonical Receptor Tyrosine Kinase Cell Signaling Cascade. Ligand binding stabilizes the RTK dimer and induces RTK trans- autophosphrylation. The adaptor proteins are recruited by phspho-RTKs and recognize the phosphor-tyrosine on the RTK, which activates the adaptor protein. The activated adaptor protein will then further activate downstream signaling proteins and transduce the signaling into cell nucleus and regulate the cellular response to RTK signal.

1.1.2 Ligand-independent RTK activation

The spatial distribution of RTK is the key step for signal transduction, and the receptors need lateral homophilic and heterophilic *cis*-interaction along with intercellular trans-interaction to form the signaling foci (Bethani et al., 2010; Casaletto and McClatchey, 2012). Accumulating evidences indicate RTKs can also form dimers or oligomers without ligand and become active or form the complex primed for ligand-dependent activation (Chung et al., 2010; Clayton et al., 2007; Harding and Hancock, 2008; Himanen and Nikolov, 2003; Inder et al., 2008; Szabo et al., 2010).

1.1.2.1 RTK Alteration

The RTK activation begins with receptor dimerization and auto-phosphorylation, besides ligand induction, the overexpression of receptor also increases the intensity of receptor leads to altered plasma membrane and receptor clustering to raise the potential for ligand-independent dimerization and activation (Chung et al., 2010; Wei et al., 2005). Also, the abnormal RTK structure can alter the receptor activation. For example, an EGFR variant is reported to form activated dimer with only single ligand instead of the ligand dimer due to the structural difference in the extracellular domain (Alvarado et al., 2010). Some mutations

occur in the RTK kinase domain and let the mutant much easier to be phosphorylated at critical

tyrosine residues (Chen et al., 2007; Moriki et al., 2001).

1.1.2.2 Redox Regulation on RTK signaling

Previous studies have shown that reactive oxygen species (ROS), such as hydrogen

peroxide (H_2O_2), participates in some physiological process, including cell growth,

transformation, cell death and senescence (Finkel, 2003; Irani, 2000).   Moreover, previous

studies also show that ROS also activate several RTKs (Martindale and Holbrook, 2002;

Nishinaka and Yabe-Nishimura, 2001).   The oxidation agent, such as H_2O_2, can oxidize and

to inhibit PTPs, which promote RTK signaling by prevent the prompt inactivation of RTK by

PTPs (Chiarugi and Cirri, 2003; Chiarugi and Giannoni, 2005).   Further, it has been reported

that H_2O_2 also trans-activate EGFR signaling through alteration of Src signaling (Zhuang and

Schnellmann, 2004) and oxidized lipid component can leads to change and distribution of

lipid cluster in local concentration to activate several RTKs including EGFR and PDGFR

(Akiba et al., 2006; Negre-Salvayre et al., 2003).

1.2   C-MET (Hepatocyte growth factor receptor)

MET, also known as hepatocyte growth factor/scatter factor (HGF/SF) receptor, is a proto-oncogenic RTK which plays crucial roles in cell proliferation and motility as well as in promoting cancer.   HGF/SF is a normal endogenous ligand for MET and is mainly produced by mesenchymal origin cells, while HGF acts as pleiotropic factor and cytokine to regulate cell proliferation, growth, mitosis and shape in different types of cells through either endocrine or paracrine mechanisms (Basilico et al., 2008; Bottaro et al., 1991; Cortner et al., 1995; Naldini et al., 1991; van der Voort et al., 2000).   First discovered in the 1980s, 170-kDa pro-MET is N-linked glycosylated and this precursor is further cleaved to form a 45-kDa α subunit and a 150-kDa glycosylated β subunit, the two subunits are then joined by di-sulfite bonds to give mature MET (Chen et al., 2013; Cooper et al., 1984).   After HGF/SF stimulation, MET forms homodimer and *trans*-autophosphorylates each other at Y1230, Y1234 and Y1235 residues, and phosphorylates Y1349 and Y1356 successively, then the specific phosphorylated residues provide docking sites for downstream messenger proteins including PI3K, Ras, STAT and Grb1 thus regulate cellular growth and survival (Fig. 3) (Ma et al., 2003; Organ and Tsao, 2011; Ponzetto et al., 1994; Rodrigues and Park, 1994).   However, MET not only forms homodimers, but also transactivated by other surface molecules such as the v6 splice variant

of hyaluronan receptor CD44/intercellular adhesion molecule 1 (ICAM1), α6β4 integrin, G-

protein couple receptors (GPCRs) and other RTKs (Trusolino et al., 2010).   While dimerizing

with these cell surface molecules, the downstream signaling were initiated through

amplification of either MET activation or its binding molecules (Trusolino et al., 2010).
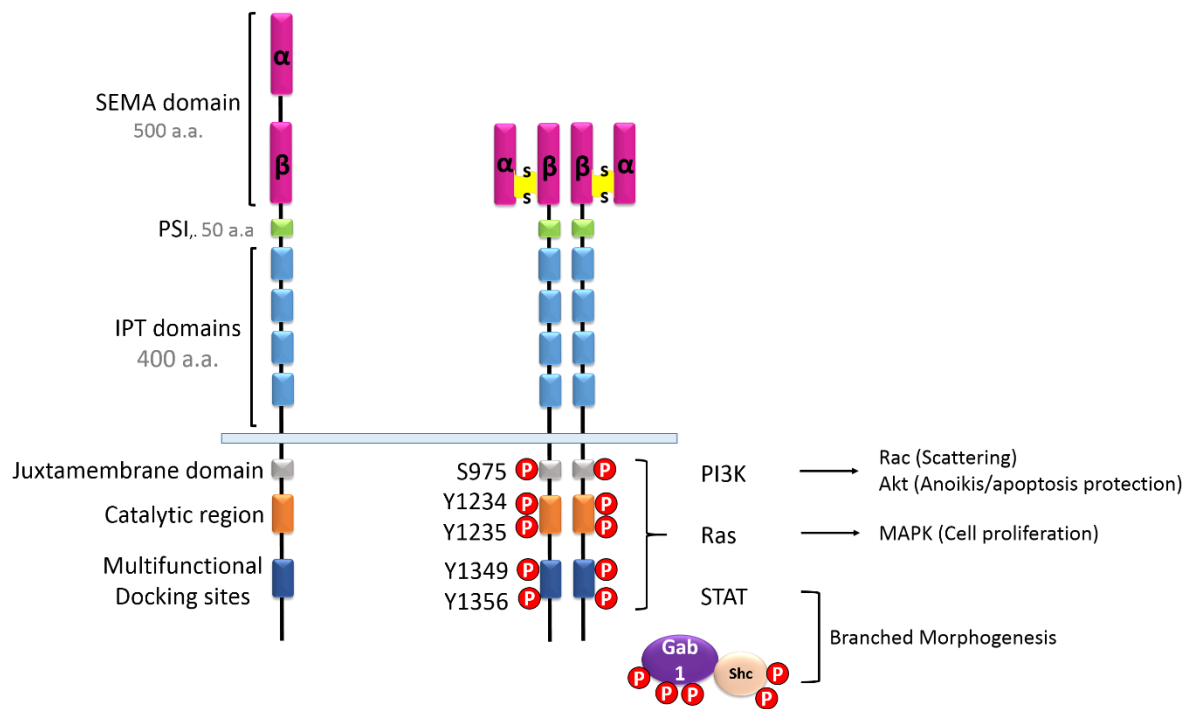
Figure 3. Structure and downstream signaling of MET.   There are many different domains in MET, the pro-MET (left panel) will be cleavage in the SEMA domain to produce α and β subunits, these subunits are linked by disulfite bond to create mature MET (right panel).

Besides activation of MET signaling pathways, down regulation of MET signaling is essential for cellular regulation, and many mechanisms are involved in the negative regulation. The protein tyrosine phosphatase (PTPs) inactivate MET activity by dephosphorylates MET at either the kinase domain or the docking tyrosine sites (Machide et al., 2006; Sangwan et al., 2008; Villa-Moruzzi et al., 1998). While HGF induces MET phosphorylation at multiple activation site, it also promotes the phosphorylation on MET negative regulation sites such as serine 985 (S985) and Y1003 residues. MET S985 is phosphorylated by protein kinase C (PKC) and leads to decreased tyrosine phosphorylation of MET, while the protein phosphatase 2A (PP2A) can dephosphorylate S985 to retain MET activity (Gandino et al., 1994; Hashigasako et al., 2004). On the other hand, E3 ubiquitin ligase casitas B-lineage lymphoma (c-CBL) is recruited by phosphorylated MET Y1003 and the MET-associated adaptor protein, Grb2 to promote receptor ubiquitination process and thus enhance MET internalization and degradation (Lefebvre et al., 2012; Petrelli et al., 2002).

1.3 MET Endosomal Trafficking — Endocytosis, Degradation and Recycling

The MET signaling not only exist on cell surface but also on endosomes before receptors were degraded (Kermorgant et al., 2004; McPherson et al., 2001; Miaczynska et al., 2004). Further, endosomal MET signaling is required for maximizing downstream signaling activation and is highly related to MET-induced tumorigenesis (Barrow et al., 2014; Joffre et al., 2011). Although the mechanisms of RTK, as well as MET, endocytosis, recycling and degradation are well-studied, little is known to the mechanism of RTKs nuclear trafficking.

Receptor endocytosis affects the physiology of cells such as motility and cell fate by regulating receptor trafficking rate to control signal duration and subcellular location, and clathrin-mediated endocytosis (CME) is a major mechanism for down-regulating activated MET signaling (Fig. 4) (Barrow et al., 2014; Clague, 2011; Goh and Sorkin, 2013; Polo and Di Fiore, 2006). The process of MET internalization begins with receptor ubiquitylation, and the process is controlled by a complex including c-Cbl, endophilins and CIN85 as well as cargo-specific adaptor proteins such as AP2 (McMahon and Boucrot, 2011; Petrelli et al., 2002). Endophilins regulates the formation of clathrin-coated vesicle through acyltransferase activity, which affects the phospholipids on cell membrane and leads to negative curvature and formation of endocytotic compartment from plasma membrane

(Schmidt et al., 1999).   Except clathrin, AP2 is the most abundant protein in the clathrin-coated cargos, while AP2 is highly conservative and specifically act on plasma membrane (Robinson, 2004).   AP2 and the receptor specific cargo accessory adaptor proteins recruited to the vesicle will determine the fate of the vesicles.   The vesicles are then send to sorting endosomes, where it is determined to travel back to the plasma membrane (recycling) or to lysosomes (McMahon and Boucrot, 2011).   MET signaling is terminated when the ubiquitylated receptor in the vesicle is recognized by the ESCRT (endosomal sorting complex required for transport) and send to lysosomal degradation (Goh and Sorkin, 2013; Jeffers et al., 1997).   The endosomal trafficking for degradation is through early endosome to late endosome and lysosome, the pH value of between compartments is gradually decreased to pH 5.0 in the lysosome for protein degradation (Goh and Sorkin, 2013; Yamashiro and Maxfield, 1984).
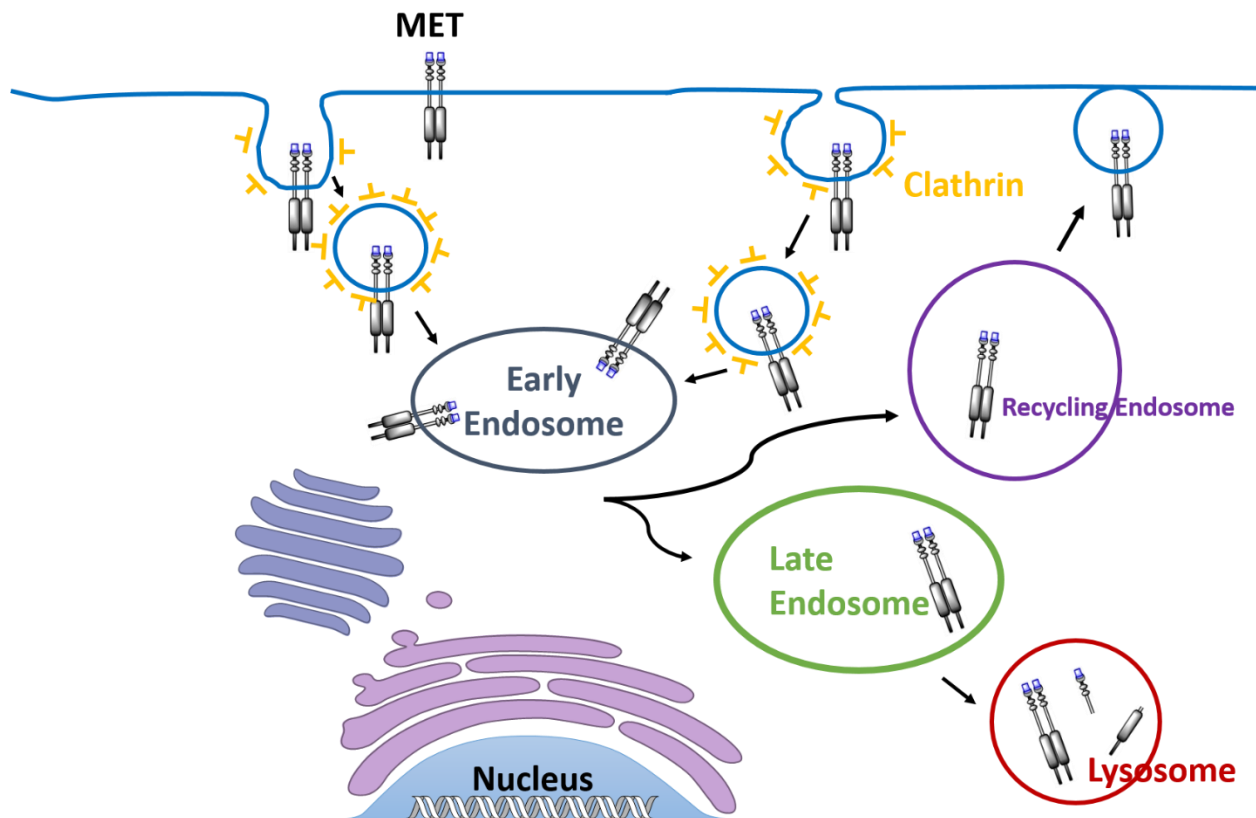
Figure 4. Endocytic trafficking of MET in response to ligand stimulation.
MET undergoes clathrin mediated endocytosis after ligand-induced dimerization. The internalized MET are transported to early endosome to determine whether recycling or degradation before send to late endosome and lysosome for lysosomal degradation.

1.4 Membrane Receptor in the Nucleus (MRIN)

Various RTKs have been reported to be shuttled from plasma membrane into cell nucleus, including EGFR, ERRB2, VEGFR, FGFR, MET and IGF1R (Carpenter and Liao, 2013; Feng et al., 1999; Kermorgant and Parker, 2008; Sehat et al., 2010; Wang et al., 2012), and had been reported to have important physiological and pathological roles in nucleus. These nuclear RTKs are now termed membrane receptors in the nucleus (MRINs) (Wang and Hung, 2009).

The nuclear function of MRINs is most studied in epidermal growth factor receptor (EGFR) and fibroblast growth factor receptor (FGFR), that includes transcriptional regulation, DNA replication and DNA repair regulation and even RNA metabolism (Planque, 2006; Wang et al., 2010b).

1.4.1 Nuclear EGFR

Every member in EGFR family, including EGFR, ErbB2, ErbB3, and ErbB4 has been reported to translocate into nucleus in different types of cells and human cancers, and nuclear EGFR has correlation with poor prognosis for many types of cancer (Carpenter and Liao, 2013; Hadzisejdic et al., 2010; Hoshino et al., 2007; Traynor et al., 2013; Wang and Hung, 2009; Wang et al., 2010b). EGFR nuclear translocation can be triggered by ligand stimulation and

cellular stress, such as ultraviolet and ionizing radiation (Chen and Nirodi, 2007; Dittmann et al., 2005; Lin et al., 2001; Oksvold et al., 2004; Xu et al., 2009).

Since both ligand and stress can stimulate EGFR nuclear translocation, the functions of nuclear EGFR cover many aspects from cell proliferation to DNA repair.   EGFR can act as an EGF-dependent transcriptional cofactor by regulating target gene expression through binding with RNA helicase A and recognize specific AT-rich sequence in the promoter region of those target genes and promote cell proliferation (Huo et al., 2010; Lin et al., 2001; Lo and Hung, 2006).   On the other hand, EGFR can also regulate gene expression through interact with other co-regulators, for example, signal transducers and activators of transcription protein 3 and 5 (STAT3 and STAT5), transmembrane mucin 1 (MUC1) and Epstein-barr virus-encoded latent membrane protein 1 (LMP1) (Bitler et al., 2010; Hung et al., 2008; Huo et al., 2010; Jaganathan et al., 2011; Tao et al., 2005).   When cells encountered cellular stress like radiation, oxidative stress or DNA-damaging reagents such as cisplatin, EGFR can translocate into nucleus in a ligand-independent manner and activate DNA repair process through phosphorylate its substrates such as DNA protein kinase (DNA-PK), Ku proteins and PCNA (Bandyopadhyay et al., 1998; Chen and Nirodi, 2007; Friedmann et al., 2006; Javvadi et al., 2012; Liccardi et al., 2011; Park et al., 2010; Yu et al., 2013).

1.4.2 Nuclear FGFR-1

It has been reported that FGFR-1 accumulates in the nucleus and plays a role as transcriptional regulator when aided by FGF-2, ribosomal S6 kinase isoform 1 (RSK1) and CREB-binding protein (CBP) (Bryant and Stow, 2005; Dunham-Ems et al., 2009; Johnston et al., 1995; Lee et al., 2013; Stachowiak et al., 1997). FGFR1 has physical interaction with RSK1, a CBP and histone phosphorylation regulator, and regulates RSK1 transcription activities (Hu et al., 2004). By recruiting the RNA polymerase II and histone acetylation on the promoters, FGFR1 up-regulate the target genes through binding with CBP (Peng et al., 2002; Stachowiak et al., 2003).

Moreover, the nuclear FGFR1 also promotes cancer invasion in different types of cancer through regulating gene expression (Chioni and Grose, 2012; Coleman et al., 2014). More and more genes are identified as targets of nuclear FGFR1, e.g., the genes of neurofilament-L, neuron-specific enolase microtubule associated protein-2 (MAP2), c-jun and cyclin D1 (Reilly and Maher, 2001; Stachowiak et al., 2003). By regulating the expression of target gene, nuclear FGFR1 is involved in cell growth and differentiation. The transactivation function of FGFR1 is independent from the surface FGFR1 signaling, suggesting the distinct function of nuclear RTK from that on plasma membrane.

1.4.3 Nuclear MET

MET is also a member of MRINs, the internalization of MET starts from clathrin and dynamin-dependent endocytosis as many other MRINs such as EGFR and ErbB-2 (Goh and Sorkin, 2013; Hammond et al., 2001; Hoffmann et al., 2006; Kermorgant et al., 2003; Kermorgant et al., 2004; Wang et al., 2012). However, the function of nuclear MET is not yet well studied. So far, *Matteucci et al.* demonstrated MET holo-receptor localization in cell nucleus is independent from HGF stimulation in MDA-MB-231 cells (Matteucci et al., 2009) and several groups have demonstrated that 60-kDa MET C-terminal fragment (CTF) form has to locate in nucleus to initiate calcium signaling and have suggested that the juxtamembrane domain preceding the tyrosine kinase domain seems indispensable for nuclear localization and transactivation activity (Gomes et al., 2008; Matteucci et al., 2009; Pozner-Moulis et al., 2006). Meanwhile, evidences reveal that nuclear localized MET is involved in non-canonical signal transduction such as accelerate STAT3 nuclear accumulation (Kermorgant and Parker, 2008). However, whether the MET holo-receptor translocates from plasma membrane into nucleus in response to stimulation and what is the nuclear trafficking mechanism utilized by MET still remains unclear.

1.5 Nuclear Trafficking mechanism of MRIN

It is important to investigate the intercellular trafficking of RTKs to clarify how spatial distribution affect endosomal RTKs signaling and further identify the potential inhibitors. There are several hypotheses regarding how membrane receptor tyrosine kinase are transported into cell nucleus. As illustrated in Figure 5, there are two main trafficking pathways regarding how MRINs translocate into nucleus: (I) Protease-dependent route and (II) holoreceptor route (II) Protease-independent route — the holo-receptor tyrosine kinase translocate into nucleus through clathrin mediated endocytic or similar mechanisms, then travel with trafficking vesicle through membrane bound mechanism (Carpenter and Liao, 2013; Du et al., 2013; Song et al., 2013; Wang et al., 2012; Wang et al., 2010c).
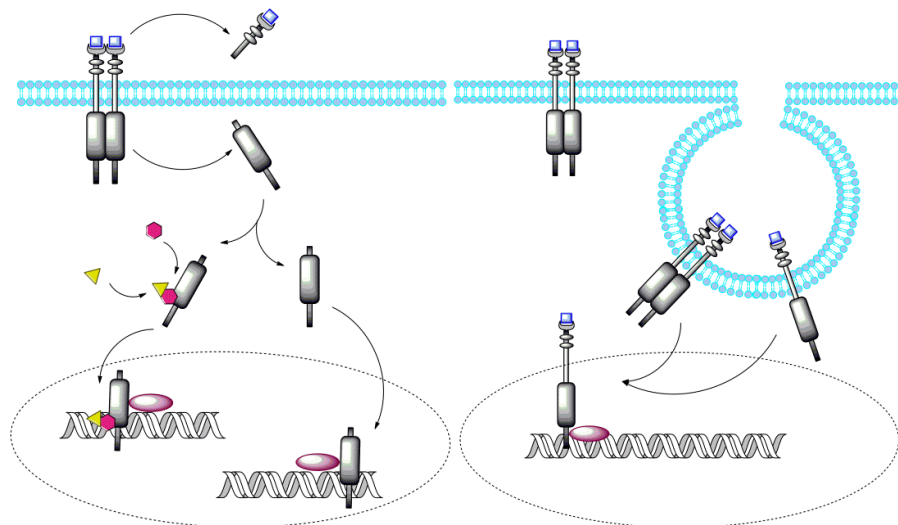
Figure 5. RTK nuclear transportation pathways in mammalian cells. There are two nuclear transportation pathways for RTKs, the protease-dependent route (left panel) and the holo-receptor route (right panel).

1.5.1 Protease-dependent RTK nuclear transportation mechanism

Generally, in the protease-dependent RTK trafficking mechanism, sequential proteolytic cleavage happened to the receptor after ligand binding. First, the receptor undergoes protease cleavage and release the extracellular domain, then the further cleavage at RTK transmembrane domain leads to production of intracellular domain (ICD), and then the ICD is released into cell cytosol to translocate into nucleus directly (Fortini, 2002; Fortini, 2009).

The mechanisms of ICD fragment varies in response to different stimulation (Carpenter and Liao, 2013), including secretase-dependent, caspase-dependent, translation-dependent, splicing-dependent and granzyme-dependent pathways. The most studied mechanism is the secretase-dependent ICD formation: the ligand-bound RTKs are first cleavage by α-secretase to release the extracellular domain, then the second cleavage is performed by γ-secretase at the juxtamembrane domain to release ICD from transmembrane domain. Currently, several receptors are reported to follow this nuclear trafficking route, including ErbB4, ephrin receptors, IGF1R, insulin receptor and VEGFR1 (Georgakopoulos et al., 2006; Ni et al., 2003).

1.5.2 Protease-independent nuclear transportation mechanism

More and more full-length RTKs has been reported to translocate in nucleus, and these RTKs are shown to appear in nucleoplasm rather than nuclear envelope (Carpenter and Liao, 2013). The RTKs are known to translocate from nuclear envelope into nucleus through the help of importin complex. Importin β, which is directly associates with nucleoporins, is a protein which can interact with the nuclear localization signals (NLSs) on the RTKs and transport RTKs into nucleus (Reilly and Maher, 2001; Walther et al., 2001; Wang et al., 2010c).

When the cell surface molecules be internalized into cytosol by endocytosis, the first organelle those molecules transported to is Golgi apparatus (Bos et al., 1995; Duncan and Kornfeld, 1988; Farquhar, 1985; Roth et al., 1985). The retrograde trafficking is different from endocytosis part, the retrograde sorting begins with cargo molecules targeting to endosomes from Golgi apparatus or plasma membrane. Illustrated in Figure 6, the cargo vesicles are retrograde transported to trans-Golgi network (TGN), Golgi membrane or ER (Bonifacino and Rojas, 2006; Johannes and Popoff, 2008).

Clathrin-binding proteins are not only essential for endocytosis, but also required for cargo transportation from early endosome to TGN (Hinners and Tooze, 2003; Shi et al., 2009). After transported to Golgi and TGN, the retrograde membrane vesicle transportation

mechanism includes the trafficking from Golgi body to ER where coat protein complex I (COPI)-coated vesicle are an essential carrier which carries MRINs between Golgi stacks and also carries MRINs from Golgi to ER (Pellett et al., 2013; Spang, 2013; Wang et al., 2010a). In addition, after MRINs reached ER, the beta subunit of Sec61 translocon (Sec61β) is indispensable for MRINs to be transported from ER into cell nucleus (Greenfield and High, 1999; Wang et al., 2010c). There are at least two typical trafficking mechanisms for RTK holoreceptor transportation from Golgi apparatus into nucleus: (I) the INTERNET mechanism, and (II) the INFS mechanism (Wang et al., 2012). However, the nuclear trafficking mechanism is not well-studied in all reported MRINs.

1.5.2.1 INFS nuclear transportation mechanism

Some RTKs, for example FGFR1 and FGFR2, translocate into nucleus through INFS (integrative nuclear FGFR-1 signaling) mechanism. Briefly, the RTKs are internalized and transported to Golgi apparatus, however, these RTKs are released from pre-Golgi or endoplasmic reticulum (ER) into cytosol and transported to the nucleus by indirectly interaction with importin β (Dunham-Ems et al., 2009; Reilly and Maher, 2001; Stachowiak et al., 2003; Stachowiak et al., 2007).

1.5.2.2 INTERNET nuclear transportation mechanism

For the INTERNET (integral trafficking from the ER to the nuclear envelope transport) mechanism, the RTKs, such as EGFR, are internalized through endocytosis and transported to the nucleus through Golgi apparatus to ER retrograde membrane-vesicle transportation route and depend on the inner nuclear membrane (INM) to translocate into nucleus.

After reaching ER, these RTKs are further translocate in a Sec61 translocon complex-dependent manner to the inner nuclear membrane (INM) through the nuclear pore complexes (NPCs) with the assistance of importin β (Wang et al., 2010c). In this study, we hypothesized that MET holo-receptor responses to cellular stress, such as oxidative stress, and transports into nucleus through the COPI and Sec61β-mediated nuclear trafficking pathway.
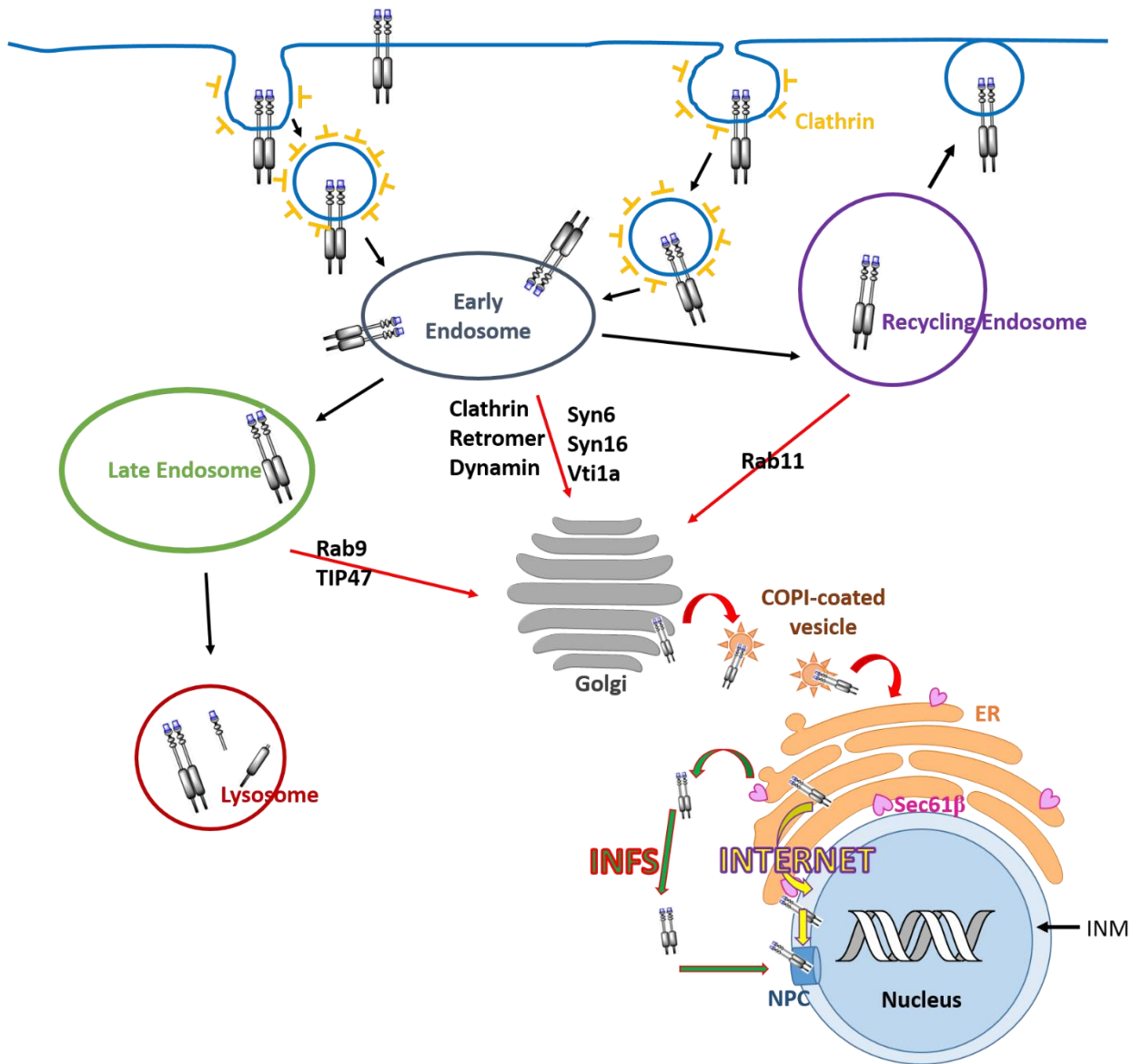
Figure 6. RTK retrograde nuclear transportation mechanisms. After surface molecules internalized by endocytosis, the cargo are sent to endosome for sorting. The molecules are then transported with the aid of different adaptor proteins to Golgi apparatus, followed by transported to ER through COPI-coated vesicles. The molecules are then translocate into nucleus through NPCs by either INTERNET or INFS mechanism.

1.6 MET Overexpression Indicates Poor Prognosis in Breast Cancer Patients

According to data revealed from the World Health Organization (WHO) in 2008, breast cancer is the most common cancer in women and also the most common cancer that leads to death among women worldwide (Jemal et al., 2011). And the data provided by Surveillance, Epidemiology and End Results (SEER) Cancer Statistics Review shows that in 2014, it is estimated that the new cases will be 232,670 people and stands for 14% of all new cancer cases (Howlader N, 2014).

So far, there are two systems in grouping breast cancer subtypes. One is based on the gene expression, the other is based on the expression level of hormone receptors. There are at least five subtypes in breast cancer carcinoma according to their gene expression pattern, denoted as luminal A and B, basal-like, HER2 enriched and normal-like (Cadoo, 2013). Another way to group the breast cancer cell is based on their expression level of three important hormone receptors, estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor (HER2). These receptors serve as biomarkers for breast cancer because their great influence on tumor growth and metastasis. Clinically, breast cancer cells can be grouped into ER/PR positive, ERBB2 (HER2) overexpression and the

breast cancer cell which lack of ER, PR, overexpressed ERBB2 is called triple negative breast

cancer (TNBC) (Riaz et al., 2013).

MET overexpression is known to be an indicator of poor prognosis in all subtypes of

breast cancer and the MET signaling also promotes drug resistance and recurrence (Eterno et

al., 2013; Inanc et al., 2014; Raghav et al., 2012; Shattuck et al., 2008; Zagouri et al., 2013).

Indeed, high MET protein expression is also found in many breast cancer cell lines, especially

in TNBC cells (Fig. 7), which suggesting that MET expression may potentially be correlated

to the malignancy of cancer cells.
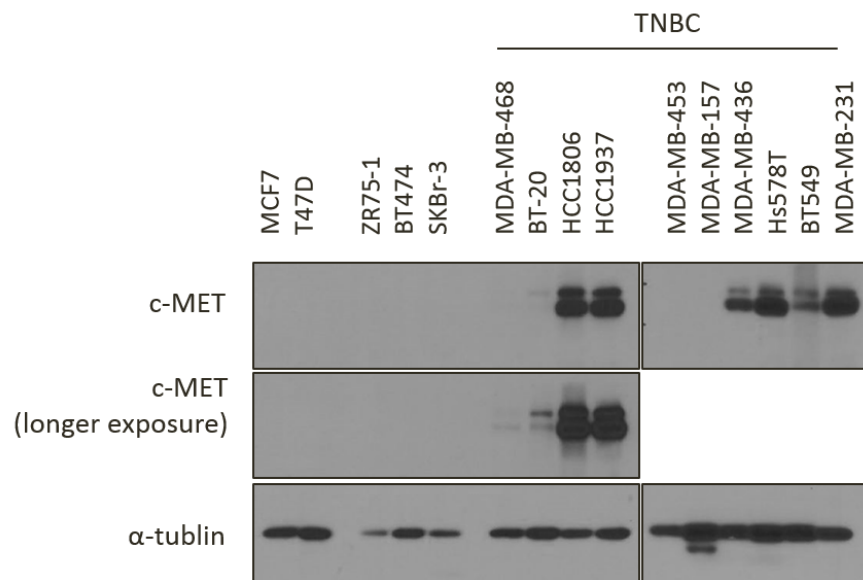
Figure 7. MET Protein Expression in Breast Cancer Cell Lines.　　The MET protein expression level is generally higher in TNBC cell lines.　　The subtypes were grouped according to ER, PGR, ERBB2 protein expression level reported by Riaz et al. (Riaz et al., 2013).

1.7 Specific Aims

Accumulating evidences show that RTK signaling and cellular function depend on the sub-cellular location. In particular, nuclear localized RTK has been reported to participate in numerous tumorigenesis pathways. The sub-cellular transportation of RTKs can be induced by both ligand and cellular stress, while cells are normally more sensitive to stress-induced transportation. Given that MET is an important RTK in various cancer types, it is important to find out the potential endosomal signaling and nuclear function of MET.

Among different cellular stress, reactive oxygen species (ROS) is one of the common stresses occur in tumor cells, either produced from metabolism or as a product while response to anti-cancer drug. This study mainly focuses on the trafficking mechanism of ligand and ROS induced MET nuclear transportation, and aimed on providing more knowledge about RTK transportation under ROS stress.

Chapter 2   Materials and Methods

2.1 Experimental reagents

The antibodies used in this study are as follows: anti-MET antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA; GeneTex, Inc., Irvine, CA, USA), anti-lamin B antibodies, anti-calregulin antibodies, anti-COPI antibodies (Santa Cruz Biotechnology Inc.), anti-Sec61β antibodies (GeneTex, Inc.). Hepatocyte growth factor (HGF) human recombinant was purchased from BioVision Inc. (San Francisco, CA, USA), hydrogen peroxide (H_2O_2) and brefeldin A were purchased from Sigma-Aldrich (St Louis, MO, USA). Brefeldin A was dissolved in methanol to generate 5 μg/mL stock solution.

2.2 Cell culture and treatments

Breast cancer cells lines were originally obtained from ATCC. MDA-MB-231, BT549, Hs578T cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 supplied with 10% fetal bovine serum and 100 U/mL penicillin and 100 μg/mL streptomycin. MCF-10A and MCF-12A cells were maintained in DMEM/F12 medium supplied with 5.2% horse serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 10 μg/mL insulin, 20 ng/mL EGF, 100 ng/mL cholera enterotoxin, 0.5 μg/mL hydrocortisone and 1.05 mM calcium chloride. Cells were treated with 100 ng/mL HGF or 10 μM H_2O_2 within culture medium

and incubated in 37℃, water-saturate incubator supplied with 5% CO_2. For Golgi body

inhibition, cells were pre-treated with 5 μg/mL BFA before other treatments.

2.3   Whole cell extract preparation

Cells were cultured until reaching 90% confluent before subjected to whole cell

extraction.   The culture medium were removed and the cells were washed with ice-cold PBS

twice before harvested by scrapping.   The cells were then pelleted by centrifuge 1800xg at 4

℃ for 5 min and re-suspended with 200   L NETN buffer (150 mM NaCl, 1 mM EDTA, 20

mM Tris pH 8.0, 0.5% Nonidet P-40), then were sonicated (Sonics Vibra-Cell, amplitude 30,

10 sec; Sonics & Materials, Newtown, CT).   The supernatant were then collected as whole

cell extract after centrifugation at maximum speed.

2.4   Cellular fractionation

To investigate the subcellular location of MET, we performed cell fractionation to

separate cell nuclear from the rest parts of cells.   The nuclear fraction and non-nuclear

fraction of cells were prepared as described (Du et al., 2013).   Briefly, cells were harvest at

90% confluent and subjected to lysed in nori lysis buffer (20 mM HEPES, pH 7.0, 10 mM

KCl, 2 mM MgCl$_2$, 0.5% Nonidet P-40) on ice for 1 h.   The cells were then homogenized by

Dounce homogenizer. After centrifuged, the supernatant were collected as non-nuclear

fraction.   The pelleted nuclei were washed with lysis buffer twice followed by re-suspended

and incubated for 10 min on ice in NETN buffer before sonication (Sonics Vibra-Cell,

amplitude 30, 10 sec).   The extract was then centrifuged and the supernatant were collected

as nuclear fraction.

2.5   Subcellular protein extraction

The extraction of cytosolic, organelle membrane and nuclear protein extraction was

performed with ProteoExtract® Subcellular Proteome Extraction Kit (Calbiochem® Merck

Millipore, MA, USA) as described (Wang et al., 2012).   Generally, the subcellular proteins

were prepared following the manufacturer's instructions.   Mitochondria, as well as cell

membrane, were removed from organelle membrane-enrich fraction by centrifugation at

20,000 x g for 30 min (Borradaile et al., 2006; Nigam et al., 1994).

2.6   RNA interference

To establish stable COPI or Sec61β knock-down clones, the plasmid were transfected into MDA-MB-231 cells with lentivirus infection.   The stable clones were cultured with 1% puromycin-containing DMEM/F12 selective medium.   The shRNA expression vectors with pLKO.1 backbone were purchased from Sigma-Aldrich, the COPI knock-down stable clones were kindly provided by Dr. Yi Du and the Sec61β siRNA targets sequence NM_006808 were purchased from GE Healthcare.   The scramble shRNA (Addgene plasmid 1864) is used as non-targeting shRNA control (Sarbassov et al., 2005).   The same procedures were used for generate stable MET knock-down clones in MDA-MB-231 cells.   The GIPZ lentiviral shRNA were purchased from Thermo Fisher Scientific (Pittsburgh PA, USA), the shMET A and B both target sequence of MET ORF while shMET C targets the sequence of MET 3'-UTR.   The RNAi sequence is listed in Table 1 and 2.

Table 1. MET shRNA Clone ID

#	Final #	Oligo ID/clone ID	Gene	Accession number	Species	Mature anti-sense Sequence
1		V2LHS_76542	HGFR/MET	NM_000245, NM_031517	human	TATTACAGGATCCACATAG
2		V2LHS_76541	HGFR/MET	NM_000245	human	ATAGATGTTAAGAGGACTTCGC
3	A	V3LHS_381509	HGFR/MET	NM_000245	human	TAGAATGACATTCTGGATGGGT
4		V3LHS_381505	HGFR/MET	NM_000245	human	TTTCTTGCCATCATTGTCCAA
5	B	V3LHS_381508	HGFR/MET	NM_000245, NM_001127500	human	TAAACACTTCCTTCTTTGT
6	C	V3LHS_646098	HGFR/MET (3' UTR)	NM_000245, NM_001127500	human	TTAATTGCATGATTTATCA

Table 2. Sec61β siRNA Probe ID

#	Final #	NCBI Probe ID	Gene	Accession number	Species
1	1	12262229	Sec61B	NM_006808	human
2	2	12262230	Sec61B	NM_006808	human
3	3	12262231	Sec61B	NM_006808	human
4	4	12262232	Sec61B	NM_006808	human

2.7   Western blotting

The Western blotting analysis was performed as described to detect the molecular weight of specific protein and the level of protein expression (Chen et al., 2011).   20 μg protein samples were boiled in protein sample buffer (12 mM Tris, pH 6.8, 0.4% SDS, 2% glycerol, 1% β-mercaptoethanol, 0.05% bromophenol blue) for 15 min before separated by 8%/12% SDS (sodium dodecyl sulfate–polyacrylamide) gel electrophoresis.   Then the proteins were transferred onto Hybond-P polyvinylidene difluoride (PVDF) membranes under 4℃.   Non-specific binding sites were blocked with 5% slim milk/TBST buffer (137 mM NaCl, 2.7 mM KCl, 25 mM Tris, 0.05% Tween-20, pH 7.4).   Primary antibody were hybridized under 4 ℃ shaking overnight and followed by HRP-conjugated secondary antibody hybridization.   To detect the antibody:antigen complexes, the blots were developed using enhanced chemiluminescence (ECL) kit (Biorad Inc., Hercules, CA, USA) according to the manufacturer's instructions.   The specificity of MET antibody was tested by comparing the signal level between MET shRNAs knock-down and MET non-targeting shRNA (shCtrl) MDA-MB-231 whole cell extract (Table1, Fig. 8).
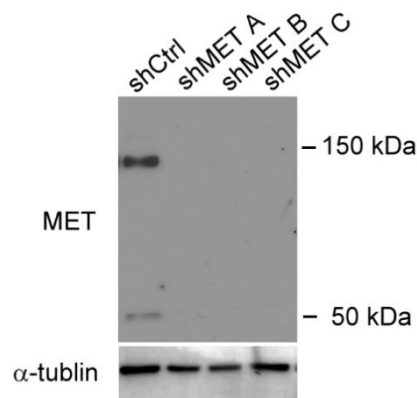
Figure 8. The specificity of MET antibody in Western blotting. 20 μg whole cell extract from MDA-MB-231 cells stable transfected with indicated shRNA were used for Western blotting. The α-tubulin is used as loading control.

2.8 Confocal microscopy

The sample for confocal microscopy is prepared as described previously (Wang et al., 2010a). In brief, cells were seeded on chamber slides at least 18 h before treatment. After treatment, the cells were then washed with phosphate buffered saline (PBS) and fixed and stained with antibodies described below. The cells were stained with primary antibodies followed by FITC or Texas Red-conjugated secondary antibody hybridization. DNA were counterstained with DAPI-containing mounting solution (Vector Laboratories Inc., Burlingame, CA, USA). The samples were then imaged with Zeiss LSM 710 laser microscope.

2.9 Cell viability assay 一 MTT assay

For cell viability assay, 1000 MDA-MB-231 cells were plated in 96-well flat bottom plates 24 h before exposed to doxorubicin for 72 h.   Then the MTT solution were added to each well at the final concentration of 1 mg/mL and incubated for 1 h.   After incubation, the MTT solution were removed and the formazan crystals were dissolved in 0.1 mL DMSO. The absorbance at 590 nm was determined with BioTech plate reader and the reference absorbance is 620 nm.   Each condition were triplicated and the viability is determined by calculating the percentage of O.D. 590 nm of each treatment to the control group.

2.10 Statistical analysis

The percentage of MET-containing cell nuclei was calculated based on the immunofluorescence images obtained by confocal microscopy.   For each experiment, at least 50 cell nucleus were counted to be the denominator, the number of nucleus which contain MET signal is counted to be the numerator.   For Western blotting analysis, each experiment were repeated at least three times independently, the signal intensity is quantified by ImageJ (http://rsbweb.nih.gov/ij/index.html) (Schneider et al., 2012).

Chapter 3

Results and Analysis

3.1 Nuclear translocation of MET

3.1.1 Ligand induced MET nuclear transportation

Previous studies have demonstrated that many RTKs translocate into nucleus in response to both ligand and cellular stress (Carpenter and Liao, 2013; Goh and Sorkin, 2013; Song et al., 2013).   To determine the subcellular location of MET after HGF treatment, we performed both cellular fractionation followed by Western blotting (Fig. 9).   Although it has been reported that full-length MET does not translocate to the nucleus upon HGF stimulation (200 ng/ml for 30 min) in MDA-MB-231 cells (Matteucci et al., 2009) , we found that prolonged HGF treatment (100 ng/mL for 1-6 hours) increased the protein levels of nuclear MET holoreceptor in both MDA-MB-231 and BT549 cells (Fig. 9).
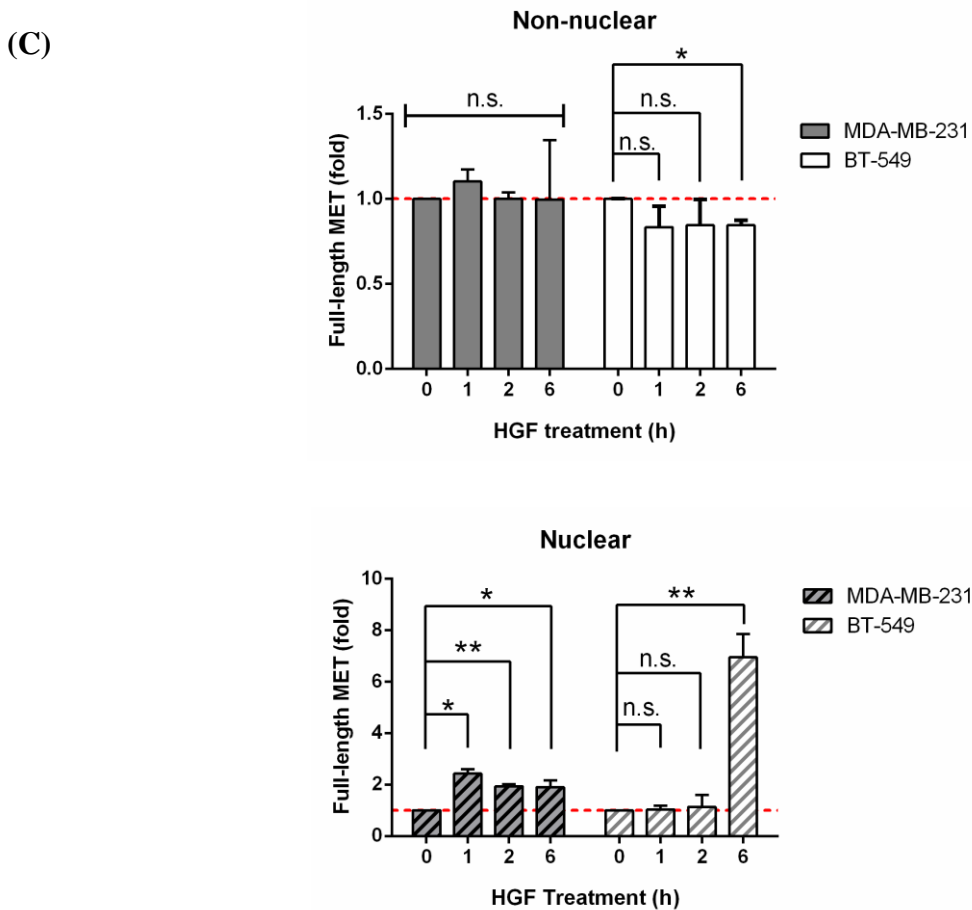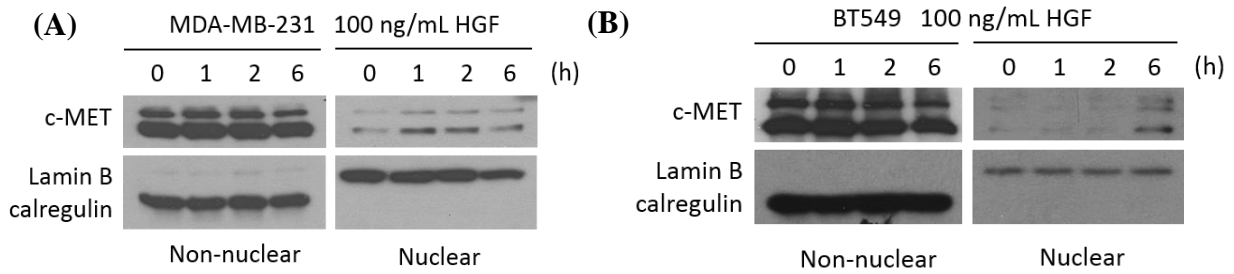
Figure 9. Full-length MET nuclear localization after ligand treatment. **A,** MDA-MB-231 **B,** BT-549 cells at 90% confluence were treated with HGF for time indicated before harvested for cell fractionation. 20 μg proteins of each sample were used for Western blotting. **C,** Quantitation of MET protein level (folds) compare to untreated (0 h) group in non-nuclear and nuclear fractions. The red dash line indicates 1 fold. * $p < 0.05$, ** $p < 0.005$, **n.s.**, non-significant difference.

3.1.2 ROS-induced MET nuclear translocation in breast cancer cells

3.1.2.1 H₂O₂-induced MET transactivation

As mentioned in Chapter 1.1.2.2, ROS can transactivate many RTKs. In this study, H_2O_2 was used to generate ROS stress. To verify if ROS can activate MET, the phosphorylation of MET Y1234/Y1235 were detected by Western blotting. As shown in Figure 10, p-Y1234/1235 can be detected in cells after treated with 10 μM H_2O_2 30 min, and this phosphorylation can be blocked by crizotinib, a clinical used MET inhibitor.
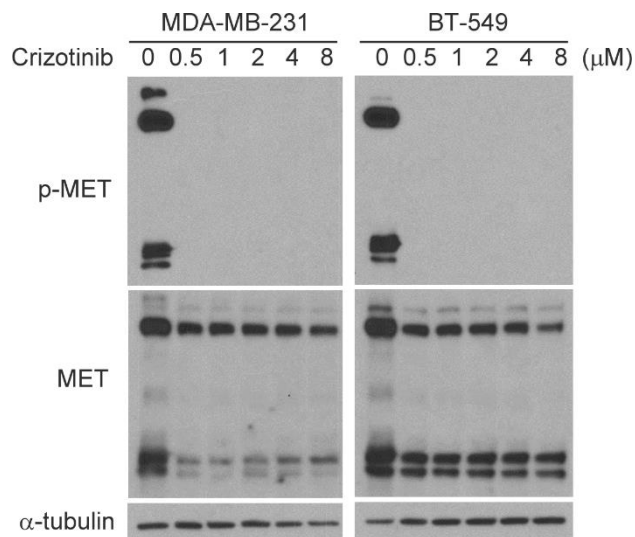
Figure 10. H₂O₂ induced MET activation can be blocked by crizotinib. MDA-MB-231 and BT-549 cells were treated with crizotinib at dose indicated for 24 h, followed by 30 min 10 μM H_2O_2 treatment. The cells were then harvested, and **A,** the phosphorylated MET Y1234/Y1235 residues **B,** total MET protein level in whole cell extracts were detected by Western blotting. **C,** α-tublin were used as loading control in Western blotting analysis.

In addition to HGF treatment, ROS also induces MET nuclear transportation in breast cancer cells. In this study, MDA-MB-231 and BT-549 cells were treated with various concentrations of H_2O_2 for different times before harvested for cellular fractionation and Western blotting. As shown in Figure 11, H_2O_2 treatment increased the levels of nuclear MET holoreceptor (145-kDa) in a dose-dependent manner. The data indicated that a 30-min treatment of 10 μM H_2O_2 was sufficient to induce MET nuclear translocation in both cell lines. Although treatment of 20 μM H_2O_2 induced MET translocation more substantially, we chose the 10 μM concentration for the time course study to avoid potential ROS-induced cell death.

(A)

(B)

(C)
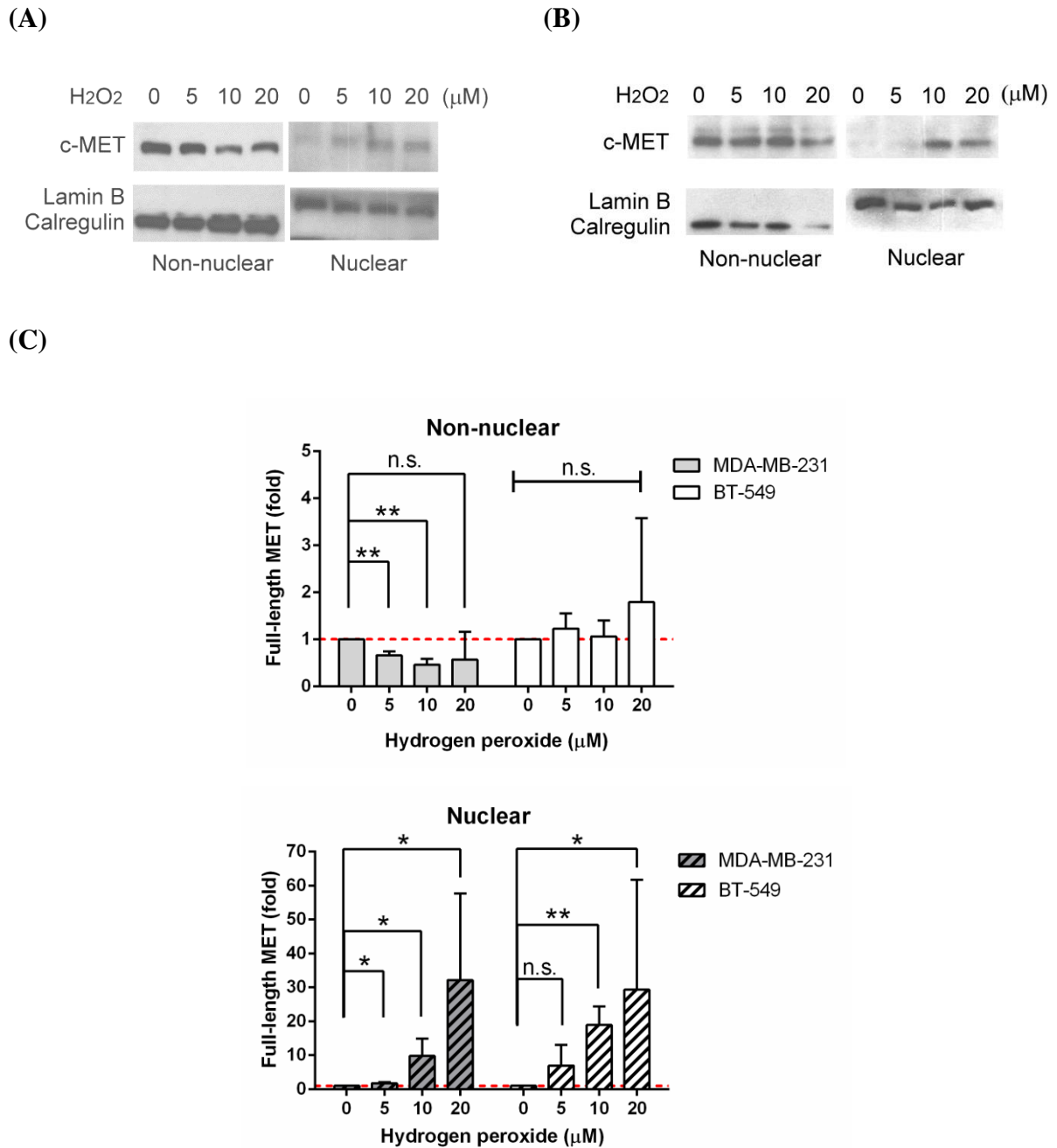
Figure 11. H₂O₂-induced MET nuclear translocation is dose-dependent. A, MDA-MB-231 B, BT-549 cells at 90% confluence were treated with H₂O₂ at dose indicated for 30 min before harvested for cell fractionation. 20 μg proteins of each sample were used for Western blotting. C, Quantitation of MET protein level (folds) compare to untreated (0 μM) group in non-nuclear and nuclear fractions. The red dash line indicates 1 fold. * p <0.05, ** p<0.005, n.s., non-significant difference.

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In order to figure out a proper condition for investigating the MET nuclear trafficking mechanism, the MET subcellular location is also detected in different time after H_2O_2 treatment. In both cell lines, nuclear accumulation of MET can be detected from 30 min to 2 h following H_2O_2 treatment (Fig. 12).

(A)

MDA-MB-231 H_2O_2 10 μM

(B)

BT-549 H_2O_2 10 μM

(C)
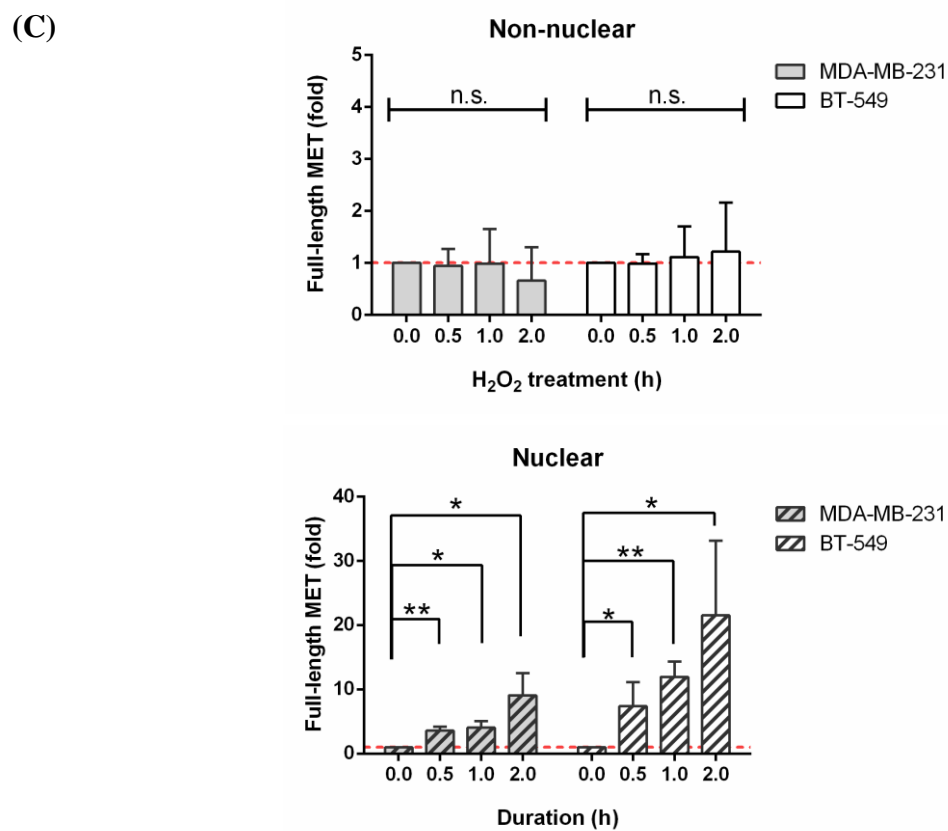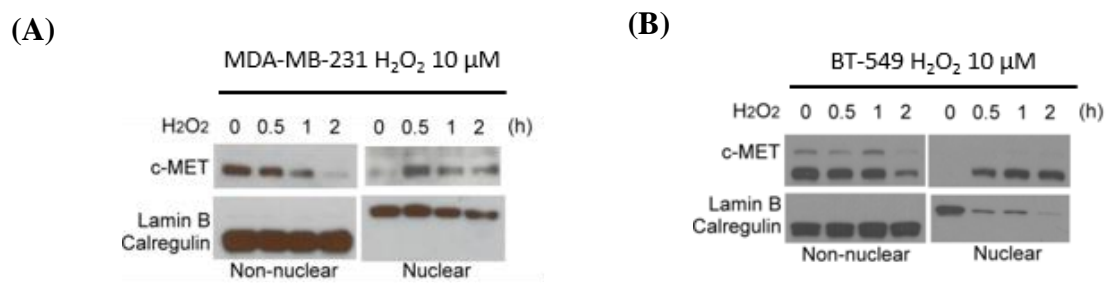
Figure 12. H₂O₂-induced MET nuclear translocation is time-dependent. **A,** MDA-MB-231 **B,** BT-549 cells at 90% confluence were treated with 10 μM H₂O₂ for indicated time before harvested for cell fractionation. 20 μg proteins of each sample were used for Western blotting. **C,** Quantitation of MET protein level (folds) compare to untreated (0 h) group in non-nuclear and nuclear fractions. The red dash line indicates 1 fold. ** p <0.05,* *** p<0.005,* ***n.s.,*** *non-significant difference.*

To minimize the effect on cellular response to H₂O₂, a 30-min 10 μM H₂O₂ treatment was used in all subsequent experiments to investigate the trafficking mechanism of MET. H₂O₂-induced MET nuclear translocation was also validated in various breast cancer cell lines (Fig. 13) in which the level of nuclear MET holoreceptor was higher in H₂O₂-treated than in non-treated cells. Interestingly, MET holoreceptor was the predominant contrary observed in the nucleus upon H₂O₂ treatment in several cell lines tested (Fig. 13).
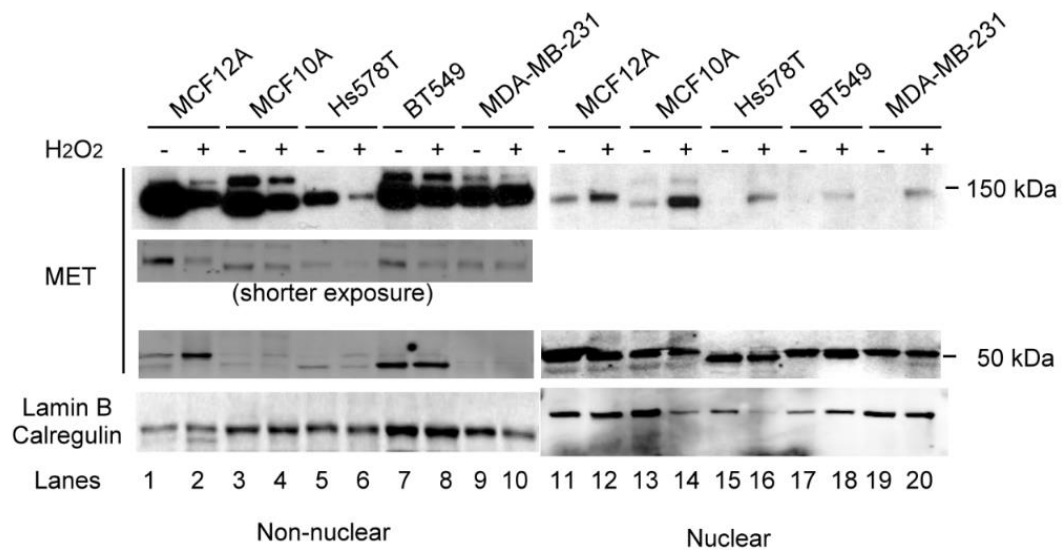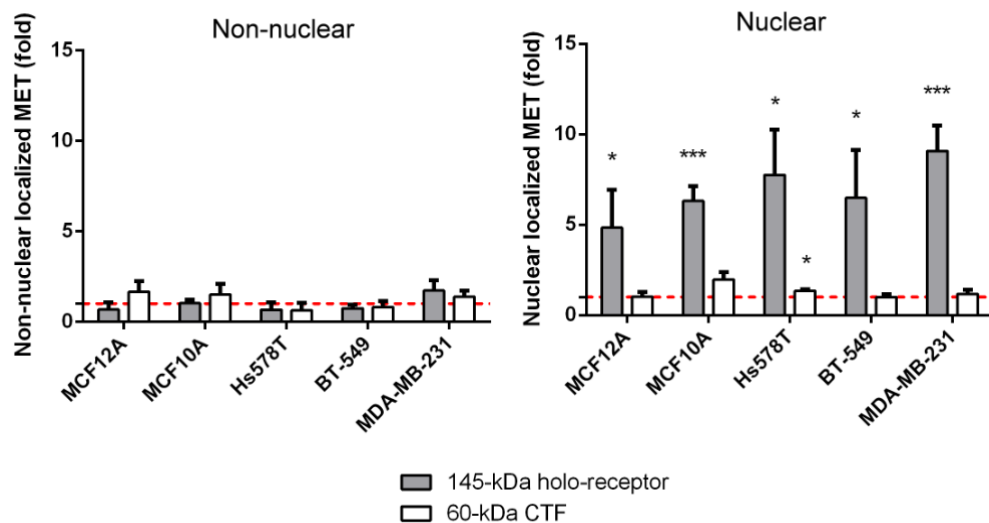
Figure 13. MET nuclear translocation after H₂O₂ treatment. Cells were treated with 10 μM H₂O₂ for 30 min before harvested for fractionation and Western blotting. The MET expression level were calculated by comparing the MET signal intensity with the loading control (calregulin for non-nuclear fraction and lamin B for nuclear fraction). The fold is calculated by the expression level of H₂O₂ treated group (H₂O₂ +) to untreated group (H₂O₂ -).

To further validate the nuclear translocated MET, immunostaining of MET was performed in both MDA-MB-231 and BT-549 cells for analysis by confocal microscopy. As shown in Figure 14, MET was mainly found in the cytosol of both cell lines under normal culture condition (control), but aggregated near the nucleus after either HGF or H₂O₂ treatment. Through counting the number of nuclear MET-containing nuclei, we observed that the percentage of MDA-MB-231 cells with MET nuclear localization following H₂O₂ treatment increased about 2-fold compared with control and HGF-treated cells (Fig. 14 B). The increase in HGF-induced nuclear MET was more substantial in BT-549 than in MDA-MB-

231 cells (Fig. 14 B).   MET nuclear location was further verified by three-dimensional

confocal microscope z-stack imaging (Fig. 15).   These results indicate that oxidative stress

elicits stronger nuclear translocation of MET than ligand stimulation.

Figure 14 (A)
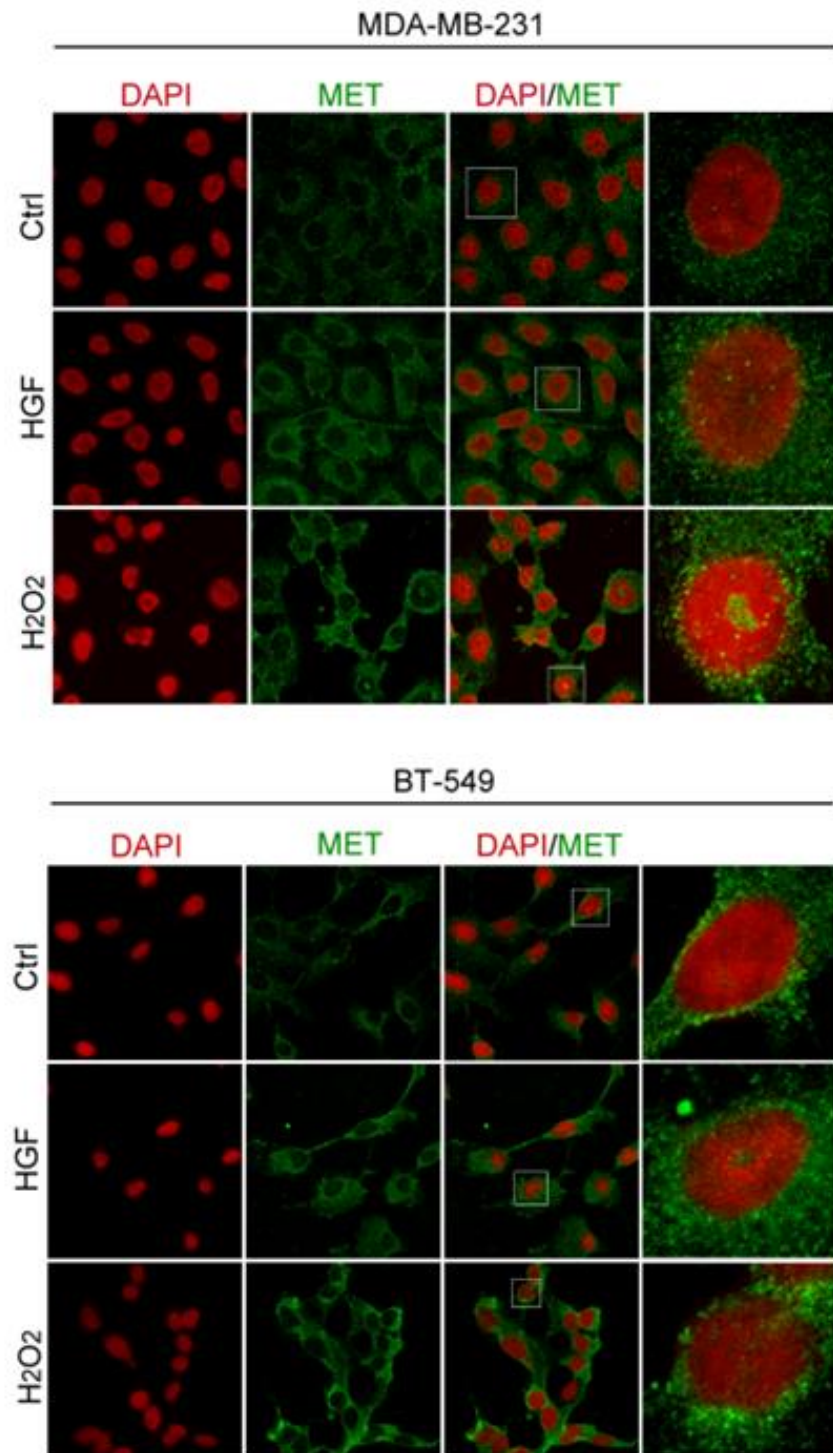
Figure 14 (B)

Figure 14. MET subcellular location after HGF or H₂O₂ treatment.
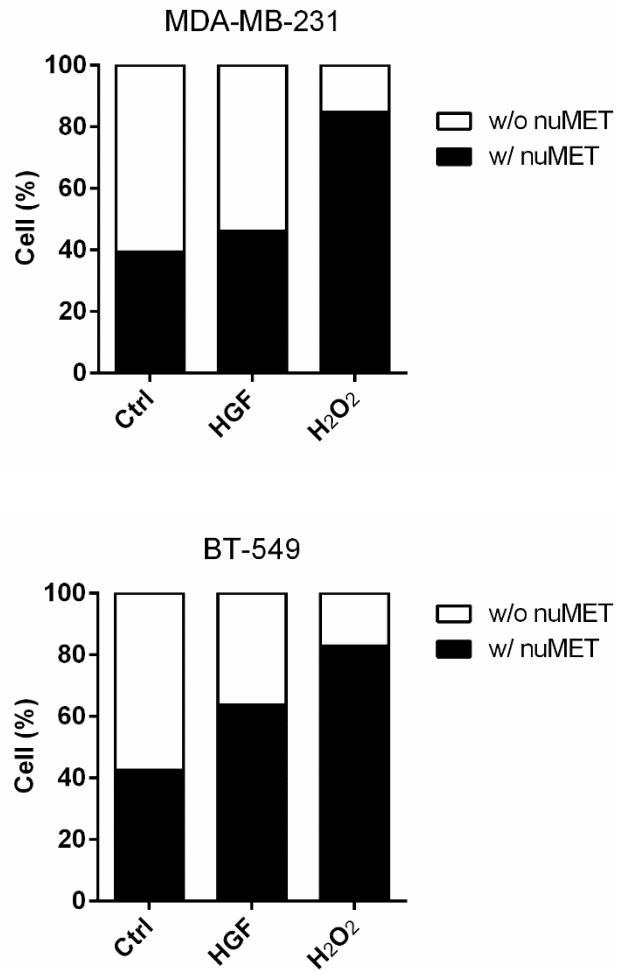
Figure 14. MET subcellular location after HGF or H₂O₂ treatment. **A,** Cells were treated with 100 ng/mL HGF for 2 h or H_2O_2 for 30 min and subjected to immunostaining with anti-MET (green fluorescence, Santa Cruz) and DAPI. **B,** The percentage of MET-containing cell nuclei in microcopy images. The nucleus were indicated by DAPI, and at least 50 nuclei were counted in each group.

Figure 15 (A)
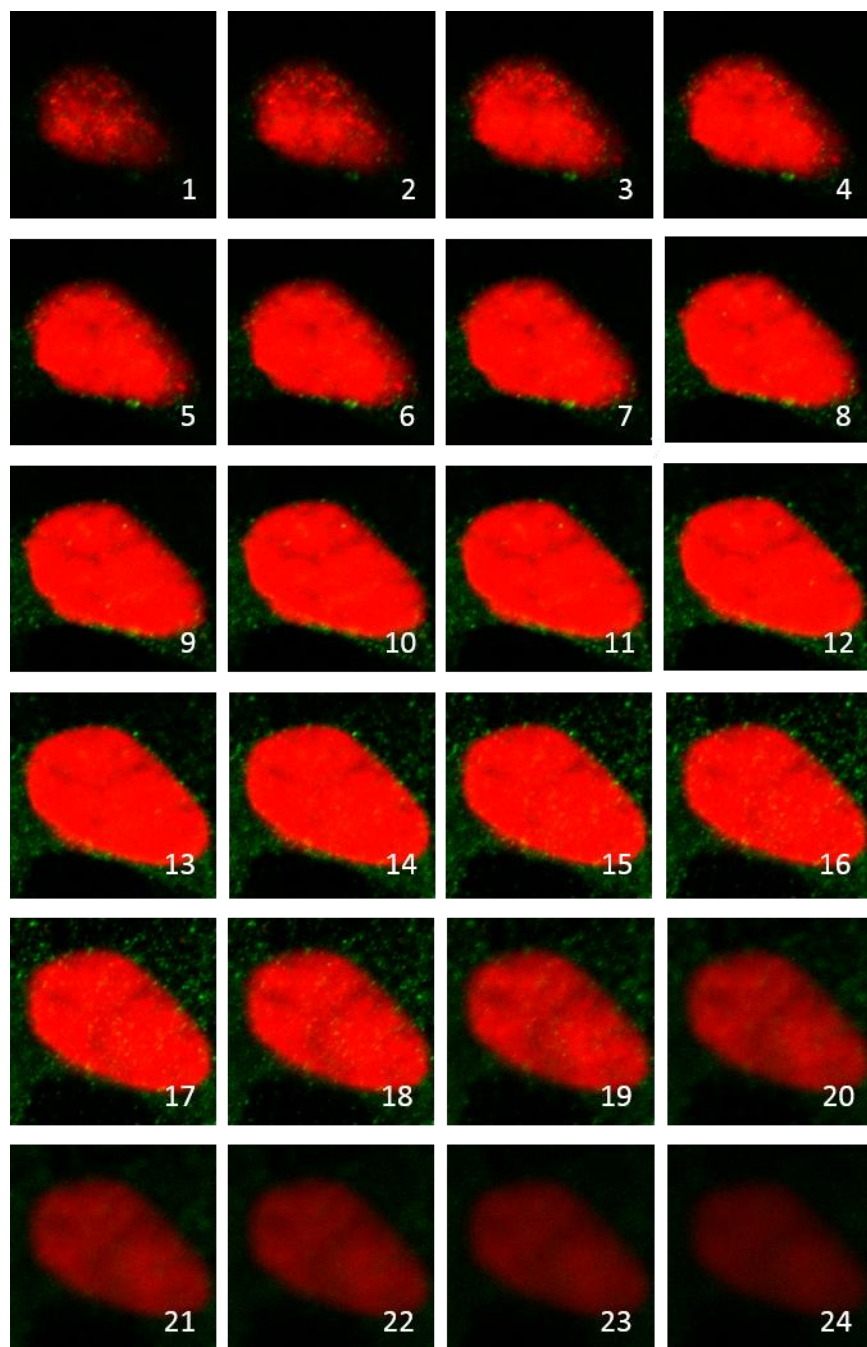
Figure 15 (B)
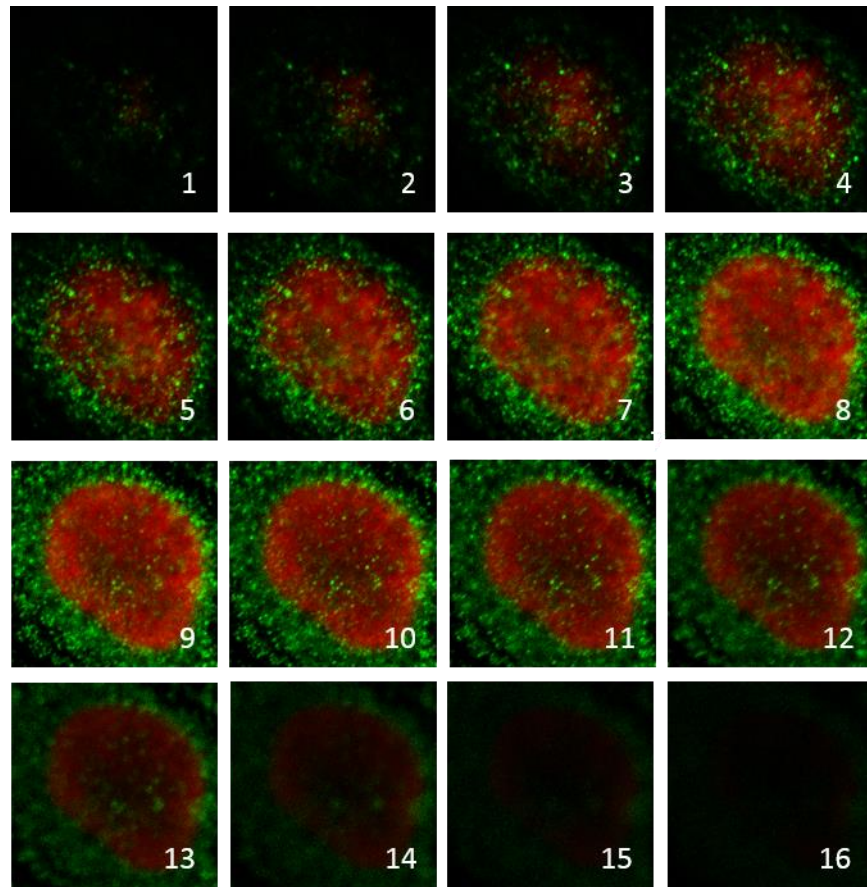
Figure 15 (C)
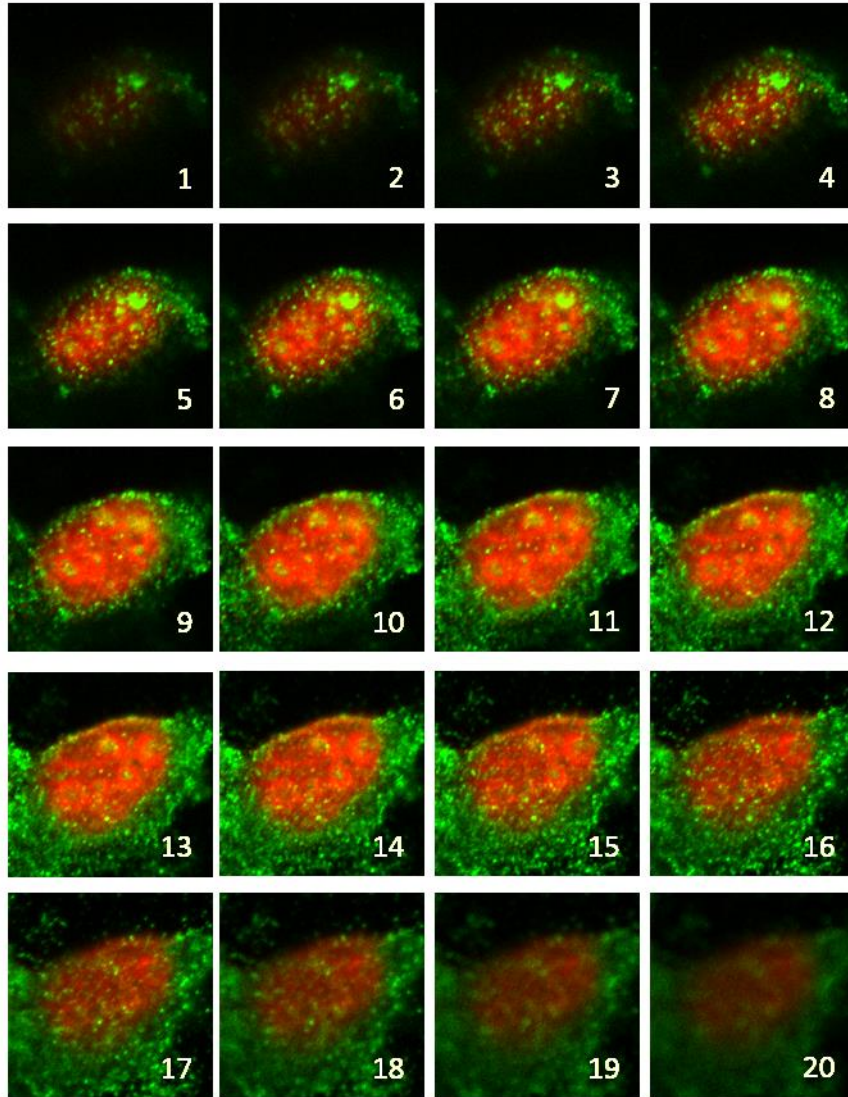
Figure 15. Z-stack confocal imaging of MET in MDA-MB-231 cells.   The cells were treated with **A,** untreated group **B,** 100 ng/mL HGF for 2 h and **C,** 10 µM H_2O_2 for 30 min before fixed and subjected to confocal microscopy.   The interval between slices is 0.30 µm.

3.2   MET Internalization from Plasma Membrane to Golgi Apparatus

Based on the detection of the MET holoreceptor in nucleus, we speculated that MET nuclear trafficking might also follow the membrane-bound trafficking mechanism of EGFR. EGFR nuclear translocation begins with receptor endocytosis and microtubule dependent cargo transportation to Golgi apparatus (Du et al., 2013).

3.2.1 MET internalization is microtubule-dependent.

Because membrane-bound cargo transport requires microtubules, we asked whether disrupting microtubule assembly also affects MET nuclear transport.   Nocodazole is an anti-mitotic drug, and it is known to inhibit microtubule polymerization through binding with tubulin (Xu et al., 2002).   Western blotting data showed that H_2O_2-induced MET nuclear translocation was reduced in cells pre-treated with nocodazole (Fig. 16), indicating microtubule is crucial to MET nuclear translocation.
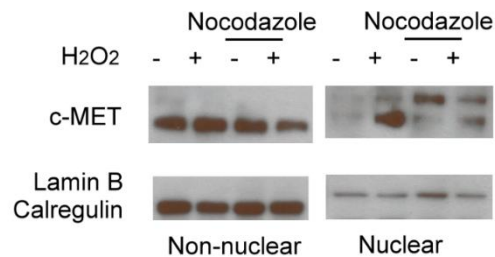
Figure 16. Nocodazole inhibits H_2O_2-induced MET nuclear translocation.   MDA-MB-231 cells were treated with nocodazole 30 min before 10 μM H_2O_2 30 min treatment.

In the cellular cargo trafficking mechanism, dynein and kinesin are two important motor proteins which carry the cargo vesicle along the microtubule (Berg JM, 2002). Moreover, dynein has been reported to affect EGFR nuclear transportation (Du et al., 2013), raising the possibility that dynein also involved in MET nuclear transportation. However, MET is widely spread in the cytosol of MDA-MB-231 and BT-549 cell, and that leads to the difficulties in investigating the trafficking cross the cytosol by confocal imaging.

3.2.2 MET nuclear trafficking mechanism is Golgi apparatus dependent.

As demonstrated by *Du et al.,* functional Golgi apparatus is essential in EGFR nuclear transportation in transfer the receptor from endocytic vesicles to ER (Du et al., 2013). To validate if Golgi apparatus is also important in MET transportation, the brefeldin A (BFA) is introduced to disrupt normal Golgi apparatus function by jeopardizing its structure. In this study, MDA-MB-231 cells were treated with BFA for 30 min prior to H_2O_2 treatment. Results from Western blot analysis showed that BFA treatment inhibited MET holoreceptor nuclear translocation (Fig.17), suggesting that MET holoreceptor translocates to the nucleus via a retrograde mechanism through the Golgi apparatus.
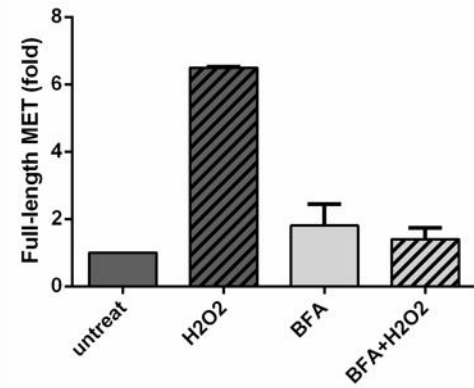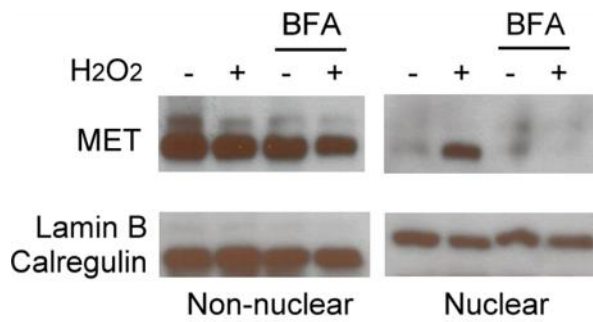
Figure 17. BFA blocks H₂O₂-induced MET nuclear transportation. MDA-MB-231 cells were pretreated with 5 μM BFA for 30 min prior to H_2O_2 treatment (10 μM) for 30 min before cellular fractionation.

3.3   COPI and Sec61-mediated Retrograde Transportation of MET

3.3.1 COPI-mediated MET retrograde transportation

To test MET membrane-bound nuclear retrograde trafficking mechanism between the

Golgi apparatus and ER, we knocked-down the COPI in MDA-MB-231 cell by shRNA to

diminish vesicle trafficking from Golgi to ER.   Of the five COPI knockdown stable clones

generated, two clones showed at least 50% inhibition of COPI protein expression as evaluated

by Western blot analysis (Fig. 18).
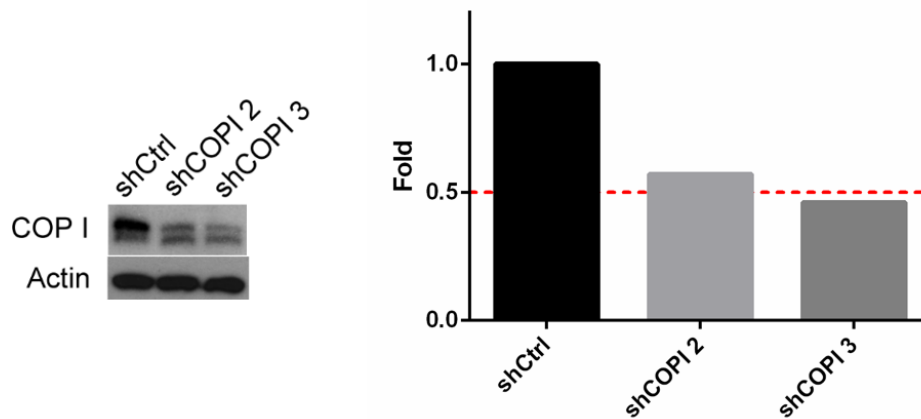
Figure 18. COPI knockdown efficiency in MDA-MB-231 cells.   The shRNA were
stable transfected to MDA-MB-231 cells through retrovirus infection.   The transfectants
were then selected with 1 µg/mL puromycin for 2 weeks to get stable clones.

As shown in Figure 19 A, the Western blotting data shows that knocking down COPI

significantly decreased H_2O_2-induced MET nuclear translocation compared with scrambled

control (shCtrl).   Meanwhile, similar phenomenon was also observed with 100 ng/mL HGF

treatment (Fig. 19 B).   These data suggest COPI-mediated vesicle retrograde transportation

mechanism is crucial to MET nuclear transportation.

(A)
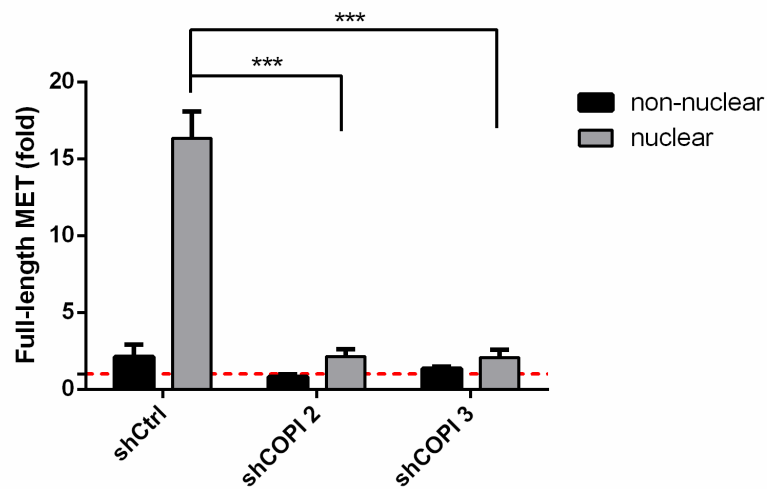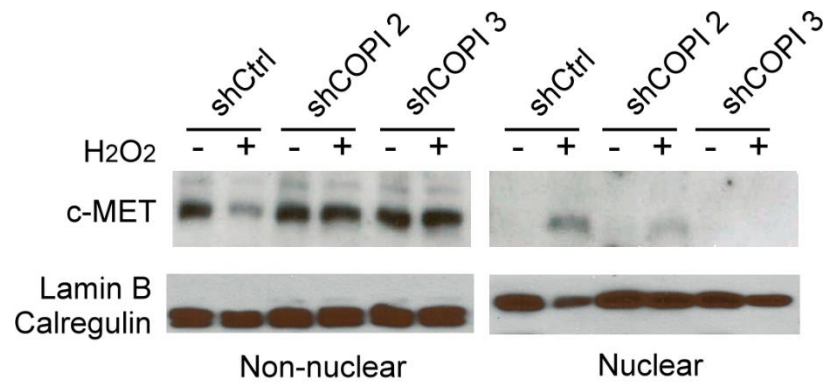
(B)
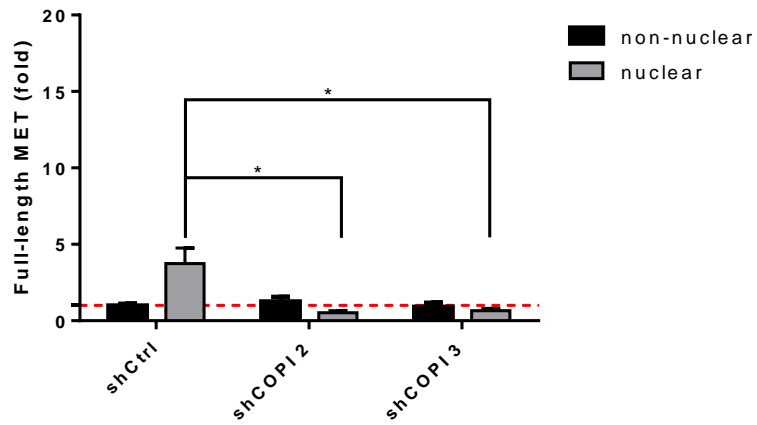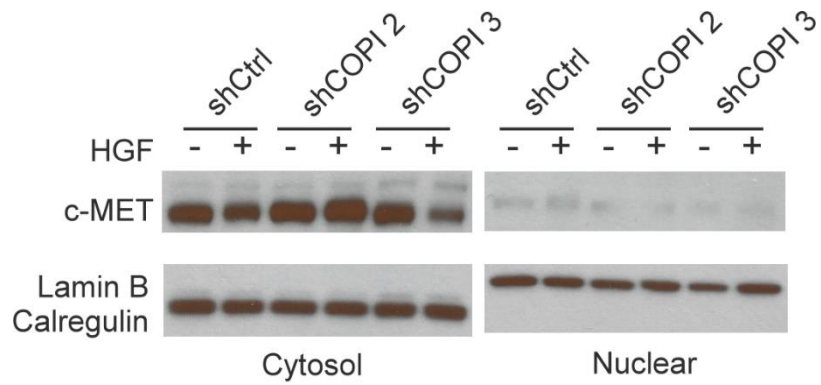
Figure 19. Knock-down COPI diminishes MET nuclear translocation. MDA-MB-231 shCOPI stable clones were treated with **A,** H_2O_2 and **B,** HGF before fractionation, and isolated fractions were subjected to Western blot analysis with the indicated antibodies.

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The immunofluorescent images also showed less MET-containing cell population after ligand and H_2O_2 treatment in COPI knocked-down cells than in control cells (Fig.22). Together, these results indicate that COPI plays an important role in MET nuclear trafficking and support our hypothesis that MET holorecpetor retrograde transports from Golgi to ER through a COPI-mediated vesicle trafficking mechanism.

3.3.2 Sec61-mediated MET nuclear transportation

MET is also speculated to translocate into nucleus through ER, which would require the Sec61 translocon complex.   According to previous studies, Sec61β is reported to be involved in membrane-bound nuclear transport of many RTKs (Wang et al., 2010c).   Here we used shRNA to knockdown Sec61β in MDA-MB-231 cells.   Among five stable knockdown clones generated, two clones showed at least 50% inhibition of Sec61β protein expression in Western blotting analysis (Fig. 20).
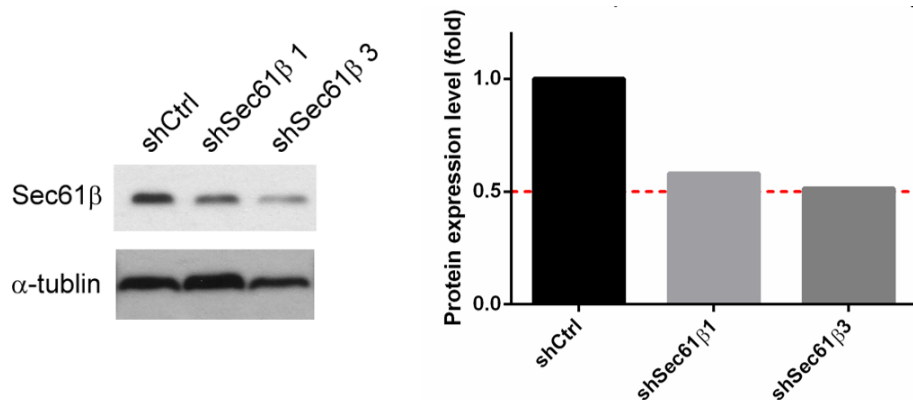
Figure 20. Sec61β knockdown efficiency in MDA-MB-231 cells. The shRNA were stable transfected to MDA-MB-231 cells through retrovirus infection. The transfectants were then selected with 1 μg/mL puromycin for 2 weeks to get stable clones.

In this study, we showed both ligand- and H_2O_2-induced MET nuclear accumulation significantly decreased in Sec61β knockdown cells in compare to the knockdown control cells (Fig. 21). The inhibition effect was further validated by confocal microscopy (Fig. 22). Together, these data provide evidence to show that MET travels through the ER for its nuclear translocation and it depends on Sec61 translocon complex to transport from ER into nucleus.

(A)
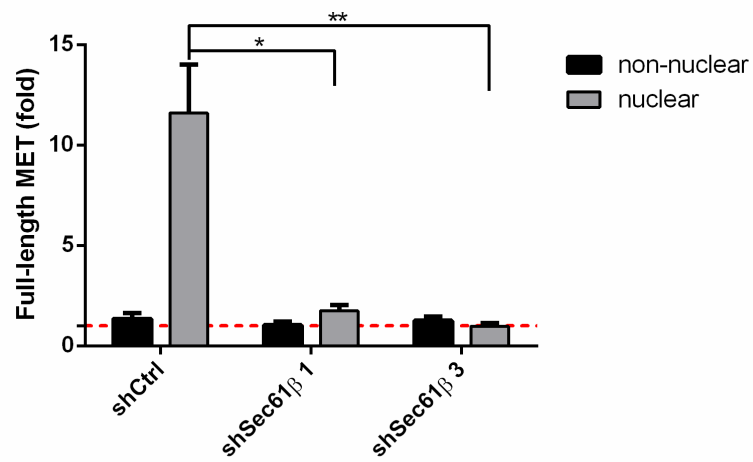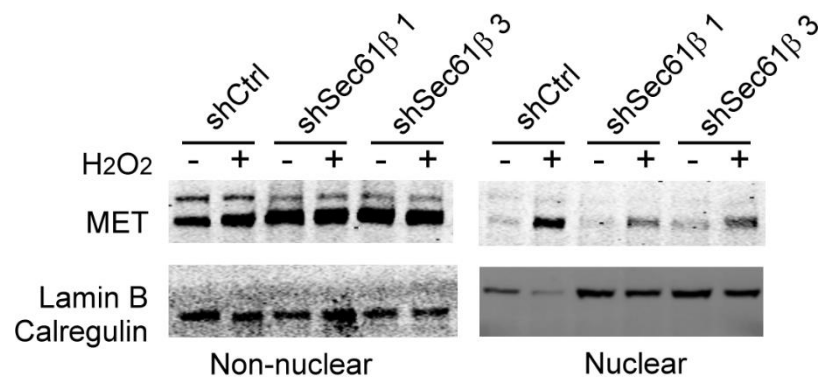
(B)
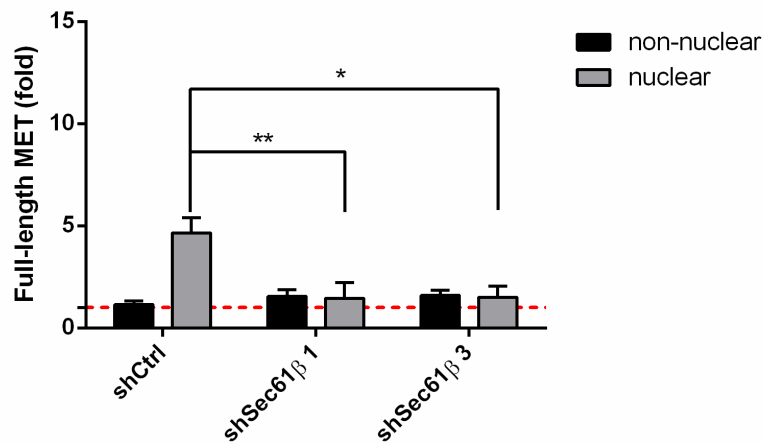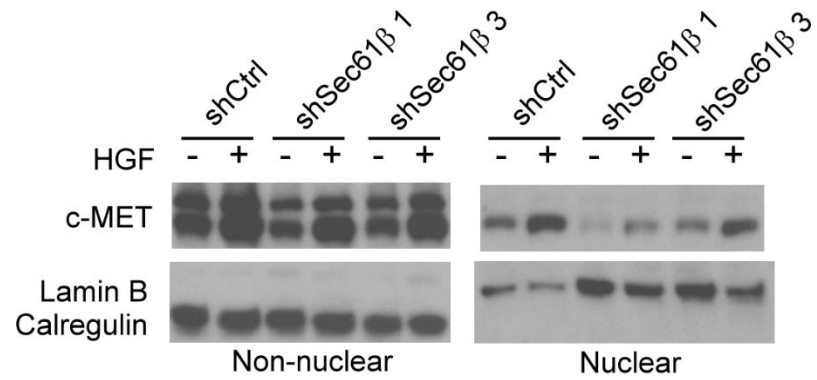
Figure 21. Knock-down Sec61β diminishes MET nuclear translocation. MDA-MB-231 shSec61β stable clones were treated with **A,** H_2O_2 and **B,** HGF before fractionation, and isolated fractions were subjected to Western blot analysis with the indicated antibodies.
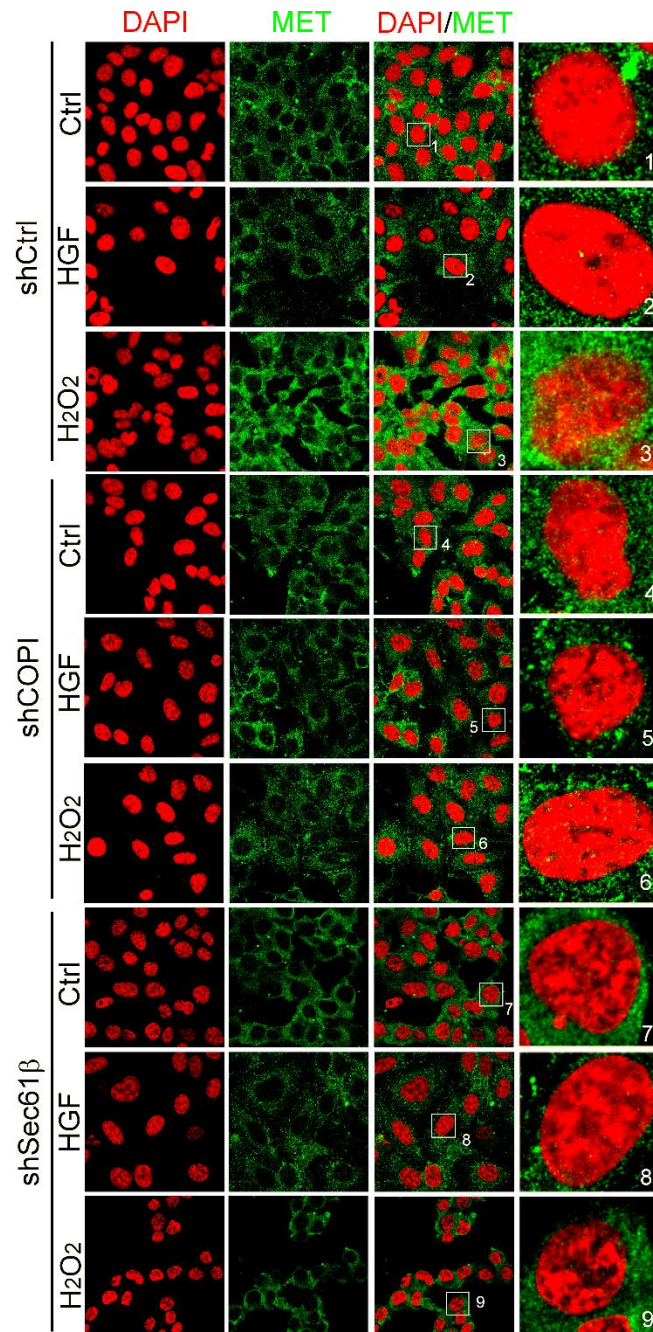
Figure 22. MET subcellular location in COPI and Sec61β knockdown MDA-MB-231 cells. Cells were treated with 100 ng/mL HGF for 2 h or H_2O_2 for 30 min and subjected to immunostaining with anti-MET and DAPI.

3.4　Membrane-bound MET trafficking through organelles

This study has demonstrated that MET is retrograde transported from plasma membrane, Golgi apparatus and ER to nucleus. The organelle membrane fraction were then examined to investigate if MET remains membrane-bound throughout the transportation process. The organelle membrane were collected by using ProteoExtract® Subcellular Proteome Extraction Kit and then analyzed by Western blotting. According to the instructions provided with the kit, the cells were swelled and the proteins that are not membrane bounded were collected as cytosolic fraction, then the organelle membrane as well as membrane bounded proteins were collected in the organelle membrane fraction. As shown in the figure, most of MET appears in the organelle fraction but not the cytosolic fraction after H_2O_2 treatment, indicating that MET remains membrane bounded during the transportation (Fig. 23).
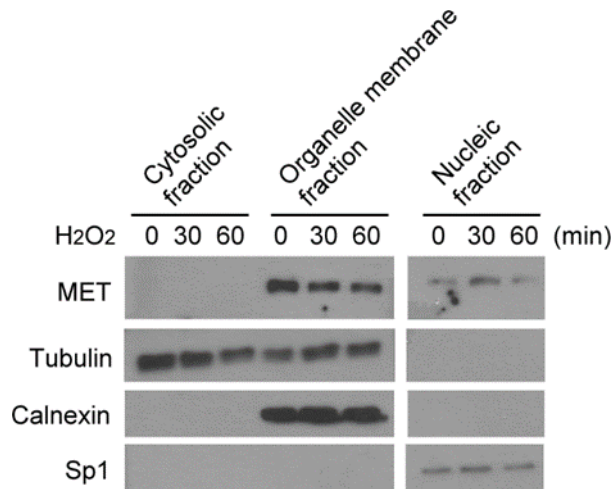
Figure 23. MET locates in the organelle membrane fraction during nuclear transportation. MDA-MB-231 cells were treated with 10 μM H_2O_2 for the time indicated and then harvested by using ProteoExtract® Subcellular Proteome Extraction Kit following the instructions provided. The protein extracts were analyzed through Western blotting. Tubulin were used to indicate the cytosol fractions, calnexin were used to indicate the organelle membrane, and Sp1 were used to indicate the nucleic fraction.

Based on the data shown by *Wang et al.,* RTKs follow the INTERNET mechanism will mainly detected in the organelle membrane fraction, while the RTKs which follow INFS transprtaion mechanism, for example, the FGFR-1, will be detected in the cytosolic fraction (Wang et al., 2012). Thus, the data shown in Figure 23 can leads to the conclusion that MET nuclear transportation belongs to the INTERNET mechanism.

Chapter 4

Conclusion and Discussion

To date, the function of nuclear MET is not well characterized as MET nuclear localization is not universally inducible by ligand (Gomes et al., 2008; Matteucci et al., 2009; Pozner-Moulis et al., 2006) and is not the main response to ligand stimulation. Furthermore, it is difficult to distinguish the function of nuclear MET from traditional RTK signaling cascade under ligand stimulation. Here, we demonstrate that both ligand- and H_2O_2-induced MET nuclear translocation follows a previously reported membrane-bound vesicle transport mechanism. However, we found that the level of MET nuclear translocation was significantly different between ligand treatment and stress stimulation.

Built on the basis of the previously identified membrane-bound EGFR nuclear trafficking mechanism (Wang et al., 2012), we show that full-length MET also utilizes the INTERNET mechanism for tis intracellular transport, indicating that this trafficking mechanism is not restricted to the EGFR family, and suggesting MET may also have biological functions in nucleus that are similar to EGFR.

The average HGF concentration in the serum of breast cancer patients has been reported to be about 0.5 ng/mL (Taniguchi et al., 1995), which suggests that MET nuclear translocation is not the primary response to HGF under physiological condition. Our results indicated that cellular stress initiates the nuclear translocation and non-canonical MET signaling more

potently than traditional ligand stimulation. Moreover, doxorubicin treatment also induced the presence of MET in the nucleus, suggesting that MET nuclear translocation may function to overcome the stress, i.e. it may contribute to cell survival through manage replication stress or even involved in DNA repair and check point regulation. Some researchers reported that inhibiting both MET and EGFR kinase activity can induce synergistic effect in cancer treatments including breast cancer treatment (Castoldi et al., 2013; Mueller et al., 2010; Takeuchi et al., 2012). Further, it has been reported that acquired MET overexpression correlates to development of secondary EGFR TKI resistance in non-small-cell lung cancer (Cappuzzo et al., 2009; Engelman et al., 2007) and the overexpression of MET in breast cancer patients and the association of EGFR/MET contribute to EGFR TKI resistance in breast cancer (Mueller et al., 2010).

In summary, the discovery of nuclear MET function may uncover a mechanism by MET overexpression promotes cell survival and drug resistance in cancer cells, while the understanding of transportation mechanism, which our study contributes, provides a potential mechanism to block nuclear transportation of MET.

Chapter 5 Bibliography

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Chapter 6 Vita

Mei-Kuang Chen was born in Changhua city, Taiwan on December 11, 1986, the daughter of Shu-Chin Wang and Shan-Trang Chen. After completing her work at National Taichung Girls' Senior High School, Taichang, Taiwan in 2005, she entered Department of Life Science, College of Life Science at National Tsing Hua University, Hsinchu, Taiwan. She finished her undergraduate thesis and received the degree of Bachelor of Science in June, 2009 with a major in Life Science. She entered Institute of Molecular Medicine, College of Life Science at National Tsing Hua University in 2009. She received the degree of Master of Science in July, 2011. Her manuscript *Delay of gap filling during nucleotide excision repair by base excision repair: the concept of competition exemplified by the effect of propolis* was published by *Journal of Toxicological Science* in May, 2011. In the next year, she worked as administration assistant and research assistant in Department of Life Science at National Tsing Hua University. In August of 2012, she entered The University of Texas Graduate School of Biomedical Sciences at Houston.

Permanent address:

5 F., No 64, Gong-Xiao St.
Changhua City, Changhua County   50075
Taiwan R.O.C.