Elucidating the role of mitochondrial-localized hepatocyte growth factor receptor in gastric oncogenesis

Sim, Kae Hwan

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**ELUCIDATING THE ROLE OF MITOCHONDRIAL-
LOCALIZED HEPATOCYTE GROWTH FACTOR RECEPTOR
IN GASTRIC ONCOGENESIS**

SIM KAE HWAN

SCHOOL OF BIOLOGICAL SCIENCES

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SIM KAE HWAN

School of Biological Sciences

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Abbreviations

2D-GE	Two-dimensional gel electrophoresis
ABB	Ammonium bicarbonate buffer
ACLY	ATP citrate lyase
ACN	Acetonitrile
AP2A2	Adaptor-related protein complex 2, alpha subunit
AP2B1	Adaptor-related protein complex 2, beta 1 subunit
APEX	Absolute protein expression
AQUA	Absolute quantification
BCA	Bicinchoninic acid protein assay
BSA	Bovine serum albumin
CA	Carbohydrate antigen 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHAPS	hydrate
co-IP	co-immunoprecipitation
COX-IV	Cytochrome C oxidase subunit IV
DAPI	4', 6-Diamidino-2-phenylindole
DAVID	The database for annotation, visualization and integrated discovery
DNAJA3	DnaJ (Hsp40) Homolog, Subfamily A, Member 3
DTT	Dithiothreitol
EEA1	Early endosome antigen 1
EGFR	Epithelial growth factor receptor
EGTA	Ethylene-bis(oxyethylenenitrilo)tetraacetic acid
emPAI	Exponentially modified protein abundance index
EPS15	Epidermal growth factor receptor pathway substrate 15
ER	Endoplasmic reticulum
ErbB2	Human epidermal growth factor receptor 2
FA	Formic acid
FASN	Fatty acid synthase
FDR	False discovery rate
GAB1	GRB2-associated binding protein 1
GO	Gene Ontology
GRB2	Growth factor receptor-bound protein 2

HCD	High-energy collisional dissociation
HER2	Human epidermal growth factor receptor 2
HGF	Hepatocyte growth factor
HGFR	Hepatocyte growth factor receptor
HIP1R	Huntingtin interacting protein 1 related
HMGA1	High mobility group AT-hook 1
HOAc	Acetic acid
HPLA	High performance liquid chromatography
HSC70	Heat shock protein 70 kDa
IAA	Indole-3-acetic acid
ICAT	Isotope-coded affinity tag
IgG	Immunoglobulin G
IMDM	Iscove's modified Dulbecco's medium
iTRAQ	Isobaric tags for relative and absolute quantification
KPNB1	Karyopherin (Importin) beta 1
LAMP1	Lysosomal-associated membrane protein 1
LC-	
MS/MS	HPLC coupled to tandem mass spectrometry
MALDI	Matrix-assisted laser desorption ionization
MAPK	Mitogen-activated protein kinase 1
MCAT	Mass-coded abundance tagging
MET	Hepatocyte growth factor receptor
MRM	Multiple reaction monitoring
MS	Mass spectrometry
mtMET	Mitochondrial-localized MET receptor
NH ₄ OH	Ammonium hydroxide
NSCLC	Non-small cell lung cancer
PFA	Paraformaldehyde
PI3KR1	Phosphoinositide-3-kinase, regulatory subunit 1
PICALM	Phosphatidylinositol binding clathrin assembly protein
PKM2	Pyruvate kinase, M2 splice isoform
PLA	Proximity ligation assay
PLCG1	Phospholipase C, gamma 1

PSAQ	Protein standard absolute quantification
QconCAT	Concatemer of standard peptides for absolute quantification
RPMI	Roswell Park Memorial Institute medium
RTK	Receptor tyrosine kinase
SDS	Sodium dodecyl sulfate
SDS-	
PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SILAC	Stable isotope labeling by amino acids in cell culture
SIn	Normalized Spectral Index
SMI	Small-molecule inhibitor
SRC	SRC proto-oncogene, non-receptor tyrosine kinase
SRM	Selected reaction monitoring
STAT3	Signal transducer and activator of transcription 3
STRING	Search tool for the retrieval of interacting genes/proteins
TCEP	Tris(2-carboxyethyl)phosphine
TFA	Trifluoroacetic acid
TKI	Tyrosine kinase inhibitor
TMT	Tandem mass tag
TOF	Time of flight
TOM20	Translocase of outer mitochondrial membrane 20
XIC	Extracted ion chromatogram

Abstract

Gastric cancer is one of the most common malignancies and leading cause of cancer-related death worldwide. Tremendous reports have demonstrated that a wide variety of receptor tyrosine kinases play a causal role in gastric cancers. Receptor tyrosine kinase MET, *aka.* hepatocyte growth factor receptor (HGFR), has been frequently found dysregulated in gastric carcinomas. MET is a single-pass receptor tyrosine kinase localizing on plasma membrane and specific for hepatocyte growth factor/scatter factor (HGF/SF)¹. Activation of MET/HGF regulates many cellular processes, such as proliferation, survival, motility, angiogenesis and morphogenesis. In gastric cancer, aberrant MET/HGF signaling pathway has been found dysregulate cell proliferation, contributing to angiogenesis, oncogenesis, tumor cell invasion and metastasis, and protection from apoptosis in cancer cells. Therapeutic strategies targeting MET/HGF thus hold promise for gastric cancer. Handfuls of studies on MET signaling in gastric cancer have looked into the underlying mechanisms of dysregulated MET signaling in gastric oncogenesis. Recently it was reported that several RTKs including MET can translocalize into mitochondria of cancer cells. An important role of mtMET (the mitochondrial-localized MET receptor is referred to as mtMET) in cancers is suggested. In this study, we aim to understand the function and substrate of mtMET of SNU5 gastric cancer cell line. Based on preliminary data from qualitative profiling of mitochondrial proteome, we identified different components of endocytosis pathway. Functional assays using different endocytic inhibitors coupled with immunofluorescence staining approach were applied to study the translocalization of MET into mitochondria. Abundance and phosphorylation of MET in mitochondria were severely disrupted when endocytosis was inhibited. We proposed that MET is activated and endocytosed, followed by translocation of a portion of MET into mitochondria of cancer cells. Moreover, our findings have suggested the participation of other mechanisms in the mitochondrial translocalization of MET. Co-immunoprecipitation and TMT-based high-

throughput quantitative proteomic approach were also adopted to identify the potential substrates of mtMET in mitochondria. Among the proteins that were identified at high confidence level, a major fraction is involved in catalytic activity and metabolic process in mitochondria. Proximity ligation assays have revealed that mtMET has direct protein-protein interaction with two novel substrates, HMGA1 and PKM2. Taken together, this report suggested a novel functional role of mtMET in regulation of energy metabolism in mitochondria. The present study has disclosed a novel paradigm of mtMET signaling in gastric cancer cells and thus contributed to the ongoing efforts to therapeutically target aberrant RTK activities in human cancers.

Chapter 1

General introduction

1.1 Introduction

1.1.1 Cancer

1.1.1.1 Cancer

Cancer is a collection of related diseases involving abnormal cell growth with the ability to invade or transmit to other normal parts of the body of patients. It is also known as malignant tumor or neoplasm. More than a hundred kinds of different cancers have been identified. Various cancers are characterized by these six common properties: (1) Cancer cells have self-sufficiency in growth signaling; (2) Cancer cells are normally insensitive to anti-growth signaling; (3) Cancer cells are often protected from apoptosis and “immortal”; (4) Cancer cells have a high proliferative rate and a limitless replicative potential; (5) Cancer cells are able to “modify” the surrounding microenvironment to induce angiogenesis for sustainable growth; (6) Cancer cells have ability to undergo metastasis and invade other parts of body². According to GLOBOCAN 2012, there are about 14.1 million cases of cancer occurred all over the world annually and the number is increasing year by year³. Overall around 8.2 million deaths are caused by cancers, which is about 14.6 % of all human deaths in one year³.

Cancer can develop in different cell types in human body. Cells grow and proliferate to generate new cells, which will then replace the old and damaged cells in human body. However, when this orderly process goes wrong, cancer cells start to form, which is known as oncogenesis or tumorigenesis (Figure 1.1).

Figure 1.1: Simple illustration of the initiation and progression of cancer.

A large number of researches have been established to study genotypes and phenotypes of various human cancers. Scientists have generated a lot of information about the dysregulation of biological processes and genetic mutations identified in human cancers. Various anti-cancer drugs and therapeutics have also been proposed. Nevertheless, after decades of “war on cancers”, the objective of totally cure a cancer still remains a highly challenging endeavor. A research group from Kiel University has recently discovered that hydra is able to form tumors like those formed in human cancers⁴. Their findings suggested that development of cancer might be “an intrinsic property” in cells and proposed to unleash individual immune system against cancer cells as a novel cancer therapy. In this regard, a vast number of future works is necessary in hope of provoking a new dialogue about the various biological connections to human cancers.

1.1.1.2 Gastric cancer

Gastric cancer, *aka.* stomach cancer, refers to the malignant tumors arising from any part of stomach. It is one of the most common malignancies in Asian countries and ranked as the fifth most common cause of cancer related death in both sexes worldwide⁵⁻¹³. Comparative studies among different countries have shown striking differences in the incidence and the overall five-year survival rate of gastric cancer patients^{3, 12-14}. According to GLOBOCAN 2012, 71 % of newly diagnosed cases of gastric cancer were found in less developed countries: Asian and European countries have the highest incidence rate of gastric cancer, while the lowest incidence rate was found in Africa and Northern America³. In Eastern Asia, mortality rates of about 28.1 per 100,000 in men and 13.0 per 100,000 in women were demonstrated^{12, 15-17}. In Africa and Northern America, even though gastric cancer is a relatively infrequent neoplasm that has low incidences, it largely contributes to the cancer related death and is the third most common and lethal gastrointestinal malignancy ranked after colorectal and pancreatic cancer^{16, 18}. It has been estimated that gastric cancer causes 740,000 deaths globally with nearly one million newly diagnosed cases each year, and with overall five-year survival rates ranging from 90 % to less than 5 %^{7, 19-21}. Statistical studies have shown that 65 % of the newly diagnosed gastric cancer cases are always identified at the advanced stage of the disease, with around 85 % of tumors accompanied by lymph node metastasis at diagnosis²². For those without metastases, only about half are suitable for a total or subtotal gastrectomy, which is the only curative modality with a potentially therapeutic effect to gastric cancer thus far^{16, 23}. Nevertheless, the recurrence rates of cancer after surgical resection are as high as 40 % to 65 %²⁴. In most countries except Japan, the overall five-year survival rates of gastric cancer patients after curative surgical resection were found ranging from about 10 % to 30 %^{25, 26}.

Nowadays, many factors that will raise the risk for occurrence of gastric cancer have been identified, for example, smoking, being overweight or obese, consuming diets high in smoked, pickled, or salty foods, chronic stomach inflammation, bacterial or virus infection, familial inheritance of certain genes, etc. However, the exact factor that causes gastric cancer cells start growing is not clear yet. Many precautionary measures are thus recommended to lower the risk for gastric cancer. It has been suggested that keeping a good lifestyle and sanitation^{14, 27}, taking diet rich in fresh fruits and vegetables²⁸, low or no alcohol and nicotine consumption^{28, 29} associate with a reduced risk of gastric cancer development. Taking good care of individual hygiene^{30, 31} also helps to lower the risk of gastric cancer as a result of lowering the infection with *Helicobacter pylori*, which is a Gram negative rod shaped bacterium classified as class I gastric carcinogen³². Regrettably, although there has been a steadily declining incidence of gastric cancer over the past few years, it has not been matched with a proportionate decrease in mortality rate worldwide^{13, 18, 33}. This bleak situation is mainly due to the lack of highly sensitive, specific and effective diagnostic or prognostic methods for gastric cancer³⁴. Also, there is no apparent symptom during the development of early-stage gastric cancer. It is expected that, if gastric cancer can be detected and cured at early stages, five-year survival rate of patients would be higher than 90 %^{33, 35}. Mass screening of asymptomatic populations and surveillance of subjects at risks are recommended in high-incidence countries³⁶. Diagnostic tools, such as endoscopic ultrasound^{37, 38}, upper gastrointestinal ³⁹ barium study⁴⁰, computed tomography⁴¹, magnetic resonance imaging⁴², abdominal ultrasound⁴³, positron emission tomography scan⁴⁴, and staging laparoscopy¹, have been developed and applied for screening of various cancers including gastric cancer. Notwithstanding their benefits of mass screening for gastric cancers, disadvantages including high cost and dependence on professional skills, varied level of detail shown, not-high-enough accuracy, and risks they could present to patients set back the popularization of these diagnostic tools³⁶. Thereby, current ongoing research aims to fully understand the underlying mechanisms of gastric

oncogenesis and to improve the efficacy of different approach to prevent, diagnose, prognose and cure gastric cancer.

1.1.2 Receptor tyrosine kinase

RTKs (Receptor tyrosine kinases)⁴⁵ are a subclass of high-affinity cell surface receptors for various growth factors, cytokines, and hormones, with intrinsic ligand-regulated tyrosine kinase activity⁴⁶. Since 1984, when the primary structure of the first RTK, EGFR (epidermal growth factor receptor)⁴⁷, was elucidated, extensive research efforts have been made towards understanding the important functions of RTKs in regulating cellular signaling pathways^{45, 48}. It is now well established that RTK activity in normal cells is tightly associated with numerous cellular processes, including cell proliferation and differentiation, cell migration, metabolism, survival, and cell-cell communication during development. However, when RTKs are mutated or structurally altered, they become potentially oncogenic⁴⁹. Irregular and dysfunctional RTK activity has been demonstrated during oncogenesis of a range of human cancers. For instance, amplification, overexpression, and/or somatic mutation of EGFR has been identified in patients with non-small cell lung cancer⁵⁰, esophageal cancer⁴⁷, gastric cancer⁵¹, liver cancer⁵², head and neck cancer⁵³, and adrenocortical carcinoma⁵⁴ amongst others. Dysregulation of other RTKs, such as fibroblast growth factor receptor, Fms-related tyrosine kinase 3, platelet derived growth factor receptor, and vascular endothelial growth factor receptor have also been observed in a wide variety of human cancers⁴⁵. As a consequence, understanding both the molecular architecture and the key roles of RTKs, together with their ligands, in tumorigenesis forms a rational goal in the development of target-specific cancer therapeutics⁴⁵.

In recent years, targeting particular RTKs has been attempted using various inhibitory compounds, including humanized monoclonal antibodies and SMIs (small-molecule inhibitors)⁵⁵. Monoclonal

antibodies, such as trastuzumab (Herceptin, Genentech, Inc.), for HER2-overexpressing metastatic breast cancer⁵⁶, and cetuximab (IMC-C225 (Erbix), ImClone Systems/Merck KGaA), for EGFR-overexpressing colorectal cancer⁵⁷, can selectively target their corresponding RTKs. Nevertheless, the use of monoclonal antibodies is restricted by a number of factors, including their sizes, the expression of heterogeneous antigen, and the expression of targeted antigens in normal cells⁵⁸. On the other hand, the development of SMIs, which typically result in fewer adverse side effects than conventional chemotherapeutics⁵⁹⁻⁶¹, has drawn considerable interest as their mechanisms of action are better understood. Significant progress has been made in the pharmaceutical development of small-molecule anticancer drugs in recent years⁶²⁻⁶⁴. In 1996, the pharmacological characteristics and inhibitory activities of both gefitinib (Iressa, ZD1839, AstraZeneca), a selective SMI of mutated EGFR in NSCLC⁶⁵, and imatinib (Gleevec, STI571, Novartis; *aka* Gleevec in USA), a potent inhibitor of the BCR-ABL oncoprotein⁶⁶, were reported. However, due to the rapid proliferation of cancer cells, selective pressure leading to resistance against anticancer SMIs and/or mutation of binding sites on RTKs, the efficacy of current therapeutic approaches is limited. Powerful inhibitors that can tolerate multiple amino acid mutations in the RTKs and/or target alternative sites on aberrant RTKs are essentially required^{67, 68}.

1.1.3 MET

1.1.3.1 MET signaling in cancers

MET, *aka*. HGFR, is a receptor tyrosine kinase encoded by *MET* proto-oncogene in human. After the primary single chain precursor (170 kDa) is synthesized and glycosylated (190 kDa), MET precursor protein is post-translationally cleaved to generate two subunits: a highly glycosylated extracellular α chain (50 kDa) and a single-pass transmembrane β chain (145 kDa)⁶⁹, which then form a disulphide-linked

heterodimer. When HGF binds to MET, MET becomes activated and undergoes trans-phosphorylation on two tyrosines (Tyrosine 1234 and 1235) locating in the catalytic site, followed by the phosphorylation of another two tyrosines (Tyrosine 1349 and 1356) locating in the carboxy-terminal tail, creating a multifunctional docking site which will recruit a wide variety of intracellular signaling effectors and transduce the extracellular signals to intracellular signaling pathways⁶⁹⁻⁷¹. MET signaling is involved in multiple significant biological processes, including cell motility, developmental morphogenesis, wound repair, angiogenesis, transcriptional regulation of numerous genes, *etc.* Multiple signaling transduction pathways are induced after MET activation, including Ras/Raf pathway, PI3k/Akt pathway, STAT pathway, Wnt pathway, Notch pathway, *etc.*⁷¹⁻⁷³. Extensive researches have been performed and much knowledge has been learnt about MET signaling pathway. While MET signaling is necessary for a large body of normal physiological activities, uncontrolled MET signaling has been strongly implicated in development and progression of various human cancers. Overexpression or mutation of MET has been identified in tumor biopsies of solid tumors, and abnormal constitutively active MET signaling was exhibited in many different human cancers⁷⁴⁻⁷⁶. As a result of its critical roles in different cancerous events, MET has been considered to be a major target in anti-cancer drug development. Several MET antagonists and SMIs are currently under clinical investigation⁷⁷⁻⁸¹. Multi-targeted therapies have also been suggested and demonstrated promise^{82, 83}. So far, the global picture of MET signaling in oncogenesis is still far from complete understanding and extensive study is required.

1.1.3.2 Mitochondrial localization of MET

All the attempts thus far to explain the efficacy of inhibitory compounds, however, are premised on blockading and targeting the conventional MET signaling pathways. The probability that MET oncoproteins may function in unrecognized, non-canonical pathways has received little

attention. Recently our group has discovered the presence of MET in mitochondria of SNU5 cells, a MET overexpressing gastric cancer cell line that is sensitive to PHA-665752, a proven highly specific ATP competitive SMI of the catalytic activity of MET⁸⁴. A recent report has shown a direct link between MET endocytosis and tumorigenesis using tumor-associated MET-activating mutations *in vitro* and *in vivo*⁸⁵. Furthermore, some recent studies demonstrated that ligand-induced receptor endocytosis and a mitochondrial localization signal are required for a directional translocation of RTK such as EGFR in endocytic vesicles towards the mitochondrial compartment⁸⁶⁻⁸⁸. Yao et al. also showed that mitochondrial translocation of EGFR might be partially caused by endocytosis followed by translocation of plasma-membrane-localized EGFR into mitochondria⁸⁹. To date, the mechanism of the mitochondrial expression of RTKs is still elusive. The previous studies taken together with our observations of the presence of MET in mitochondria and perturbed mitochondrial functions by MET selective inhibitor prompt us to hypothesize that MET is activated and phosphorylated on plasma membrane, subsequently internalized through endocytic pathway, from which a subpopulation of internalized activated MET is translocated into the mitochondria, modifying the mitochondrial functions and promote gastric oncogenesis, cancer cell survival and proliferation.

1.1.4 Mass spectrometry-based quantitative proteomics

Mass spectrometry is an analytical chemistry technique that can be used to determine the kind and amount of molecules present in a sample by measuring the mass-to-charge (m/z) ratio detected in a mass spectrometer. This powerful technique can also be applied in the elucidation of biological states of cells in discovery-driven research. At present, both relative and absolute mass spectrometry-based quantitative proteomic profiling have been extensively used to address the important biological and biomedical problems in cancer research⁹⁰⁻⁹⁵.

Moreover, both labeling and label-free approaches are available^{96, 97} (Figure 1.2).

Figure 1.2: Schematic representation of quantitative proteomics methods. Quantitative proteomic methods can be divided into absolute and relative quantitation. Each method can be further classified into labeling-based and label-free approach. AQUA, absolute quantification; SRM, selected reaction monitoring; MRM, multiple reaction monitoring; PSAQ, protein standard absolute quantification; QconCAT, concatemer of standard peptides for absolute quantification; emPAI, exponentially modified protein abundance index; APEX, absolute protein expression; SILAC, stable isotope labeling by amino acids in cell culture; ICAT, isotope-coded affinity tag; MCAT, mass-coded abundance tagging; iTRAQ, isobaric tags for relative and absolute quantification; TMT, tandem mass tags.

1.1.4.1 Label-free quantitative proteomic approach

For label-free quantitative proteomic methods, no stable isotope-containing compound is used to chemically tag the protein or peptide. As compared to labeling based quantitative methods, these techniques have a relatively low quantification precision. Despite the disadvantage, they have several benefits including low cost, suitability for any type of sample, applicability to unlimited number of samples, and providing higher sequence coverage of quantified proteins⁹⁶. These techniques give results of high analytical depth and dynamic range. Label-free quantification can be performed based on ion intensity of XIC (extracted

ion chromatogram), spectral counting, SIn (normalized Spectral Index) or on protein abundance based quantification techniques such as emPAI (exponentially modified protein abundance index) and APEX (absolute protein expression index)⁹⁸⁻¹⁰². In present study, emPAI is utilized to estimate the protein abundance in the co-immunoprecipitation experiments. During Mascot database search, emPAI value for each protein is calculated automatically according to the algorithm developed by Ishihama et al^{98, 103, 104}. The detailed description of emPAI value calculation will be discussed in Materials and Methods part of Chapter 3.

1.1.4.2 Labeling based quantitative proteomic approach

Different from label-free approaches, labeling based quantitative proteomic approaches use stable isotope-containing compounds to chemically label the sample at protein or peptide levels. Commonly used methods include SILAC (stable isotope labeling by amino acids in cell culture), iTRAQ (isobaric tags for relative and absolute quantification), ICAT (isotope-coded affinity tag), TMT (tandem mass tag), *etc*¹⁰⁵⁻¹⁰⁸. Labeling based approaches normally provide data of higher quantitative precision and less sensitive to experimental bias than label-free quantitative approaches. In present study, TMT 6-plex isobaric tag based quantitative approach is performed in peptide level to simultaneously quantify 6 biological samples in different states (Chapter 3). TMT 6-plex reagents are sets of isobaric compounds that are amine-reactive and NHS-ester-activated for covalent, irreversible labeling of the peptide amino terminus and free amino termini of lysine residues (Figure 1.3).

Figure 1.3: Schematic illustration of the isobaric tagging chemistry of TMT reagents.

As shown in Figure 1.3, each TMT 6-plex reagent has the same nominal precursor mass and shares an identical structure that consists of an amine-reactive NHS-ester group, a spacer arm and an MS/MS reporter. Upon collisionally activated dissociation, unique MS/MS reporter ions will be cleaved and released from the TMT-tagged peptides into the low mass regions, which will be used for subsequent relative quantification. This labeling quantitative technique has been successfully applied in our quantitative proteomic studies reported in Chapter 3.

1.1.5 Objective and overview of project

In this project, we aim to elucidate the unidentified molecular mechanism of MET translocation into mitochondria, and to identify the potential substrates and functions of mitochondria-localized MET in SNU5 gastric cancers. To achieve these objectives, different approaches were developed and utilized. Based on preliminary data from mitochondrial proteomic profiling of several cancer cell lines, we have identified the evidences of participation of clathrin-mediated endocytosis in mitochondrial-translocalization of MET. Using biochemical manipulations with specific inhibitors targeting different endocytic pathways, combined with the fluorescence microscopy and proteomic analyses, the role of endocytosis in the translocation of MET to mitochondria was studied and revealed. In subsequent phase, we aim to understand the global picture of the proteins associated with mtMET (mitochondrial-localized MET) in mitochondria and to identify the potential substrate(s) and thus the function of mtMET in gastric oncogenesis. Qualitative and quantitative proteomic profiling of the mitochondrial proteome of SNU5 gastric cancer cells were performed. We purified SNU5 mitochondria and performed co-immunoprecipitation of mtMET by applying a specific approach termed rapid

immunoprecipitation mass spectrometry of endogenous proteins with some modification¹⁰⁹. Isobaric tag-based quantitative proteomic profiling of SNU5 mitochondrial proteome was subsequently established using differentially labeled tags TMT coupled with multidimensional liquid chromatography and tandem mass spectrometry. Overall, four significantly identified proteins were potentially shortlisted as the candidate proteins of the novel putative substrate of mtMET in mitochondria. In current study, we applied proximity ligation assay to visualize and verify the protein-protein interactions between mtMET and two candidate proteins, individually. These two proteins include HMGA1, and PKM2. Taken all information together, two common potential target proteins were identified. We proposed that mtMET interacts and regulates HMGA1 and PKM2 in SNU5 mitochondria. Moreover, through bioinformatics analysis of the mitochondrial proteome, we showed that the mitochondrial metabolism activities were seriously influenced when activation of mitochondrial-localized MET was suppressed. Our study is the first to discover the novel substrates of MET in mitochondria of SNU5 gastric cancer cells. Our works are believed to uncover a novel paradigm of MET signaling in gastric cancer cells and thus contribute to the ongoing efforts to therapeutically target aberrant RTK activities in human cancers.

Chapter 2

**Elucidating the molecular mechanism of translocation of
hepatocyte growth factor receptor (MET) into the mitochondria in
SNU5 gastric cancer cells**

2.1 Abstract

Dysregulated cell growth and proliferation, angiogenesis, cancer cell invasion and metastasis are critical hallmarks of cancer. Tremendous reports have demonstrated that a wide variety of RTKs play a causal role in all of these processes in different human cancers. MET, *aka.* HGFR or c-MET, have been found to be amplified and overexpressed in gastric cancers, contributing to the development and progression of tumors, angiogenesis, invasiveness, and metastasis. Therapeutic strategies targeting MET thus hold promise for gastric cancer and handfuls of anti-MET studies were reported as of now. However, we are still far away from a complete understanding of the mechanisms underlying the aberrant MET activities in cancer cells. Recently MET is found localizing in mitochondria, which is an unconventional subcellular localization of this plasma-membrane-localized RTK, and an important role of mtMET in cancer cells is suggested. Based on preliminary data from qualitative profiling of mitochondrial proteome, different components of endocytosis pathway were identified. We proposed that MET is activated and endocytosed, followed by translocation into mitochondria of cancer cells to execute yet-to-identify functions. Herein functional assays using different endocytic inhibitors coupled with immunofluorescence staining approach were applied to study the translocalization of MET into mitochondria. Our results revealed that abundance and activation of MET in mitochondria were severely disrupted after endocytosis inhibition. Our findings also suggested that participation of other mechanisms in the translocalization of MET into mitochondria. The present study proposes a novel functional role of MET signaling in regulation of gastric oncogenesis.

2.2 Introduction

2.2.1 Roles of receptor tyrosine kinases in cancer

Since the establishment of primary structure of EGFR⁴⁷, the first identified RTK in 1984, infinite research efforts have been made to comprehensively understand the significance and roles of RTKs in intra- and extracellular signaling network^{45, 48}. RTKs have been found to be tightly associated with cellular processes including cell proliferation and differentiation, cell migration, metabolism, cell survival, and cell-cell communication. On the other hand, RTKs are known to be potentially oncogenic when they are genetically mutated, structurally altered, or transcriptionally or translationally overexpressed⁴⁹. Dysfunctional activities of different RTKs have been shown in the development and progression of cancers, for instance, the amplification, overexpression, and/or somatic mutation of EGFR in non-small cell lung cancer⁵⁰, esophageal cancer⁴⁷, gastric cancer⁵¹, liver cancer⁵², head and neck cancer⁵³, and adrenocortical carcinoma⁵⁴ have been reported.

Targeting particular RTKs has been attempted using various inhibitory compounds, including humanized monoclonal antibodies and small-molecule inhibitors (SMIs)⁵⁵. The use and development of SMIs are comparatively successful and have drawn considerable interest because of fewer undesired side effects, and also better understanding of the mechanisms of action^{58,59-61,62-64}. Gaumann *et al.* have recently proposed RTKs as the most promising therapeutic targets to inhibit tumor neoangiogenesis or to normalize the tumor vasculature using RTK inhibitors (RTKIs)¹¹⁰. However, because of the rapid proliferation of cancer cells, selective pressure leading to resistance against anticancer SMIs or other RTKIs, and/or mutation of binding sites on RTKs, powerful inhibitors that can tolerate multiple amino acid mutations in the RTKs and/or target alternative sites on aberrant RTKs are urgently required^{67, 68}. Moreover, targeting single RTK or signaling pathway alone with the presently available inhibitors may not to be

effective for lasting tumor therapy. In a review of drugs targeting RTKs, the author has illustrated that common pathways to those regulated by RTKs were also activated synergistically by some other non-receptor tyrosine kinase, such as Bcr-Abl, leading to enhanced cell proliferation, tumorigenesis, invasion and metastasis¹¹¹. Further studies are thus warranted to unravel the currently incomplete molecular architecture of RTKs, together with their ligands, in tumorigenesis and to form a rational goal in the development of personalized medicine⁴⁵.

2.2.2 Dysregulated receptor tyrosine kinases in gastric cancer

2.2.2.1 Dysregulation of receptor tyrosine kinases in gastric cancer

Numerous RTK studies have implicated abnormal RTK activities in gastric cancer. Tanner *et al.* and Akamatsu *et al.* demonstrated the presence of HER2 gene amplification in 12.2% of gastric and 24% of gastroesophageal junction adenocarcinomas, respectively^{112, 113}. Elevated levels of serum VEGF, the ligand of VEGFR, have been detected in gastric cancer patients with distant metastases¹¹⁴. Overexpression of EGFR, one of the most well studied RTKs, has been detected in 27.4% gastric carcinomas¹¹⁵. In a study of patients with late stage gastric cancer, overexpression of both EGFR and type 1 insulin-like growth factor receptor were identified in 55% of surgically resected primary gastric tumors¹¹⁶. Several studies have revealed that EGFR is linked with poor prognosis and is strongly associated with the advanced stages of gastric cancer^{51, 117, 118}.

2.2.2.2 MET in gastric cancer

MET (mesenchymal epithelial transition factor, *aka.* c-MET, HGFR, and scatter factor ¹ receptor) has been found to be highly amplified and overexpressed in gastric cancer^{73, 119-122}. MET is a disulfide-link heterodimeric product of the *MET* proto-oncogene, and acts as a high-

affinity RTK for HGF/SF¹. Following MET activation by binding to HGF/SF, the activated protein kinase recruits and interacts with downstream effectors, including GAB1, GRB2, PIK3R1, PLCG1, SRC, and STAT3, which leads to the activation of several signaling cascades, such as RAS/ERK/MAPK and PI3K/AKT⁶⁹. MET is associated with a wide variety of biological processes, including morphogenesis, coordination of prosurvival effects, gastrulation, development and migration of muscle and neuronal precursors, angiogenesis, and organ regeneration and tissue remodeling¹²³⁻¹²⁵. Dysfunctional MET kinase and aberrant signaling of MET pathways were found to contribute to tumor progression, angiogenesis, invasiveness, and metastasis in various human cancers^{76, 119, 121}. Mutations in *MET* protooncogene, overexpression of MET and/or HGF/SF, and uncontrolled MET activation have been shown to correlate with tumorigenesis and metastasis⁷⁴. Recently, Drebber *et al.* proposed MET as a novel prognostic factor and potential drug target for gastric cancer, after showing that it was overexpressed in gastric cancer¹²⁶. Bachleitner-Hofmann *et al.* identified that MET-overexpressing gastric tumors co-overexpressed EGFR and/or HER3¹²⁷. Interestingly, about 20% of cancers with acquired resistance to EGFR-selective tyrosine kinase inhibitors (TKIs) showed evidence of having undergone an oncogene kinase switch due to amplification of *MET* protooncogene that developed during EGFR TKI treatment¹²⁸⁻¹³⁰. Thus, understanding the entire repertoire of MET functions in cancers would be clinically useful.

As a result of the overwhelming evidence showing the pivotal role of abnormal MET signaling in human cancers, MET has become an attractive target for molecular-based targeted therapy in gastric cancer. Several MET-selective inhibitory compounds have been tested in the laboratory and in clinical trials, with the ultimate goal of producing clinically useful MET-targeted drugs for malignancies¹³¹⁻¹³³. All attempts thus far to explain the efficacies of inhibitory compounds are premised on the blockade of conventional MET signaling pathways. The possibility

that MET oncoprotein may function in non-canonical pathways has received little attention. We recently detected the presence of MET in the mitochondria of SNU5 cells, a MET over-expressing gastric cancer cell line that is extremely sensitive to PHA-665752, a highly specific ATP-competitive SMI of the catalytic activity of MET⁸⁴. In contrast, mtMET were not detected in cancer cell lines that are resistant to the cognate RTK inhibitor. This striking finding of novel subcellular localization of MET in mitochondria of inhibitor-sensitive cancer cells suggests that mtMET may play critical roles in oncogenesis and susceptibility to specific drugs.

2.2.3 Endocytosis of receptor tyrosine kinases in cancers

In addition to our own discovery, other RTKs, such as EGFR and ErbB2, have been demonstrated to translocate to the mitochondria of cancer cells and are associated with tumor proliferation, progression, and drug resistance^{134, 135}. Given that the regulation of RTKs is significant for different aspects of cellular functions, the expression and degradation of RTKs, and the activation and deactivation of RTK signaling are therefore tightly controlled by endocytosis¹³⁶⁻¹³⁸. RTK signaling continues from within the endocytic compartment and is able to trigger distinct intracellular signaling pathways through this unconventional mechanism^{139, 140}. Casaletto *et al.* presented specific examples of spatially deregulated RTKs in cancer and suggested that spatial deregulation of RTK activity might in fact drive cancer tumorigenesis¹⁴¹. It has been demonstrated that there is a direct link between MET endocytosis and tumorigenesis using tumor-associated MET-activating mutations in both cell lines (*in vitro*) and animal models (*in vivo*)¹⁴². Furthermore, recent studies demonstrated that ligand-induced receptor endocytosis and a mitochondrial localization signal are required for directional translocation of RTKs, such as EGFR in endocytic vesicles, to the mitochondrial compartment^{143, 144}. Yao *et al.* also showed that

endocytosis might contribute to mitochondrial translocation of EGFR using a juxtamembrane-truncated EGFR mutant¹⁴⁵, although the mutant used in this study might have altered the mitochondrial targeting signal in return, affecting the interpretation of their results.

2.2.4 Localization of MET in mitochondria

2.2.4.1 Knowledge gap

To date, the mechanism of mitochondrial RTK expression is still unclear. The combined observations of the presence of MET in mitochondria and perturbed mitochondrial function by MET selective inhibitors prompted us to hypothesize that MET is activated and phosphorylated on the plasma membrane and subsequently internalized through the endocytic pathway. This would result in the translocation of a subpopulation of internalized MET to the mitochondria, modifying mitochondrial function and promoting gastric oncogenesis, cell survival, and proliferation. Inhibition of MET activity by monoclonal antibodies or SMIs, or blockade of MET endocytosis and/or translocation to mitochondria would therefore promote gastric cancer cell death.

2.2.4.2 Specific aims in the study

This project aims to elucidate the unidentified molecular mechanism of plasma-membrane-localized MET translocation to mitochondria, and to identify the mitochondrial protein substrates and functions of mtMET in gastric oncogenesis. In current chapter, we performed biochemical manipulations using specific inhibitors of different endocytic pathways to analyze the participation of endocytosis in the translocation of plasma-membrane-localized MET into mitochondria. Together with , combined with the results of fluorescence confocal microscopy and proteomic analyses,, we confirmed the role of endocytosis in regulation of mitochondrial translocalization of plasma-membrane-localized MET.

Current study to uncover the mechanisms of mtMET translocalization in gastric cancer cells are believed to contribute to the ongoing efforts to understand the aberrant RTK activities in various human cancers.

2.3 Materials and Methods

2.3.1 Chemicals

High quality reagents were used in all experiments. Water was prepared using a Milli-Q system (Millipore, Bedford, MA). All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Reagents for silver staining were bought from Bio-Rad (Bio-Rad Laboratories, Cambridge, MA). The selective MET inhibitor PHA-665752 was obtained from Pfizer Global Research and Development (La Jolla Laboratories, San Diego, CA). Protease inhibitor cocktail EDTA-free and phosSTOP were from Roche Diagnostics (Indianapolis, IN, USA). Trypsin-EDTA, phosphate buffered saline (PBS) and penicillin-streptomycin for cell cultures were from GIBCO-Invitrogen (Carlsbad, CA, USA), while fetal bovine serum (FBS) was from Thermo Scientific HyClone (Waltham, MA, USA) and NuSerum was purchased from BD Biosciences (Bedford, MA, USA). HGF recombinant human protein (cat. no. PHG0254) was bought from Invitrogen (Carlsbad, CA, USA). Alexa Fluor® 488 antibody labeling kit (Life Technologies, cat. no. A-20181) was a gift from Dr. Renyan. Mitochondrial protein extracts from A431, H2444, HCC827, Hs746T and MKN-45 cells were gifts from Dr. Guo Tiannan. Stock solutions of PHA-665752, dynasore, cytochalasin D, nocodazole, and phenothiazine were prepared in DMSO, stored in -80 °C, and diluted with fresh medium before use. In all experiments, the final concentration of DMSO was < 0.1 %.

2.3.2 Cell culture

The human gastric cancer cell lines, SNU5 and SNU1, were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured as recommended. Briefly, SNU5 cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) containing 20 % FBS/NuSerum and 100 units/ml penicillin-streptomycin; SNU1 cells were cultured in Roswell Park Memorial Institute 1640 medium (RPMI-1640) containing

10 % FBS and 100 units/ml penicillin-streptomycin. Cells were maintained in a humidified atmosphere of 37 °C with 5 % carbon dioxide (CO₂).

2.3.3 Western blotting

Western blotting was performed using the following primary antibodies at the indicated dilutions: 1:1000 MET (clone C-12), 1:1000 phospho-MET (Y1234/1235), 1:1000 phospho-MET (Y1349), 1:1000 E-cadherin (clone G-10), 1:4000 actin (Clone C4), 1:1000 integrin α L (clone C-17), 1:1000 COX-IV (clone Q-17), 1:1000 TOM20 (clone FL-145), 1:1000 EEA1 (ab2900), and 1:1000 LAMP1 (ab24170). EEA1 and LAMP1 antibodies were purchased from Abcam (Abcam (Hong Kong) Ltd., Hong Kong, China), MET and phospho-MET antibodies were bought from Cell Signaling (Danvers, MA, USA), actin antibody was from Millipore (Billerica, MA, USA), while the other primary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Briefly, 40 μ g of mitochondrial proteins were resolved on 10% polyacrylamide gels by SDS-PAGE. Proteins were then transferred onto nitrocellulose membranes (Bio-Rad) using a transblot apparatus in buffer containing 20 mM Tris-HCl (pH 8.3), 200 mM glycine, and 20 % methanol. After blocking in 4 % BSA, the membranes were incubated overnight with primary antibodies at 4 °C, followed by incubation with the appropriate secondary antibody at 1:4000 dilutions for 1 h at room temperature. Protein bands were detected using the enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL, USA) according to the manufacturer's protocol.

2.3.4 Mitochondria isolation and purification

A two-step procedure was used to isolate mitochondria of high yield and maximal purity. The method consisted of a differential centrifugation step, followed by further purification of the crude mitochondrial fraction

using a commercial mitochondria isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol with some modifications. Briefly, 1×10^8 SNU5 cells were collected and washed twice in ice-cold PBS. Cells were homogenized on ice in cell lysis buffer (10 mM Tris-MOPS, 200 mM sucrose, 1 mM EGTA/Tris supplemented with Complete Protease Inhibitor Mixture Tablets and phosSTOP) using a 30-gauge Sterican insulin needle (Braun, Kronberg, Germany) until 95 % of cells were broken. The crude cell lysate was centrifuged at $2 \times 600\text{ g}$ for 10 min to pellet nuclei, unbroken cells and large debris, and the supernatant was centrifuged at $2 \times 7000\text{ g}$ for 10 min at 4 °C to pellet the mitochondrial fraction. The crude mitochondrial fraction was incubated with anti-TOM22 MicroBeads in the $1 \times$ separation buffer provided for 1 h at 4 °C with gentle agitation. Subsequently, the suspension was loaded onto a pre-conditioned MACS LS column and washed with $3 \times 3\text{ mL}$ separation buffer before removing the column from the magnetic field and eluting the mitochondrial fraction with 1.25 mL separation buffer. The mitochondrial pellet was washed with $2 \times 500\text{ }\mu\text{L}$ storage buffer followed by $2 \times 500\text{ }\mu\text{L}$ ice-cold PBS

2.3.5 Bicinchoninic acid (BCA) protein assay

Protein concentration was measured using the BCA protein assay in a 96-well plate format. A standard curve was prepared from a known concentration of bovine serum albumin (BSA) at concentrations of 0.1, 0.2, 0.4, 0.6, 0.8, and 1 mg/mL. The plate was incubated at 37 °C for 25 min after the sample/standard and working reagent (50 bicinchoninic acid solution: 1 $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ solution, v/v) were mixed at a ratio of 1:20. Thereafter, the optical density was measured at 562 nm using the Infinite® M1000 Microplate Reader (Tecan, Switzerland) and exported to Microsoft Excel to calculate protein concentrations from the standard curve.

2.3.6 Immunofluorescence

HGF recombinant human protein was complexed to Alexa Fluor® 488 according to manufacturer's instruction and supplemented in IMDM medium. SNU5 cells were cultured to 70 % confluency and treated with dynasore as indicated in Results part. 5×10^3 SNU5 cells were washed with ice-cold PBS for three times and incubated in 20 % FBS/PBS, followed by cytocentrifuging onto superfrost plus microscope slides for 5 min at 120 g. Cells were stained as previously described¹⁴⁶. Briefly, cells attached on microscope slides were washed with 1 × PBS once and fixed in 3.7 % paraformaldehyde (PFA) for 20 min at room temperature. After PFA fixation, cells were washed with 3 × 1 mL PBS, and then permeabilized with 0.2 % Triton X-100/PBS for 10 min, and blocked in 4% BSA (in 0.1 % Triton X-100/PBS) for 1 h at room temperature. Cells were incubated with 1 µg/mL rabbit anti-TOM20 polyclonal antibody (clone FL-145, Santa Cruz Biotechnology) in 1 % Triton X-100/PBS at 4 °C overnight. After that, washed away primary antibody solution with 0.1 % Triton X-100/PBS for three times. Then cells were incubated with 1:1000 goat anti-rabbit IgG H&L (Alexa Fluor® 647, ab150079, Abcam) in 1 % Triton X-100/PBS at room temperature for 1 h. Each sample was counterstained and coverslips were mounted with Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA). Edges of the coverslips were sealed with nail polish. Slides were stored at 4 °C until visualizing the results. Images were captured using a Zeiss LSM 710 confocal microscope (Carl Zeiss, Germany) and analyzed using Image J software.

2.3.7 Mitochondrial protein digestion

Mitochondrial protein extracts from each cell line were resuspended in 200 µL of PBS containing 2 % SDS, supplemented with complete protease inhibitor mixture tablet and phosSTOP. The suspension was sonicated for 20 × 3 s at 4 °C with 5 s rest in between using output 30 %. Protein concentrations were measured using BCA protein assay before

SDS-PAGE. 40 µg of mitochondrial proteins were separated in a 10 % polyacrylamide gel and stained with silver stain following the manufacturer's protocol. Gel bands were excised and diced into 1 mm × 1 mm, followed by reduction with 10 mM dithiothreitol (DTT) at 56 °C for 1 h, and then alkylation with 55 mM iodoacetamide (IAA) at room temperature in dark for 45min. Proteins were digested with 10 ng/µl of sequencing grade modified trypsin for 4 hr at 37 °C. Same amount of trypsin was added for a second time tryptic digestion overnight at 37 °C. Peptides were extracted from gel pieces with 50 % isopropanol, 5 % acetic acid (HOAc) followed by 50 % acetonitrile (ACN), 5 % formic acid (FA). Peptide extracts from each band were vacuum-dried with a SPD 2010 SpeedVac system (Thermo electron, Waltham, MA, USA) and desalted with SEP-PAK C18 cartridges 50 mg (Water Corp., Milford, MA, USA). Desalted peptides were subsequently vacuum-dried again.

2.3.8 LC-MS/MS

The resultant peptides were dried with vacuum concentrator and analyzed by LC-MS/MS. The dried peptides were firstly re-dissolved in LC-MS/MS compatible buffer (3 % ACN, 0.1 % FA). Peptides were separated and analyzed on a Dionex Ultimate 3000 RSLC nano system coupled to a Q Exactive (Thermo Fisher, MA) as previously described with minor modification¹⁴⁷. Approximately 1.2 µg of peptides from each sample were injected into an Acclaim peptide trap column (Thermo Fisher, MA) via the autosampler of the Dionex RSLCnano system. Peptides were separated in a capillary column (75 µm x 10 cm) packed with C18 AQ (5 µm, 300 Å; Bruker-Michrom, Auburn, CA, USA) at room temperature. The flow rate was at 300 nL/min. Mobile phase A (0.1 % formic acid in 5 % ACN) and mobile phase B (0.1 % formic acid in 90 % ACN) were used to establish a 60 min gradient: 30 min of 7-18 % B, 15 min of 18-30 % B, 4 min of 30-50 % B, 1 min of 50-80 % B, 2 min of 80 % B, 5 min of 80-5 % B, followed by re-equilibration at 7 % for 3 min. Peptides were then analyzed on Q Exactive with a nanospray source

(Thermo Fisher, MA) at an electrospray potential of 1.5 kV. A full MS scan (350–1600 m/z range) was acquired at a resolution of 70,000 at m/z 200 and a maximum ion accumulation time of 100 ms. Dynamic exclusion was set as 30 s. Resolution for HCD spectra was set to 17,500 at m/z 200. The AGC setting of full MS scan and MS2 were set as 1E6 and 1E5, respectively. The 10 most intense ions above a 1000 counts threshold were selected for HCD fragmentation with a maximum ion accumulation time of 100 ms. Isolation width of 2 Th was used for MS2. Single and unassigned charged ions were excluded from MS/MS. For HCD, normalized collision energy was set to 28 %. The underfill ratio was defined as 0.1 %.

2.3.9 Data Analyses

The raw data files were converted into the dta format using the extract_msn program (version 4.0) in Bioworks Browser 3.3 (Thermo Electron, Bremen, Germany), and then the dta files were converted into Mascot generic file format using Proteome Discoverer 1.4.1.14 (Thermo Electron, Bremen, Germany). Protein identification was performed by querying against the extracted Uniprot *Homo sapiens* database (released on 29 Nov, 2015; containing 70,141,034 residues and 176,946 sequences as well as the reverse sequences for decoy search) using an in-house Mascot server (version 2.4.1, Matrix Science, Boston, MA) with peptide precursor mass tolerances of 10 ppm and fragment ions mass tolerance of 0.02 Da¹⁴⁷. Two missed cleavage sites of trypsin and # 13 C of 2 were allowed in the search. Carbamidomethylation of cysteine residues was set as a fixed modification, while oxidation of methionine, deamidation of asparagine and glutamine residues, and phosphorylation of serine, threonine and tyrosine residues were set as variable modifications. Only proteins with at least two unique peptides identified and with ion scores $\geq$ identity or homology scores were considered.

2.3.10 Data Annotation

Open source online bioinformatics software tools, including Panther v9.0^{148, 149}, DAVID v6.7¹⁵⁰⁻¹⁵² and GeneCards®, were used to analyze and interpret the data.

2.4 Results

2.4.1 Profiling of mitochondrial proteome to uncover the correlation of endocytosis

Clathrin-mediated endocytosis plays a critical role in regulating RTK signaling pathways. Upon activation of RTKs by binding of respective ligands, activated RTKs are internalized into the cells through clathrin-coated vehicles. Signaling of RTKs is subsequently inhibited and RTKs are either recycled back to cell surface or degraded in the ubiquitin proteasome pathway. To determine the involvement of clathrin-mediated endocytosis in translocation of RTKs to mitochondria in different cancer cells, mitochondrial proteomic data of a panel of RTK inhibitor-sensitive cancer cell lines, including SNU5, SNU1, Hs 746T, MKN-45, A431, H2444, and HCC827, were analyzed. A stringent inclusion criteria was employed to filter the qualitative proteomics dataset, *i.e.* only proteins quantified from at least two unique peptides, having ion scores ≥ 20 , and having ion scores $\geq$ identity/homology scores were considered as confident protein hits and advanced to the next phase of analysis (<https://drive.google.com/file/d/0BzFz90GtH20RbUY3NIFrTERxR3M/view?usp=sharing>). As shown in the dataset and Table 2.1, several proteins associated with the clathrin-mediated endocytosis pathway were detected in the mitochondrial proteomes of all cell lines tested, including clathrin, adaptor-related protein complex 2 alpha 2 subunit (AP2A2), adaptor-related protein complex 2 beta 1 subunit (AP2B1), heat shock 70kDa protein 8 (HSC70), EGFR pathway substrate 15 (EPS15), dynamin, phosphatidylinositol binding clathrin assembly protein (PICALM), synaptojanin, huntingtin-interacting protein 1-related protein (HIP1R), cortactin, and endophilin. The atypical presence of these proteins in the mitochondria of RTK inhibitor-sensitive cell lines suggests that endocytosis via clathrin-coated vesicles may form a common molecular mechanism for translocation of activated RTKs, including MET, from the cell surface into mitochondria.

Table 2.1: Components of clathrin-mediated endocytosis were identified by mitochondrial proteomic profiling of RTK TKI-sensitive cell lines. Only proteins with at least two unique peptides identified and with ion scores $\geq$ identity/homology scores were considered. Functions of components were confirmed manually using the online PANTHER classification tool, Gene ontology and GeneCards® with literature reviews.

Cell line	A431	H2444	HCC827	Hs746T	MKN-45	SNU1	SNU5
Core components							
Clathrin		✓	✓	✓	✓	✓	✓
AP2	✓	✓	✓	✓	✓	✓	✓
EPS15-EPS15R					✓		
AP180, CALM			✓	✓	✓	✓	✓
Dynamin	✓	✓	✓	✓	✓	✓	✓
HSC70	✓						
Cargo-specific adaptors							
Synaptojanin		✓				✓	
Actin nucleation at clathrin-coated vesicles							
HIP1-HIP1R					✓		
Cortactin	✓						
Other proteins potentially involved in clathrin-mediated endocytosis							
Endophilin					✓		

2.4.2 Inhibition of endocytosis using endocytic inhibitors

To confirm the role of clathrin-mediated endocytosis in translocation of plasma membrane-localized MET to the mitochondria, we specifically inhibited clathrin-mediated endocytosis using several commonly used inhibitors. The first inhibitor employed was dynasore, a rapidly acting reversible GTPase inhibitor that selectively targets dynamin and blocks endocytosis. SNU5 cells were selected as the model cell line in subsequent functional studies because it is a MET-dependent gastric cancer cell line with confirmed mtMET⁸⁴. Cells were cultured in complete IMDM medium to 70 % confluency and then incubated in 80 μ M dynasore (in serum-free medium) for 24 h. Cells grown in complete IMDM medium for 24 h without any drug treatment were used as a positive control. For the negative control, cells were grown in 50 nM PHA-665752 (in serum-free medium) for 24 h to suppress phosphorylation and catalytic activity of MET. These conditions were previously shown to inhibit MET activation without inducing substantial SNU5 cell death⁸⁴. In the optimization of experimental conditions, we found that dynasore binds to serum proteins and loses its activity. To maintain the efficacy of dynasore, dynasore and PHA-665752 were dissolved in medium lacking albumin and serum; for positive control, cells were grown in complete IMDM medium with 20 % FBS (data shown in Figure 2.1A). However, long-term serum-free condition was expected to cause undesired effect and stress to SNU5 cells and influence the level of phosphorylation of METs. Therefore, to standardize the cell culture conditions, complete IMDM medium with 20 % NuSerum (BD Biosciences, Bedford, MA) was utilized in subsequent cell culture experiments in place of traditional FBS.

(A)

(B)

(C)

Figure 2.1: Immunoblotting evidences of MET and phosphor-MET in SNU5 mitochondria. (A) Western blot result showed the expression level of MET in mitochondria, phosphorylation of mtMET on tyrosine 1234/1235 and tyrosine 1349, and actin. (B) No contamination of integrin- α L and E-cadherin from plasma membrane in the high-purity mitochondrial extract. TOM20 was selected as the internal control in subsequent experiments. (C) Neither EEA1 nor LAMP1 was identified in the high-purity mitochondrial extract, respectively. Control, control; PHA, PHA-665752; Dyna, dynasore; (1), control, 24 h; (2), PHA-665752, 24 h; (3), dynasore, 2 h; (4), dynasore, 12 h; (5), dynasore, 24 h; (6), cytochalasin D, 24 h; (7), nocodazole, 24 h; (8), phenothiazine, 24 h.

Consistent with our hypothesis, mtMET was found in high abundance in the control mitochondrial fraction, while treatment of SNU5 cells with PHA-665752 reduced the expression level of mtMET. Inhibition of endocytosis using 80 μ M dynasore considerably decreased the abundance of mtMET, indicating the endocytic origin of mtMET. Auto-phosphorylation of mtMET was downregulated, as shown by the decreased level of phosphorylation of mtMET on tyrosine 1234/1234 [pMET (Y1234/1235)] and tyrosine 1349 [pMET (Y1349)] when compared to the controls (Figure 2.1A). The results strongly support the hypothesis that endocytosis is involved in the translocation of MET to the mitochondria in SNU5 cells.

The possibility that the presence of mtMET in mitochondrial fraction of SNU5 cells was caused by contamination of MET proteins from other sources, such as the plasma membrane or cytosol, was tested by immunoblotting for the presence of proteins localized to specific cellular components. As shown in Figure 2.1A, actin could not be detected, indicating that no cytosolic contamination in the mitochondrial protein fraction. Similarly, integrin- α L and E-cadherin, both of which are plasma membrane-localized proteins, were not detected (Figure 2.1B). EEA1 (early endosome antigen 1, a protein found in endosome and necessary for endosomal trafficking) and LAMP1 (lysosomal-associated membrane protein 1, a membrane glycoprotein and a hallmark of lysosomes) were also not detected in the high-purity mitochondrial fractions (Figure 2.1C). These controls demonstrated that only mitochondria, but not any other organelles, were enriched using the two-step mitochondrial isolation method employed throughout the project. Thus MET protein trafficking within other intracellular compartments did not affect or alter the study results.

To determine which mitochondrial protein was suitable as the internal control of loading amount of mitochondrial proteome, the abundance of TOM20 and COX IV, two well-studied mitochondrial hallmark proteins, were probed. The expression levels of TOM20 in each mitochondrial fraction were very consistent. By contrast, the expression level of COX IV was slightly upregulated in the dynasore-treated mitochondrial fraction (Figure 2.2B). TOM20 was therefore employed as an internal control to confirm equal loading amount of mitochondrial proteins in subsequent experiments.

To further verify the practicability and efficiency of the experimental design of mitochondrial purification, mitochondrial proteome obtained from two-step mitochondrial isolation approach was compared with the total cell lysate of SNU5 cells. As described in Materials and Methods,

the two-step procedure is consisted of differential centrifugation step and anti-TOM22 antibody-based mitochondrial pull down assay. In differential centrifugation step, a crude mitochondrial pellet was firstly collected. The second step then pulled down mitochondria using anti-TOM22 magnetic microbeads to ensure the removal of any potential contaminants from the crude mitochondrial samples. The whole procedure took around 3 hours and technical replicates were performed using same batch of cells. The total cell lysate and mitochondrial proteome were subsequently immunoblotted for the landmark proteins of different organelles, including EEA1, LAMP1, actin, integrin α L, E-cadherin and TOM22 (Figure 2.2). In addition, irrelevant antibodies, including calnexin (ER-associated protein), GM130 (Golgi protein), and GAPDH (cytosolic protein), were used as negative controls to indicate the high quality of mitochondrial prep (Supplementary Figure 1). These results clearly illustrated that the two-step mitochondrial isolation procedure can extract mitochondria of very high purity in short duration. No contamination of proteins from other organelles and cytosolic fractions were identified in individual replicate of mitochondrial proteome.

Figure 2.2: Immunoblotting evidences showing high purity of mitochondrial fractions. To ensure the purity of mitochondrial fractions, two-step mitochondrial isolation methods were utilized in the project. By comparing to SNU5 total cell lysate, the mitochondrial fractions showed no contamination from endosome (EEA1), lysosome (LAMP1), and cytosolic fraction (actin, integrin α L, and E-cadherin). (1), SNU5 total cell lysate_1st replicate; (2) SNU5 total cell lysate_2nd replicate; (3) SNU5 mitochondria_1st replicate; (4) SNU5 mitochondria_2nd replicate.

2.4.3 Time course study of inhibition of endocytosis using different endocytic inhibitors

To study the effect of inhibition of endocytosis on the abundance and activity of mtMET in the immediate and short-term, SNU5 cells were treated with dynasore for 2 h, 12 h, and 24 h. In addition to dynasore, several endocytosis inhibitors known to inhibit endocytosis through targeting distinct pathways were applied to analyze the role of clathrin-

mediated endocytosis in MET translocation. These inhibitors were 50 μ M cytochalasin D (a potent inhibitor of actin polymerization), 20 μ M phenothiazine (a hydrophobic amine that affects the function of clathrin and clathrin-coated vesicles), and 10 μ M nocodazole (an inhibitor of polymerization of the microtubule cytoskeleton).

Figure 2.3: Immunoblotting evidences showing the expression level of mtMET and phosphor-mtMET under endocytic inhibition. Expression level of mtMET, phosphorylated mtMET on tyrosine 1234/1235 and tyrosine 1349 under different conditions was reduced greatly. (1), control, 24 h; (2), PHA-665752, 24 h; (3), dynasore, 2 h; (4), dynasore, 12 h; (5), dynasore, 24 h; (6), cytochalasin D, 24 h; (7), nocodazole, 24 h; (8), phenothiazine, 24 h. pMET(Y1234/1235), phosphorylation of mtMET on Tyrosine 1234/1235; pMET(Y1349), phosphorylation of mtMET on Tyrosine 1349.

Treatment of dynasore for 2 h remarkably decreased the mtMET abundance, which was further reduced after 12 h. For 24-h inhibition of endocytosis using dynasore, no further reduction of mtMET expression level was observed. Phosphorylation of mtMET on Y1234/1235 and Y1349 was also reduced in the presence of dynasore. As shown in Figure 2.3, longer duration of inhibition caused a lower abundance of phosphorylated mtMET on both Y1234/1235 and Y1349. On the other

hand, inhibition using cytochalasin D and phenothiazine for 24 h resulted in a similar reduction of mtMET expression levels. Phosphorylation of mtMET was also suppressed by these two inhibitors, producing comparable results to those of PHA-665752 and dynasore. Unexpectedly, treatment with nocodazole for 24 h had little effect on the expression of MET protein in mitochondria or MET activation and phosphorylation (Figure 2.3).

2.4.4 Immunofluorescence analysis of localization of mtMET of SNU5 gastric cancer cells

Based on our hypothesis, plasma-membrane-localized MET is bound and activated by HGF, leading to the endocytosis and translocation of MET/HGF protein complex into mitochondria. Thus, HGF recombinant human protein complexed to Alexa Fluor® 488 was used as the target to visualize and study the internalization and translocation of MET into SNU5 mitochondria. Confocal microscope was employed to determine the colocalization (same Z coordinate) of MET and mitochondria. Inhibition of endocytosis was performed by culturing SNU5 cells in 80µM-dynasore medium (IMDM medium supplemented with 80 µM dynasore, 20 % Nuserum and 50 ng/mL Alexa Fluor® 488 HGF complex) for 24 h. Endocytosis was subsequently re-activated by culturing the cells in fresh dynasore-free medium (IMDM medium supplemented with 20 % FBS and 50 ng/mL Alexa Fluor® 488 HGF complex). The negative control remained inhibited without changing the medium, and untreated SNU5 cells served as a positive control.

Referred to Figure 2.4, several points of colocalization of MET/HGF and mitochondria (left-hand panel, indicated by white arrows) could be located in SNU5 cells in the untreated control. However, after 24-h inhibition of endocytosis, no spot of colocalization could be identified (middle panel). When the inhibition was removed and endocytosis was

reactivated, an increased level of colocalization of MET/HGF and mitochondria were found inside SNU5 cells (right-hand panel). SNU5 cells also grew more aggressively after the replenishment of nutrient (complete IMDM medium) compared to SNU5 cells grown under the positive control conditions (data not shown).

Figure 2.4: Fluorescence microscopic imaging showing the effect of dynasore inhibition in SNU5 cells. When inhibition of endocytosis was removed and endocytosis was re-activated, more endocytosis and translocation of plasma membrane-localized MET to mitochondria was detected. The white arrows indicate the points of colocalization of mtMET/HGF and mitochondria. SNU5_Control, SNU5 cells grown in IMDM without treatment for 24 h; SNU5_Negative-control, SNU5 cells grown in IMDM medium with 80 µM dynasore for 24 h; SNU5_Endo-reactivated, SNU5 cells grown in fresh IMDM medium after 24-h inhibition of endocytosis. Red: mitochondria; green: MET/HGF; blue: DAPI.

As shown in Figure 2.4, when endocytosis of cells was inhibited using dynasore (negative control), plasma membrane-localized MET could not be internalized into intracellular compartments and did not colocalize with mitochondria. However, when inhibition was removed and endocytosis was re-activated, internalization of MET resumed. In

addition, as compared to the positive control, there was an increased level of colocalization identified in SNU5 cells after endocytosis pathway was reactivated. This observation reinforced our hypothesis that the presence of RTKs *i.e.* MET in mitochondria is due to endocytosis and translocation of plasma membrane-localized MET into the mitochondria.

2.5 Discussion

Accumulating evidence in cancer research shows that some cancers appear to be highly dependent on a single oncogene/oncoprotein for cancer oncogenesis and cell proliferation¹⁵³⁻¹⁵⁵. Although it is known that MET plays critical roles in both normal and cancerous cells, the complete blue-print of MET in gastric oncogenesis remains elusive^{76, 121}. Using a combination of quantitative proteomic approaches and mitochondrial functional assays, our lab have recently demonstrated the presence of activated MET proteins in a MET-dependent SNU5 gastric cancer cell line and uncovered a novel mechanism through which kinase inhibitors could act directly on mitochondrial RTKs⁸⁴.

It has been well established that MET is present and functioning on the plasma membrane, and MET selective inhibitors act through plasma membrane-localized MET⁷⁶. Nevertheless, MET and other RTKs, such as EGFR, have been found to be capable of translocating and residing in other organelles, including the nucleus^{156, 157}. Recently, both MET and EGFR were also found in the mitochondria of cancerous cells, where they actively modulate mitochondrial proteins^{84, 158}. To dissect the underlying mechanism of translocation of MET to mitochondria, we have established a model to inhibit clathrin-mediated endocytosis activity using selective endocytosis inhibitors, including dynasore, cytochalasin D, nocodazole, and phenothiazine, in SNU5 gastric cancer cells. The concentrations of each inhibitor, based on those previously established in the literature, are not expected to result in lethal or off-target effects¹⁵⁹⁻¹⁶². Dynasore specifically targets dynamin, a protein which functions in endocytic coated vesicle formation, and inhibits the pinching-off of clathrin-coated vesicles from the plasma membrane¹⁶². Dynasore rapidly inhibits endocytosis in approximately 30 min for various cell types. As demonstrated in the western blotting and microscopy data, the abundance of MET in mitochondria was greatly reduced after 24-h of dynasore inhibition. Importantly, cytochalasin D

and phenothiazine, which targeted different proteins implicated in the endocytic pathway, produced consistent results to dynasore. These pieces of evidence suggested that mtMET originates from the plasma membrane: when endocytosis is suppressed at either phases, mtMET cannot be “replenished” through translocation of plasma-membrane-localized MET, the abundance of mtMET starts to decrease as a result of mitophagy and degradation of mitochondria. When the blockade on endocytosis was removed, endocytosis re-activated and an increased level of translocation of MET was identified (Figure 2.4). Interestingly, inhibition with nocodazole did not affect the expression of MET on mitochondria. Nocodazole acts primarily on microtubules to disrupt the endocytic pathway of large particles¹⁶³. The small-sized MET/HGF complexes could be endocytosed via a clathrin-mediated endocytic process independently of microtubule activity. Hence, treatment with nocodazole would not influence the translocation of MET to mitochondria.

A number of mitochondrial proteins, such as the components of the electron transfer chain (ETC) and mitochondrial permeability transition pore (mPTP), are closely regulated by protein kinases, including PI3K/AKT/PKB, RAF/MEK/ERK, and MAPK^{164, 165}. Although not yet clearly demonstrated, phosphorylated mtMET is believed to play a pivotal role in modulating mitochondrial function. Significantly, we discovered that mtMET is functionally inactive following inhibition of endocytosis (Figure 2.1A and 2.3). This finding credibly links RTK-targeted therapeutics with the inhibition of endocytosis. It has been posited that therapeutic efficacy and treatment versatility could be promoted by inhibition of signals alternative to the main target and located either along the same pathway or in parallel pathways that synergize with respect to an essential function¹⁶⁶. Because suppression of endocytosis could selectively inhibit the activation of MET localized in mitochondria of TKI-sensitive gastric cancer cells, this has potential implications in personalized cancer therapeutics.

However, the origin of mtMET is still not completely understood. Caveolin-mediated endocytosis and other mechanisms of internalization cannot be ruled out. Based on the data reported here, internalization and translocation of activated plasma membrane-localized MET forms a significant component of mtMET. Yet, a baseline level of mtMET proteins could still be detected by western blotting when endocytosis was inhibited for up to 24 h. It is also possible that a fraction of newly synthesized MET is directly targeted to mitochondria from the cytoplasm or ER/Golgi. Some mitochondrial outer membrane proteins, such as TOM20 and Bcl-xL, contain a noncanonical mitochondrial localization sequence consisting of highly hydrophobic and basic amino acid-rich sequences in their transmembrane domains and membrane flanking regions. We performed an *in silico* study on MET and other RTKs by using online BLASTP system (basic local alignment search tool-protein). Surprisingly, several RTKs were found contain amino acid sequence of high hydrophobicity and basicity around their transmembrane regions. These RTKs included MET, EGFR, VGFR2, and PGFRB (Supplementary Figure 2). Demory *et al.* have also reported that EGFR contain a highly hydrophobic sequence located around its transmembrane region (transmembrane domain (amino acid 622-644) plus amino acid 645-666), which is sufficient and necessary for EGFR mitochondrial localization. Together, these evidences suggest that some membrane-dependent event is involved in mitochondrial localization of MET. Furthermore, the substrates of mtMET remain to be determined. Future work is required to unravel the role of mtMET in gastric cancer oncogenesis and to evaluate mtMET as a predictive biomarker for sensitivity to molecular-targeted cancer therapeutics.

2.6 Conclusions and Future Work

In an effort to understand the molecular mechanism underlying mitochondrial localization of MET in MET-dependent SNU5 gastric cancer cells, we have uncovered the involvement of clathrin-mediated endocytosis in the translocation of MET to the mitochondria. Inhibition of endocytosis using various inhibitors affected the abundance as well as the activation and phosphorylation of mtMET. The data presented shows the critical role of endocytosis in mtMET activity, which might form one of the direct targets in molecular-targeted gastric cancer therapeutics and could be targeted in a synergic relationship with MET-selective inhibitors. Further work is needed to disclose the underlying mechanisms and importance of mtMET, and to consider whether this novel paradigm may apply to other oncogenic protein kinases. In the next chapter, we will employ quantitative proteomic techniques, *i.e.* TMT (tandem mass tag) labeling, to identify the potential substrates of mtMET. Co-immunoprecipitation will be performed to pull down mtMET-substrate(s) complexes from mitochondria of SNU5 gastric cancer cells to identify its binding partners in order to deduce a potential function for mtMET.

Chapter 3

**Quantitative proteomic profiling to identify novel substrates of
mitochondria-localized MET (mtMET) SNU5 gastric cancer cells**

3.1 Abstract

Gastric cancer is one of the most common malignancies and the fifth leading cause of cancer-related death worldwide. MET has been frequently found dysregulated in gastric carcinomas. Amplification and overexpression of MET as well as the uncontrolled MET/HGF signaling pathway contribute to the cancer cell proliferation, angiogenesis, invasiveness, metastasis, and also protection from apoptosis in cancer cells. A large number of studies on MET in gastric cancer have looked into the underlying mechanisms of dysregulated MET signaling in gastric oncogenesis. Recently it was reported that several kinds of RTK including MET are localized in mitochondria of cancer cells. Yet, the substrate and function of MET in mitochondria of cancer cells remain unknown. In present study, using SNU5 gastric cancer cell as model cell line, we adopted co-immunoprecipitation and TMT based high throughput quantitative proteomic approach to identify potential substrate of mtMET in mitochondria. Among the proteins that were identified at high confidence, a major fraction is involved in catalytic activity and metabolic process in mitochondria. Proximity ligation assays has also revealed the direct protein-protein interactions between mtMET with HMGA1 and PKM2, respectively. Our findings have suggested a novel functional role of mtMET in regulation of energy metabolism in mitochondria. This study is believed to have disclosed a novel paradigm of mtMET signaling in gastric cancer cells and thus contributed to the ongoing efforts to therapeutically target aberrant RTK activities in various human cancers.

3.2 Introduction

3.2.1 Gastric cancer

3.2.1.1 Gastric cancer

Gastric cancer has been ranked as the fifth most common cause of cancer related death in both sexes worldwide. Even though the incidence of gastric cancer varies greatly across populations and geographic environments, it has been estimated to cause 740,000 deaths globally with nearly one million newly diagnosed cases each year, and with overall five-year survival rate ranging from 90 % to less than 5 %^{5-21, 33}. The fact that gastric cancer causes so many deaths annually has called the attention to improve the efficacy of current approaches to prevent, diagnose and treat this disease. Yet a steadily declining incidence of gastric cancer in the past few years has not been matched with a proportionate decrease in mortality rate worldwide^{13, 18, 33, 158}. Despite progression in the diagnosis and treatment of advanced gastric cancer, the prognosis of gastric cancer patients remains poor, in part due to the low rate of diagnosis during its early stages. Also, there is no apparent symptom during the progression of early-stage gastric cancer. It has been expected that if gastric cancer is detected and cured at early stage, the five-year survival rate would be higher than 90 %³³⁻³⁵.

3.2.1.2 Advances of genomic and proteomic methods in gastric cancer diagnosis

During the past few decades, the advances in sciences and technologies are promising to improve and transform our understanding of oncogenesis that ultimately describe a tumor genotype as well as phenotype¹⁶⁷. It is now evident that different cancers can vary extremely from each other with numerous molecular abnormalities and interactions^{168, 169}. Recent applications of genomic tools, such as oligonucleotide or cDNA microarray-based gene expression profiling, identification of metastasis-related genes, and selection of differentially

expressed gene sets, generate a large number of information on the differential gene expression patterns related with gastric cancer^{34, 168, 170-174}. For instance, recently microRNA and DNA hypomethylation have been proposed as novel biomarkers¹⁷⁵. Moreover, the completion of human genome sequencing, together with the advanced evolution of high-throughput genomic techniques, provides priceless data to the comprehensive understanding of oncogenic mechanisms. These achievements have helped to pilot the systematic investigation of molecular tumor taxonomies and also the documentation of different prognostic groups, creating molecular staging systems and models in the development of clinically targeted therapeutics¹⁷⁶⁻¹⁷⁸. It is therefore obviously shown that the applications of genomic tools have the potential ability to refine the way of gastric cancer diagnosis and prognosis.

While it is clear that no single gene could ever completely illustrate the biology of cancer and cancerization, protein-protein interactions, which is the direct executors of life activities constructing the intra- and inter-cellular functional networks, play pivotal roles in gastric oncogenesis¹⁷⁹. Nowadays, the majority of clinically validated biomarkers as well as the effective molecular targets in targeted cancer therapeutics are proteins^{180, 181}. Numerous studies have investigated the molecular basis of gastric cancer, involving the alteration of pathogenesis, and invasion and metastasis. With the development of modern technologies, various novel biomarkers had been identified that appear to possess diagnostic and prognostic value. Several biomarkers for diagnosis and for follow-up in gastrointestinal stromal tumor and gastric cancer patients have been suggested and reported in detail recently. Standard biomarkers used for gastric cancer diagnosis, including p53³⁹, E-cadherin¹⁸², CD-34¹⁸³, c-ErbB2 [*aka* human epidermal growth factor receptor 2 (HER2)]¹⁸⁴, carbohydrate antigen 72-4 (CA 72-4)^{185, 186}, cancer antigen 19-9 (CA 19-9)^{185, 186} and carcinoembryonic antigen^{186 186 186}, have been implicated in increased local recurrence and decreased survival in gastric cancer

patients. These proteins have thus been regarded as putative tumor markers and potential prognostic indicators of gastric cancer. Current achievements strongly motivate the study of changes and transformation of protein inventory in gastric cancer, aiming to accurately predict various tumor behaviors and thoroughly cure the disease one day.

3.2.2 Proteomics and gastric cancer

Proteomics, which refers to the techniques for systematic analysis of proteins in discovery-based research, permits large-scale analyses of proteins for unveiling the novel biological mechanisms of protein-protein interactions. Over the years, the field of proteomics has experienced tremendous technical improvement, including the protein separation technique [for example, two-dimensional gel electrophoresis (2D-GE), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and high performance liquid chromatography (HPLC)] and mass spectrometry¹⁸⁶ analysis [for example, matrix-assisted laser desorption/ionization (MALDI)-time-of-flight (TOF) or MALDI-TOF/TOF, quadrupole (Q)-TOF, HPLC coupled to tandem mass spectrometry (LC-MS/MS), and multiple reaction monitoring (MRM) MS analysis]¹⁸⁷. In Singapore, our laboratory pioneers the introduction and development of proteomics. By combining qualitative and quantitative proteomics analysis together with protein database search, we have been gaining unprecedented comprehensive information and understanding on the study of various diseases including gastric cancer. By applying an unbiased quantitative proteomics approach and several biochemical studies, a previously unrecognized presence of MET kinase in mitochondria of gastric cancer cell line SNU5 was recently discovered⁸⁴. This discovery has enlightened a pave to understanding of gastric oncogenesis and evaluated mtMET (mitochondria-localized MET) as a predictive biomarker for molecularly targeted gastric cancer therapeutics.

3.2.3 MET: structure, function and dysregulated activation

c-MET is located on chromosome 7q21-31. The transcription of *c-MET* is regulated by E-twenty six (Ets), paired box 3 (Pax3), activator protein-2 (AP2) and transcription factor 4 (Tcf-4)¹⁸⁸⁻¹⁹¹. It is expressed as multiple mRNA transcripts of 1.5, 3, 4.5, 7 and 8 kilobases¹⁹² and the protein product is the MET tyrosine kinase protein¹⁹³. After proteolytic processing of the precursor in post-Golgi compartment, MET receptor tyrosine kinase is formed as a single-pass, disulphide-linked α/β heterodimer locating on plasma membrane¹⁹⁴. Extracellularly, MET consists of three domain types: (i) a large semaphorin¹⁹⁵ domain,¹⁹⁶ a PSI domain (found in plexins, semaphorins and integrins), followed by (iii) four immunoglobulin-like domains which are connected to the transmembrane helix. Intracellularly, MET has a tyrosine kinase catalytic domain flanked by distinctive juxtamembrane and carboxy-terminal sequences. When HGF, the ligand for MET, binds and activates MET, trans-phosphorylation on Tyrosine 1234 and 1235 happens, followed by phosphorylation of Tyrosine 1349 and 1356 in the carboxy-terminal tail. Different adaptor proteins and signaling effectors are then recruited to the phosphorylated tyrosines, regulating a wide variety of biological responses unique to the receptor including promoting cell proliferation, survival, motility, scattering, differentiation and morphogenesis^{75, 195, 197-199}. In 1980s, *c-MET* was firstly identified as an oncogene in a human osteosarcoma cell line treated with the carcinogen *N*-methyl-*N*-nitro-*N*-nitrosoguanidine²⁰⁰. Knowledge gained from decades of research using *in vitro* and *in vivo* tumor models has identified three mechanisms of dysregulated MET signaling operating during tumor growth and cancer progression: (i) the occurrence of abnormal genetic lesions such as translocations, gene amplifications and activating mutations,¹⁹⁶ transcriptional upregulation of MET protein, and (iii) ligand-dependent autocrine and paracrine mechanisms²⁰¹. Despite much work has been done to map out the details of MET signaling, understanding of the greater MET network remains incomplete. Our lab has recently reported an unconventional presence of MET kinase in mitochondria of SNU5

gastric cancer cells and proposed mtMET as a versatile candidate for targeted therapeutic intervention⁸⁴. Yet, the role of mtMET in mitochondria of gastric cancer cells remains unclear.

3.2.4 Specific aims in the study

In the present study, we aim to understand the global picture of the proteins associated with mtMET in mitochondria and to identify the potential substrate(s) and thus the function(s) of mtMET in gastric oncogenesis. We applied both qualitative and quantitative proteomic profiling of the mitochondrial proteome of SNU5 gastric cancer cells. We purified SNU5 mitochondria and performed coIP (co-immunoprecipitation) of mtMET by applying a specific approach termed rapid immunoprecipitation mass spectrometry of endogenous proteins¹⁰⁹. Isobaric tag-based quantitative proteomic profiling of SNU5 mitochondrial proteome was subsequently performed using differentially labeled tags TMT (Tandem Mass Tags) coupled with multidimensional liquid chromatography and tandem mass spectrometry. Overall four significantly identified proteins were potentially shortlisted as the candidate proteins of the novel putative substrate of mtMET in mitochondria. Biochemical approaches were subsequently applied and have confirmed protein-protein interactions of two candidate proteins, HMGA1 and PKM2, with mtMET. We are the first to discover the novel substrates of mtMET in mitochondria of SNU5 gastric cancer cells. Our works are believed to uncover a novel paradigm of mtMET signaling in gastric cancer cells and thus contribute to the ongoing efforts to therapeutically target aberrant RTK activities in human cancers.

3.3 Materials and Methods

3.3.1 Chemicals

The highest quality reagents were used in experiments. All water used was prepared using a Milli-Q system (Millipore, Bedford, MA). All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. The selective MET inhibitor PHA-665752 was from Pfizer Global Research and Development (La Jolla Laboratories, San Diego, CA). Protease inhibitor cocktail EDTA-free and phosSTOP was ordered from Roche Diagnostics (Indianapolis, IN, USA). Trypsin-EDTA, PBS and penicillin-streptomycin for cell cultures were obtained from GIBCO-Invitrogen (Carlsbad, CA, USA), while FBS was purchased from Thermo Scientific HyClone (Waltham, MA, USA) and NuSerum was bought from BD Biosciences (Bedford, MA, USA). Stock solutions of PHA-665752 and dynasore were prepared in DMSO, stored in -80 °C and diluted with fresh medium before use. In all experiments, the final concentration of DMSO was <0.1 %.

3.3.2 Cell culture

Human gastric cancer cell line SNU5 was obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured as recommended. SNU5 cells were cultured in IMDM medium containing 20 % FBS, and 100 units/ml penicillin-streptomycin until 70 % confluency before sub-culturing or drug treatment. In MET inhibition and endocytosis inhibition experiments, SNU5 cells were cultured in IMDM medium supplemented with 20 % Nuserum (BD Biosciences) with 50 nM PHA-665752 and 80 μ M dynasore, respectively. All cultures were maintained in a humidified atmosphere of 37 °C with 5 % CO₂ and 95 % air.

3.3.3 Co-IP

Co-IP of mtMET protein complexes from SNU5 mitochondria was performed according to the Mohammed *et al.*'s protocol with some modification (Mohammed et al. 2013) (Figure 3.1). Briefly, 2×10^7 SNU5 cells, with or without treatment, were harvested and washed twice with ice-cold PBS. Cells were re-suspended and cross-linked in pure IMDM medium containing 0.6 % formaldehyde (EM grade; tebu-bio) for 6 min with constant rotation. Crosslinking were quenched by adding glycine to a final concentration of 0.2 M. Cells were washed twice with ice-cold PBS supplemented with 0.2 M glycine and once with pure ice-cold PBS buffer. The mitochondrial fraction was extracted by re-suspending and homogenizing the cell pellet in lysis buffer I [1X lysis buffer from mitochondria isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany), supplemented with complete protease inhibitor mixture tablet and phosSTOP]. Mitochondria were then isolated using the mitochondria isolation kit according to the manufacturer's instruction. Purified mitochondrial pellets were lysed in lysis buffer II [50 mM Tris-HCl, pH 7.0, 0.1 mM EGTA, 2 % CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate), 1 % Triton X-100, supplemented with complete protease inhibitor mixture tablet and phosSTOP] by vigorously vortexed at 4 °C for 1 h followed by sonicating in a waterbath sonicator (Diagenode Bioruptor) for 30 sec. The mitochondrial lysate was centrifuged for 10 min at 20,000 g to remove the debris. Subsequently, co-IP of mtMET complex were performed with 10 µg of anti-MET rabbit monoclonal antibody (target, clone D1C2, Cell Signaling Technology), 10 µg of anti-HMGA1 rabbit polyclonal antibody (target, clone FL-95, Santa Cruz Biotechnology), 10 µg of anti-PKM2 rabbit polyclonal antibody (target, clone H-60, Santa Cruz Biotechnology), and 10 µg of normal rabbit IgG (control, clone SC-2027, Santa Cruz) using the classic magnetic co-IP kit (Pierce, Thermo Scientific) according to the manufacturer's protocol. Protein extracts were in-gel digested and analyzed using mass spectrometry as described below.

3.3.4 BCA protein assay

Protein concentration was measured using BCA protein assay on a 96-well plate. Standard curve was made from standard bovine serum albumin solution of concentrations of 0.1, 0.2, 0.4, 0.6, 0.8, and 1 mg/mL, respectively. The plate was incubated at 37 °C for 30 min after the sample/standard and working reagent (50 bicinchoninic acid solution: 1 CuSO₄·5H₂O solution, v/v) were mixed. Thereafter, the optical density was measured at 562 nm with Infinite® M1000 Microplate Reader (Tecan, Switzerland) and exported to Microsoft Excel for calculations of protein concentration.

3.3.5 Mitochondrial protein digestion

Mitochondrial protein extracts, with or without treatment, were resuspended in 200 µL of PBS containing 2 % SDS, supplemented with complete protease inhibitor mixture tablet and phosSTOP. The suspension was sonicated for 20 × 3 s at 4 °C with 5 s rest in between using output 30 %. Protein concentrations were measured using BCA protein assay before SDS-PAGE. 40 µg of mitochondrial proteins were separated in a 10 % polyacrylamide gel and stained with silver stain following the manufacturer's protocol. Gel bands were excised and diced into 1 mm × 1 mm, followed by reduction with 10 mM DTT at 56 °C for 1 h, and then alkylation with 55 mM IAA at room temperature in dark for 45 min. Proteins were digested with 10 ng/µL of sequencing grade modified trypsin for 4 h at 37 °C. Same amount of trypsin was added for a second time tryptic digestion overnight at 37 °C. Peptides were extracted from gel pieces with 50 % isopropanol, 5 % HOAc followed by 50 % ACN, 5 % FA. Peptide extracts from each band were vacuum-dried with a SPD 2010 SpeedVac system (Thermo electron, Waltham, MA, USA) and desalted with SEP-PAK C18 cartridges 50 mg (Water Corp., Milford, MA, USA). Desalted peptides were subsequently vacuum-dried again.

3.3.6 Protein digestion and TMT labelling

TMT kit (Pierce, Idaho, ID, USA) was used in quantitative proteomic profiling of SNU5 mitochondrial proteome in different conditions (Figure 3.6). One hundred micrograms of each sample was precipitated in cold acetone (1:6), at -20°C overnight. Six samples were reconstituted in 100 μL 8 M urea, 100 mM ammonia bicarbonate buffer (ABB) (pH 8.0). Then, each sample was reduced by 10 mM Tris(2-carboxyethyl)phosphine hydrochloride solution (TCEP) for 3 h at 37°C and alkylated by 20 mM IAA for 45 min at room temperature in the dark. Six samples were digested with 2.5 μg of sequencing grade modified trypsin for 4 h at 37°C . Same amount of trypsin was added for a second time tryptic digestion overnight at 37°C . TMT reagents were reconstituted according to the manufacturer's instructions. Briefly, each tube containing the different isobaric chemical tags (0.8 mg each) was added with 41 μL of anhydrous acetonitrile at room temperature. Reagents were dissolved by vortexing for 5 min, and then spun down to concentrate the labeling reagents. All the 41 μL of TMT isobaric tag was added into each sample and incubated at room temperature for 1 h. Then, the labeling reaction was quenched with 8 μL of 5 % hydroxylamine at room temperature for 15 min. Finally, six samples were pooled together and subsequently vacuum-dried. The dried TMT sample would be fractionated using HPLC prior to mass spectrometry analysis.

3.3.7 Reverse phase HPLC fractionation

The pooled TMT-labelled peptides were fractionated using a high pH reverse phase C18 column (4.6×200 mm; 5 μm , 300 $\text{\AA}$; Waters X-Bridge) on a Prominence HPLC system (Shimadzu, Kyoto, Japan). The UV detection was monitored at a wavelength of 280 nm. Mobile phase A [0.02 % ammonia hydroxide (NH_4OH) in HPLC water] and mobile phase B (0.02 % NH_4OH in 80 % ACN) were used to established a 65-min gradient at a flow rate of 1 mL/min. Briefly, 3 % buffer B for 5 min, 3 - 35 %

buffer B for 40 min, 35 – 70 % buffer B for 5 min, 70 – 100 % buffer B for 5 min, 100 % buffer B for 5 min, and finally re-equilibrated the column with 0 % buffer B for 5 min. A total of 63 fractions were collected. Then the fractions were pooled in concatenated way into 30 fractions and dried in vacuum concentrator.

3.3.8 Desalting peptide samples

Pre-equilibrated SEP-PAK C18 cartridges 50 mg with 1 mL of ACN followed by 3 mL of 0.1 % trifluoroacetic acid (TFA). Resuspended vacuum-dried samples in 1mL of 0.4 % TFA and loaded the suspension onto pre-equilibrated C18 cartridges. Washed the cartridges with 4 mL of 0.1 % TFA and eluted peptides with 1mL of 70 % ACN, 0.1 % FA. Eluate was subsequently vacuum-dried.

3.3.9 LC-MS/MS

The resultant peptides were dried with vacuum concentrator and analyzed by LC-MS/MS. The dried peptides were firstly re-dissolved in LC-MS/MS compatible buffer (3 % ACN, 0.1 % FA). Peptides were separated and analyzed on a Dionex Ultimate 3000 RSLC nano system coupled to a Q Exactive (Thermo Fisher, MA) as previously described with minor modification¹⁴⁷. Approximately 1.2 µg of peptides from each sample were injected into an Acclaim peptide trap column (Thermo Fisher, MA) via the autosampler of the Dionex RSLCnano system. Peptides were separated in a capillary column (75 µm x 10 cm) packed with C18 AQ (5 µm, 300 Å; Bruker-Michrom, Auburn, CA, USA) at room temperature. The flow rate was at 300 nL/min. Mobile phase A (0.1 % formic acid in 5 % ACN) and mobile phase B (0.1 % formic acid in 90 % ACN) were used to establish a 60 min gradient: 30 min of 7-18 % B, 15 min of 18-30 % B, 4 min of 30-50 % B, 1 min of 50-80 % B, 2 min of 80 % B, 5 min of 80-5 % B, followed by re-equilibration at 7 % for 3 min. Peptides were then analyzed on Q Exactive with a nanospray source

(Thermo Fisher, MA) at an electrospray potential of 1.5 kV. A full MS scan (350–1600 m/z range) was acquired at a resolution of 70,000 at m/z 200 and a maximum ion accumulation time of 100 ms. Dynamic exclusion was set as 30 s. Resolution for HCD spectra was set to 17,500 at m/z 200. The AGC setting of full MS scan and MS2 were set as 1E6 and 1E5, respectively. The 10 most intense ions above a 1000 counts threshold were selected for HCD fragmentation with a maximum ion accumulation time of 100 ms. Isolation width of 2 Th was used for MS2. Single and unassigned charged ions were excluded from MS/MS. For HCD, normalized collision energy was set to 28 %. The underfill ratio was defined as 0.1 %.

3.3.10 Data Analyses

The raw data files were converted into the dta format using the extract_msn program (version 4.0) in Bioworks Browser 3.3 (Thermo Electron, Bremen, Germany), and then the dta files were converted into Mascot generic file format using Proteome Discoverer 1.4.1.14 (Thermo Electron, Bremen, Germany). Protein identification was performed by querying against the extracted Uniprot *Homo sapiens* database (released on 29 Nov, 2015; containing 70,141,034 residues and 176,946 sequences as well as the reverse sequences for decoy search) using an in-house Mascot server (version 2.4.1, Matrix Science, Boston, MA) with peptide precursor mass tolerances of 10 ppm and fragment ions mass tolerance of 0.02 Da¹⁴⁷. Two missed cleavage sites of trypsin and # 13 C of 2 were allowed in the search. Carbamidomethylation of cysteine residues was set as a fixed modification, while oxidation of methionine, deamidation of asparagine and glutamine residues, and phosphorylation of serine, threonine and tyrosine residues were set as variable modifications. For TMT experiments, TMT 6-plex modification of lysine residues and peptide N terminals was set as the additional variable and fixed modification, respectively. The extracted Mascot results for each sample were exported to csv file format and further processed with in-

house scripts and Microsoft Excel. Unpaired Student's *t*-test was subsequently performed to examine the statistical significance change of expression level of protein by comparing ratios of 24-h drug treatment to those of control by using online GraphPad QuickCalcs: *t*-test calculator (<http://graphpad.com/quickcalcs/ttest1.cfm>)

EmPAI-based label-free quantification method was applied for the comparison of protein abundance in co-IP experiments. emPAI values were calculated according to the stated equation 1 to 3, as shown below^{98, 104}. The N_{obsd} and N_{obsbl} represented the number of observed peptides per protein and the number of observable tryptic peptides per protein, respectively. Only peptides with peptide score equal to and/or higher than homology threshold and identity threshold were collected and used for emPAI values calculation by Mascot server during the database search procedure. The normalization of emPAI value was performed by dividing the emPAI value of each fraction with sum of the respective fractions of each individual protein as stated in Equation 3. After completion, peptide and protein lists were exported to csv file format and further processed using in-house scripts and Microsoft Excel for in-depth analysis.

Equation 1,

$$PAI = \frac{N_{obsd}}{N_{obsbl}}$$

Equation 2,

$$emPAI = 10^{PAI} - 1$$

Equation 3,

$$Normalized\ emPAI_i = \frac{emPAI_i}{\sum_{i=1}^x emPAI_i}$$

To minimize the experimental bias, the protein abundances were normalized on protein median. False discovery rate of peptide identification was set to be less than 1 % [FDR = $2.0 \times (\text{decoy_hits}/\text{total_hits}) \times 100 \%$]. Identified peptides were sorted from smallest to largest Mascot peptide expectation value, and largest to smallest ion score. Only peptides fulfilled these two requirements were selected for further analysis: (1) Peptides with ion scores greater than 20; and (2) peptides with expectation value smaller than 0.001.

3.3.11 Data Annotation

Several open source online bioinformatics software tools, including STRING v10.0²⁰², Panther v9.0^{148, 149}, Genemania v3.1.2^{203, 204} and DAVID v6.7¹⁵⁰⁻¹⁵², were used to help to analyze and interpret the data.

3.3.12 *In situ* proximity ligation assay, microscopy and data handling

The *in situ* PLA (proximity ligation assay) experiments were performed using reagents and instructions found in commercially available kits from Sigma-Aldrich; Duolink® *in situ* PLA® Probe Anti-Rabbit MINUS/PLUS (DUO92005/DUO92002), Duolink® *in situ* PLA® Probe Anti-Mouse MINUS/PLUS (DUO92004/DUO92001) and Duolink® *in situ* Detection Reagents Orange (DUO92007) (Olink Bioscience). Briefly, 5,000 SNU5 cells were washed in ice-cold PBS thrice and incubated in 20 % FBS/PBS, followed by cytocentrifugating for 5 min at 1,000 rpm onto superfrost plus microscope slide. Cells attached on microscope slide were washed with 1 × PBS once and fixed in 3.7 % PFA for 20 min at room temperature. After fixing, cells were washed with PBS thrice and permeabilized with 0.2 % Triton X-100/PBS for 10 min followed by blocking in Duolink *in situ* PLA blocking solution for 30 min at 37 °C. Primary antibodies were diluted in 2 % BSA in 1 % Triton X-100/PBS to final concentrations of 1 µg/mL and added to corresponding slides and

incubated in a humidity chamber overnight at 4 °C, followed by washing the slides in 1 × TBS 0.05 % Tween (TBS-T) for 3 × 5 min with gentle agitation. Secondary probes were diluted to final concentrations of 1:5 in antibody diluent (supplied in the kit) and added to each slide. The slides were incubated in a pre-heated humidity chamber for 1 h at 37 °C and then washed in 1 × TBS-T for 3 × 5 min with gentle agitation. Thereafter pre-mixed ligation-ligase solution was added to each sample and slides were incubated in a humidity chamber for 30 min at 37 °C. Ligation solution was removed and slides were washed with 1 × Wash Buffer A for 2 × 2 min under gentle agitation. Mixture of amplification solution (MilliQ H₂O, Amplification/detection mix, polymerase) was added to each sample and slides were incubated in a humidity chamber for 100 min at 37 °C. Slides were washed in 1 × Wash Buffer B for 2 × 10 min and then in 0.01 × Wash Buffer B for 1 min under gentle agitation. Each sample was counterstained and coverslips were mounted with Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA). Edges of the coverslips were sealed with nail polish. Slides were stored at 4 °C until visualizing the results. The results were viewed with an Axio Observer microscope (Carl Zeiss, Germany) and stacked TIFF files were captured using AxioVision acquisition software (Carl Zeiss, Germany), with a maximum slice distance of 0.5 µm in order to image every PLA signal. At least five images were taken for each sample at various sites on the slide. A total number of 50-70 cells were imaged from each sample to account for the cell-to-cell variation in the number of PLA signals per cell. Images obtained were analyzed and processed using ImageJ and Microsoft PowerPoint. The quantifications were exported to Microsoft Excel and the numbers of PLA signals were carefully counted using Adobe Photoshop manually. Student's *t*-test was performed to visualize the significance level of experiments.

3.4 Results

3.4.1 Discovery of novel putative substrate of mtMET using a label-free quantitative strategy

In the study we would like to identify the candidate protein associated with mtMET in mitochondria, aiming to discover the potential substrate of mtMET and its role in SNU5 gastric oncogenesis. Since MET is initially known as a RTK that executes its functions from and locates on plasma membrane and other intracellular compartments¹⁴², it is possible that, the putative substrate of mtMET on mitochondria will be a cytoplasmic protein and, like mtMET, may have no conventional mitochondrial targeting peptide signal. We approached this by combining the selective co-IP of mtMET with a label-free, quantitative proteomics method. We modified and applied a previously published protocol, named RIME (rapid immunoprecipitation mass spectrometry of endogenous proteins)¹⁰⁹, to isolate the protein complex associated with mtMET from SNU5 mitochondrial extracts (Figure 3.1).

As described in Figure 3.1, mtMET and associated proteins were co-immunoprecipitated using anti-MET rabbit monoclonal antibody or normal rabbit IgG. Protein extracts pulled down by anti-MET antibody (target) were analyzed by gel electrophoresis and mass spectrometry, and then we subsequently compared the result to that of protein extracts pulled down by normal rabbit IgG (control). To ensure the accuracy and reliability of proteome detected by the LC-MS/MS, biological replicates of co-IP were performed and protein extracts were pooled together to minimize sample-to-sample variation. Then technical replicates of the protein extracts from both control and target were analyzed in triplicate on a high mass resolution Q Exactive instrument. The fragment ion spectra acquired from 36 LC-MS/MS runs were searched against the Uniprot *Homo sapiens* database in a concatenated target and decoy strategy. We assigned a total of 65,258 (21,558 + 21,970 + 21,730) and 146,392 (48,832 + 48,496 + 49,064) MS/MS spectra for control and

target pull-down, respectively, with an estimated FDR lower than 1 %. Overall the assigned spectra were matched to 190 unique proteins (total 257 proteins) for IgG-coIP (IgG pull-down) and 1,227 unique proteins (total 2,223 proteins) for MET-coIP (MET pull-down), with more than one unique peptide identified for each protein (95 % confidence).

Figure 3.1: Schematic representation of the experimental design of mtMET co-IP. mtMET and associated proteins were isolated according to the workflow, and protein extracts were eventually subjected to LC-MS analysis.

A stringent inclusion criteria was employed to filter the qualitative proteomics dataset, *i.e.* only proteins quantified from at least two unique peptides, and having ion scores $\geq$ identity/homology scores were considered as confident protein hits and advanced to the next phase of analysis. In the triplicate runs of MET-coIP, 382 proteins were commonly detected in three fractions (Figure 3.2A). The commonly immunoprecipitated proteins were matched to the list of proteins detected in IgG-coIP. In total, 342 candidate proteins were uniquely identified in the MET-coIP fractions (Figure 3.2B). Reproducibility of co-

IP experiments was examined by comparing the normalized emPAI values of each candidate protein between pairs of technical replicates and calculating the Pearson correlation coefficients. For all of the comparisons, we obtained high R^2 values (ranging around 0.8), as illustrated in Figure 3.3, indicating the high reproducibility and thus the high reliability of our results. These 342 proteins were further analyzed using an online PANTHER classification system database in terms of the molecular functions and biological processes (Figure 3.4A and 3.4B).

Figure 3.2: Comparison of the identified proteins between IgG-colPs and MET-colPs. (A) In the triplicate runs of MET-colP, 382 proteins were commonly detected after 36 runs of mass spectrometry analyses. (B) In the protein list of MET-colP, total 342 proteins were uniquely identified as compared to the list of proteins identified in IgG-colP fractions.

Figure 3.3: Scatter plots showing the normalized emPAI values in the technical triplicate measurements. Blue dash lines demonstrated the linear regression of the data and the calculated Pearson correlation coefficient R^2 values were indicated in the bottom. Proteins from triplicate runs of MET-colP were plotted in pairs: (A) 1st vs 2nd replicate of MET-colP, (B) 2nd vs 3rd replicate of MET-colP, and (C) 1st vs 3rd replicate of MET-colP.

Figure 3.4: Classification analysis of 342 uniquely identified proteins in MET-colPs. List of proteins in triplicate runs of MET-colP were subjected to different Gene Ontology classification tools using the online PANTHER classification system database: (A) molecular functions, (B) biological processes, and (C) cellular components.

In the classification of molecular functions, the analysis pointed out that major fraction of the identified proteins involved in the catalytic activity (43.6 %) including kinase activity, phosphatase activity, transferase activity, peptidase activity, oxidoreductase activity, etc (Figure 3.4A). As expected, when the proteins were classified according to the biological process, majority of the proteins were found involving in the metabolic process (35.9 %) and cellular process (18.1 %) since one of the most prominent roles of mitochondria is to regulate the cellular metabolism (Figure 3.4B).

A bioinformatics classification based on the Gene Ontology subcellular localization information was also performed to determine proteins coming from which intracellular compartments had been extracted in the MET-colP experiments (Figure 3.4C). Up to about 32 % of the 342 proteins found locating in mitochondrion. This result pointed out the possibility that the potential substrate of mtMET might not be a native or nucleus-encoded mitochondrial protein.

On the other hand, the list of candidate proteins was submitted to the online biological database, STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database, to disclose the known and predicted protein-protein interactions in our dataset. A medium confidence (score 0.40) cut-off threshold was set in order to connect the mtMET with any proteins pulled down in our study based on previous published works. A highly complex protein-protein interactions network was shown in Figure 3.5. Table 3.1 summarized the proteins that have been shown interacting or connecting to MET in previous works through data and text mining.

Figure 3.5: Protein-protein interactions network extracted from STRING database. A complex interacting network of the uniquely identified proteins in MET-colPs was illustrated. A medium confidence cut-off threshold was applied to remove any node of protein-protein interaction with STRING combined score lower than 0.40. Outliers, which do not show any interaction to other proteins, have also been excluded from the list.

Table 3.1: Identification of proteins interacting with MET in co-IP data. List of 342 proteins were searched in STRING *Homo sapiens* database. A medium confidence (score 0.40) cut-off threshold was set.

Node1	Node2	Neighborhood	Experimental	Database	Textmining	Score
MET	GAPDH	--	--	--	0.559	0.559
MET	YWHAZ	--	0.337	--	0.138	0.404
MET	NSUN2	0.462	--	--	0.157	0.527
MET	ACLY	--	--	--	0.457	0.457
MET	KPNB1	--	--	0.9	--	0.9
MET	FASN	--	--	--	0.834	0.834
POTEF	MET	--	0.228	--	0.261	0.405
EZR	MET	--	--	--	0.417	0.416
PCNA	MET	--	--	--	0.513	0.513

MET was found interacting with GAPDH, YWHAZ, NSUN2, ACLY, KPNB1, FASN, POTEF, EZR and PCNA protein through data mining in previous experimental data, computational prediction and public text collections (Table 3.1). Since mtMET was a RTK, it is likely that the novel putative substrate of mtMET would be a protein able to bind to and/or to be activated by mtMET through phosphorylation. We performed manual literature revision to study the 9 proteins in Table 3.1 and all of them have been shown could be phosphorylated by kinase proteins. Nevertheless, at this stage we could not draw a conclusive decision regarding the potential substrate of mtMET in mitochondrion yet.

3.4.2 TMT labeling of SNU5 mitochondrial proteome under different conditions

Quantitative proteomic profiling of SNU5 mitochondrial proteome treated with different drugs was performed using differentially labeled tags TMT coupled with multidimensional liquid chromatography and tandem mass spectrometry. The workflow of the experiment is shown below (Figure 3.6). Briefly, six SNU5 mitochondrial samples were reduced with TCEP, alkylated with IAA, digested with trypsin, differentially labeled at peptide-level with TMT 6-plex reagents, pooled together, fractionated using RP-HPLC, and finally subjected to analyses by LC-MS/MS using an Q Exactive instrument (Figure 3.6). Of these, one mitochondrial sample without any treatment (control) was labeled with TMT⁶-126 (TMT with reporter ions at $m/z = 126$); one mitochondrial sample activated with 100 ng/mL HGF for 24 h was labeled with TMT⁶-127; two mitochondrial samples inhibited with 50 nM PHA-665752 for 24 h and 12 h were labeled with TMT⁶-128 and TMT⁶-129, respectively; and two mitochondrial sample treated with 80 μ M dynasore for 24 h and 12 h were labeled with TMT⁶-130 and TMT⁶-131, respectively. Technical replicate analyses of the samples were performed on the high mass resolution Q Exactive instrument. The raw data files obtained after LC-MS/MS analyses were processed in Proteome Discoverer version 1.4.1.14. After searching against Uniprot *Homo sapiens* database and filtering with a 1 % FDR, proteins were identified and quantified according to the intensity of reporter ions and normalized on the protein median. Ratios of protein abundance were calculated for each sample by comparing with that of control sample (TMT⁶-126). By combining all the data, a total of 1657 proteins, which have quantitative information and detected in both technical replicates, were obtained. Distribution of physiochemical parameters such as molecular weight and calculated isoelectric point of identified proteins (Figure 3.7) showed well distribution of these parameters, indicating that proteomics technique used for this experiment was suitable for all types of protein

identification. The combined original dataset was demonstrated in Supplementary Table 2.

Figure 3.6: Schematic representation of the experimental design to perform the quantitative proteomics analysis of SNU5 mitochondrial proteome using TMT 6-plex isobaric tags. SNU5 cells were treated with HGF, PHA-665752 or dynasore for different durations prior to mitochondrial protein extraction and TMT labeling.

Figure 3.7: Physiochemical characteristics of identified protein. Scatter plot showed the well distribution of the intrinsic properties of identified proteins, including the molecular weight (MW) and calculated isoelectric point (calc. pI). Molecular weights were calculated in log space.

Figure 3.8: Distribution of the relative expression levels of temporal proteome. When SNU5 cells were inhibited with PHA665752_24h (128/126) or Dynasore_24h (130/126), the relative expression levels of proteins were normally distributed. Top 10% of proteins which were most perturbed by inhibitors were selected. For the fold change of protein abundance, TMT ratio above 1.200 was considered as upper cut-off (increased protein expression) and ratio below 0.833 was regarded as lower cut-off (decreased protein expression). Ratio was calculated in log space before converting into linear space.

Table 3.2: Data analysis of identified proteins in different drug treatment of SNU5 mitochondria digests.

	Number of proteins with significant changes in TMT ratio				
Sample	HGF_24h	PHA665752_24h	PHA665752_12h	Dynasore_24h	Dynasore_12h
TMT ratio	127:126	128:126	129:126	130:126	131:126
Ratio >1.200	307	248	24	331	333
Ratio <0.833	312	291	5	350	303

The cutoff for defining perturbed and unperturbed protein expression in TMT experiments depends on the characteristics of biological samples as well as MS instruments. To avoid setting the cutoffs arbitrarily, we examined the distribution of the expression levels of the 1657 proteins. When SNU5 cells were treated with inhibitors, *i.e.* PHA665752_24h (128/126), and Dynasore_24h (130/126), the relative expression levels of proteins were all normally distributed, indicating that sublethal treatment of either one of these compounds only modulated a small percentage of the SNU5 mitochondrial proteome. We thus focused on the top 10% proteins whose expression was most perturbed by PHA-665752 and/or dynasore. With this criterion, protein ratios <0.833 were regarded as underexpressed, whereas ratios >1.200 were considered overexpressed, thereby narrowing the reliable differentially expressed proteins to a small number (Figure 3.8 and Table 3.2), which reflected significant effects of mtMET inhibition in SNU cells. Proteins with significant TMT ratio change at least in one condition were selected for further analysis (Table 3.2). However, we noticed that, when cells were inhibited with 12 h of 50 nM PHA-665752, the change in the mitochondrial proteins expression level was relatively low as compared to other conditions. Only 24 and 5 proteins showed significant fold change of protein abundance above 1.200 and below 0.833, respectively. Thereafter we decided to take account of the proteins confidently detected in HGF_24h (127/126), PHA665752_24h (128/126) and Dynasore_24h (130/126), so that we could have a much more reliable study and better comparison among samples of same duration

of drug treatment. Herein we found that the ratio of mtMET protein abundance in SNU5 mitochondria decreased when the cells were inhibited with 24 h of PHA-665752 (128/126) or dynasore (130/126) (Table 3.3). Hence it was reasonably believed that the abundance of the potential substrate of mtMET would respond in the same way, which has a decreased fold change of protein expression when SNU5 cells were treated with either kinds of inhibitor. In the mitochondria digestion coupled isobaric tags based quantitative proteomic study, we confidently identified 91 proteins that have shown reduced protein abundance when the cells were inhibited using PHA-665752 or dynasore for 24 h (Table 3.3). Proteins were analyzed with the Gene Ontology classification using the online PANTHER classification tool. Although only 56 of the 91 confidently identified proteins were available in GO annotation, we found that major fraction of the identified proteins involved in the catalytic activity (38 %, Figure 3.9A) and metabolic and cellular process (37.7 % and 20.5 %, Figure 3.9B) in the classification of molecular function and biological process, respectively. The result of TMT quantitative proteomics study was in complete accord with that of the co-IP label-free quantitative proteomics study described above. Together, both results suggested that mtMET and its putative substrate in mitochondria play a critical role involved in the catalytic activity and/or metabolic and cellular biological processes which would contribute to the SNU5 gastric oncogenesis.

We manually studied and revised these significantly identified proteins through extensive data mining on previously published literatures. Importantly, 33 proteins, *i.e.* 36.3 % of the 91 proteins have been previously established and found playing a crucial role in oncogenesis, cancer development and metastasis and/or apoptosis of various human cancer cells. Brief descriptions and related publications of the 33 proteins were listed systematically in Table 3.4.

A**GO Molecular Function** **B****GO Biological Process**

Figure 3.9: GO analysis illustrates the classes of proteins with significantly reduced expression. Proteins with significant decreased fold change at least in one 24 h of inhibition conditions were subjected to GO

classification in terms of molecular function (A) and biological process (B).

Table 3.3: List of proteins showing significantly reduced protein expression level in the PHA665752_24h (128/126) and Dynasore_24h (130/126). Ratios are quantified according to the intensity of the reporter ions and normalized on the protein median. p value < 0.05.

Accession	Description	%Cov	#Pept	#PSMs	#AAs	calc.pl	127/126		128/126		129/126		130/126		131/126	
							Ratio	CoV	Ratio	CoV	Ratio	CoV	Ratio	CoV	Ratio	CoV
A8K202	5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase, isoform CRA_g	20.14%	12	33	591	6.71	0.703	0.5	0.782	11.2	0.995	4	0.688	0.3	1.254	0.2
B1AK13	3-hydroxymethyl-3-methylglutaryl-Coenzyme A lyase (Hydroxymethylglutaricaciduria), isoform CRA_b	26.33%	9	11	300	7.61	0.764	8.8	0.898	21.7	1.012	10.3	0.714	19	0.791	2.7
B1AKM8	Phosphatidylserine decarboxylase (Fragment)	11.84%	3	6	228	9.82	0.955	11.7	0.945	0.6	1.088	8.5	0.746	11.4	0.649	7.1
B3KRM2	Serine/threonine-protein phosphatase	11.97%	3	5	309	5.43	0.775	26.9	0.768	4	1.074	8.5	0.727	6.7	1.420	4.5
B3KS15	6-phosphofructokinase	16.88%	12	20	776	8.59	0.823	6.7	0.879	16.8	1.021	0.1	0.822	22.1	1.207	3.9
B4DE36	Glucose-6-phosphate isomerase	10.38%	6	13	530	8.15	0.878	11.8	0.858	4.3	0.966	0.6	0.818	8.8	1.129	9.4
B4DFY5	Mitogen-activated protein kinase kinase 1, isoform CRA_d	13.48%	5	10	371	6.62	0.981	2.5	0.797	1.6	0.982	3.7	0.973	27.3	1.164	2.8
B4DHJ7	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3	12.78%	2	4	180	6.29	0.848	8.5	0.640	1.8	1.023	4	0.959	6	0.619	13
B4DHN5	Syntenin-1	13.81%	4	13	239	6.64	0.853	5.6	0.729	0.7	0.961	1.6	0.751	9.4	0.998	1.3
B4DJ11	L-lactate dehydrogenase	25.90%	9	27	305	8.46	0.936	7.4	0.714	2.8	1.019	2.2	0.831	6	1.033	0.4
B4DLR8	NAD(P)H dehydrogenase [quinone] 1	24.26%	5	21	202	8.5	0.746	2.2	0.823	4.6	1.017	3.7	0.711	4.6	1.020	2
B4DP21	Prostaglandin E synthase 3	30.00%	3	15	130	4.77	0.741	3	0.820	2	0.967	3.5	0.761	3.9	1.407	1
B5BUB1	RuvB-like 1 (Fragment)	16.45%	6	12	456	6.42	0.776	3.5	0.788	9.9	1.066	9.8	0.800	7	0.908	5.5
B8ZZG1	MAGUK p55 subfamily member 6	11.68%	5	8	428	8.76	1.183	2.6	0.850	1.6	1.053	5.1	0.788	7.4	1.080	7
C9J6B1	Ras-related protein Ral-B (Fragment)	17.96%	3	10	167	4.93	1.218	2.6	0.758	10.2	0.916	6.2	0.810	6.1	0.924	6.2
C9JFR7	Cytochrome c (Fragment)	42.57%	6	21	101	9.66	0.722	2.6	0.792	0.1	0.925	1.1	0.805	4.4	0.604	3.3
D3DUZ3	Interferon, gamma-inducible protein 16, isoform CRA_a	25.92%	19	47	733	9.32	0.527	2.6	0.535	11.5	0.974	3.2	0.503	14.2	0.740	3.1

Accession	Description	%Cov	#Pept	#PSMs	#AAs	calc.pl	127/126		128/126		129/126		130/126		131/126	
							Ratio	CoV	Ratio	CoV	Ratio	CoV	Ratio	CoV	Ratio	CoV
D6R938	Calcium/calmodulin-dependent protein kinase (CaM kinase) II delta, isoform CRA_e	10.84%	5	8	498	7.25	0.692	2.6	0.816	4.6	0.991	3.2	0.825	5.7	1.283	5.3
E7ESV4	Ras-related protein Rap-1b (Fragment)	29.30%	6	20	157	4.79	1.005	2.6	0.723	6.2	1.012	3	0.684	3	1.060	0.5
E9PF23	SUN domain-containing protein 1	21.53%	15	47	785	6.93	0.497	2.6	0.563	2.2	1.057	0.8	0.510	21.7	0.925	4.6
F8WBJ6	RNA-binding protein PNO1	32.35%	4	5	136	9.82	1.003	2.6	0.801	2.6	0.903	1.6	1.003	17.3	1.031	2.2
G2XKQ0	Sumo13	18.81%	2	12	101	5.52	0.660	2.6	0.751	4.7	1.004	0.8	0.783	2.2	1.060	4.7
G3V130	MTERF domain containing 1, isoform CRA_c	11.82%	4	6	296	8.44	0.765	2.6	0.851	2.7	1.037	8.4	0.723	1.8	0.900	17.3
H0UID5	Adaptor-related protein complex 2, beta 1 subunit, isoform CRA_c	12.31%	13	24	934	5.34	1.006	2.6	0.874	0.8	0.989	2.2	0.769	1.8	0.816	0.4
H3BRY5	Sulfotransferase 1A1 (Fragment)	14.95%	4	8	281	6.62	0.933	2.6	0.816	9.8	0.952	1.7	0.752	2.4	0.542	9.2
J3KPE3	Guanine nucleotide-binding protein subunit beta-2-like 1	23.81%	6	13	273	7.65	0.663	2.6	0.713	3.3	0.953	6	0.738	0.2	1.449	1.5
K7EM91	Kunitz-type protease inhibitor 2	16.83%	3	13	202	7.46	1.188	2.6	0.889	2.6	1.009	8.3	0.819	5.3	0.817	7
K7ENL3	Signal transducer and activator of transcription 3	12.74%	8	19	722	7.12	0.993	2.6	0.842	4	0.944	3.5	0.784	2.1	0.906	6.5
O60762	Dolichol-phosphate mannosyltransferase	21.15%	6	12	260	9.57	0.814	2.6	0.771	2.1	1.029	5.3	0.849	6.7	1.126	1.6
O60884	DnaJ homolog subfamily A member 2	11.41%	6	10	412	6.48	0.779	2.6	0.611	9.2	0.993	1	0.661	17.5	1.307	2.3
O75083	WD repeat-containing protein 1	16.17%	9	13	606	6.65	1.105	2.6	0.783	11.6	0.917	6.7	0.650	7.1	0.904	3.8
O94776	Metastasis-associated protein MTA2	12.43%	9	16	668	9.66	0.689	2.6	0.649	1.9	0.984	0.5	0.682	15.8	0.855	4.1
O95407	Tumor necrosis factor receptor superfamily member 6B	21.33%	6	14	300	8.24	0.710	2.6	0.383	6.2	0.977	4.1	0.424	2.4	0.689	4.8
P00558	Phosphoglycerate kinase 1	27.82%	13	17	417	8.1	0.843	2.6	0.690	6.6	1.037	1.9	0.828	3.1	1.155	3.8
P04075	Fructose-bisphosphate aldolase A	54.12%	18	103	364	8.09	0.852	2.6	0.798	0.5	1.030	3.2	0.962	5.3	1.057	3.2
P04181	Ornithine aminotransferase, mitochondrial	16.63%	6	13	439	7.03	0.764	2.6	0.849	8.8	1.052	4.8	0.774	2.2	0.744	6.7
P04406	Glyceraldehyde-3-phosphate dehydrogenase	50.75%	20	182	335	8.46	0.561	2.6	0.598	4.1	1.031	0.7	0.707	2.9	1.234	2.2
P05161	Ubiquitin-like protein ISG15	15.15%	3	10	165	7.44	0.713	2.6	0.786	5.9	0.965	3.3	0.681	7.7	1.000	2.8

Accession	Description	%Cov	#Pept	#PSMs	#AAs	calc.pl	127/126		128/126		129/126		130/126		131/126	
							Ratio	CoV	Ratio	CoV	Ratio	CoV	Ratio	CoV	Ratio	CoV
P08581	Hepatocyte growth factor receptor	22.01%	31	122	1390	7.33	1.158	2.6	0.818	4.2	1.060	2.6	0.734	1.7	0.900	0.9
P09429	High mobility group protein B1	26.51%	7	14	215	5.74	0.354	2.6	0.353	6.5	0.993	4.6	0.406	8.3	0.904	2.3
P0C7P0	CDGSH iron-sulfur domain-containing protein 3, mitochondrial	28.35%	5	12	127	10.55	0.727	2.6	0.838	8.2	1.089	6.5	0.775	10.3	0.587	10.1
P12956	X-ray repair cross-complementing protein 6	36.29%	20	74	609	6.64	1.068	2.6	0.908	2.6	0.980	3.1	0.782	2.4	0.922	2.7
P13674	Prolyl 4-hydroxylase subunit alpha-1	17.42%	8	14	534	6.01	0.836	2.6	0.832	28.9	0.863	10.9	0.961	7.2	1.226	8.3
P14618	Pyruvate kinase	38.16%	21	82	511	7.5	0.861	2.6	0.789	0.7	1.031	1.3	0.822	1.7	0.896	2.8
P17096	High mobility group AT-hook 1	39.58%	4	20	96	10.32	0.822	2.6	0.387	30.3	0.990	3.2	0.450	24.6	0.781	9.4
P21912	Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial	45.71%	19	90	280	8.76	0.906	2.6	0.844	1.9	0.982	0	0.819	1.9	0.699	2.7
P27348	14-3-3 protein theta	50.20%	15	106	245	4.78	0.965	2.6	0.872	6.2	0.970	5.8	0.804	8.8	1.027	0.4
P29317	Ephrin type-A receptor 2	23.46%	24	50	976	6.23	1.141	2.6	0.821	2.9	1.019	1.2	0.855	0.2	1.171	6
P30043	Flavin reductase (NADPH)	15.05%	3	15	206	7.65	0.613	2.6	0.785	10.3	0.977	2.3	0.719	1	1.125	9
P46977	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit STT3A	10.64%	9	18	705	8.07	0.897	2.6	0.821	3.8	1.037	1.5	0.801	4.1	1.234	3.1
P49755	Transmembrane emp24 domain-containing protein 10	15.07%	3	12	219	7.44	0.857	2.6	0.790	9.6	0.980	1.8	0.869	5.2	1.234	5.7
P50552	Vasodilator-stimulated phosphoprotein	15.79%	5	11	380	8.94	0.946	2.6	0.905	10.5	0.954	3.2	0.699	4.4	1.205	4.7
P51153	Ras-related protein Rab-13	31.53%	7	18	203	9.19	0.951	2.6	0.773	1	0.996	6.2	0.955	9.7	1.285	2.5
P53396	ATP-citrate synthase	13.99%	15	22	1101	7.33	0.753	2.6	0.818	1.4	1.009	0.1	0.762	13.5	1.175	12.9
P62136	Serine/threonine-protein phosphatase PP1-alpha catalytic subunit	22.42%	8	23	330	6.33	0.949	2.6	0.822	4.1	1.069	0.5	0.826	0.8	1.147	8.5
P62993	Growth factor receptor-bound protein 2	23.04%	5	9	217	6.32	1.311	2.6	0.760	8	1.079	0.4	0.658	14	0.822	0.2
P63104	14-3-3 protein zeta/delta	55.92%	18	113	245	4.79	1.104	2.6	0.901	4.4	0.929	2	0.831	0.9	1.021	2.5
P78527	DNA-dependent protein kinase catalytic subunit	22.21%	92	223	4128	7.12	0.827	2.6	0.717	1.8	1.015	1.5	0.631	5.4	0.860	0.7
Q04917	14-3-3 protein eta	26.42%	7	74	246	4.84	1.041	2.6	0.856	3.3	1.031	2	0.702	9.5	0.999	6.3
Q05932	Folypolyglutamate synthase, mitochondrial	10.05%	5	10	587	7.94	0.859	2.6	0.669	16.1	1.013	10.5	0.548	25.7	0.780	37.9
Q08380	Galectin-3-binding protein	12.65%	5	8	585	5.27	0.847	2.6	0.753	5	0.954	2.9	0.792	2.4	0.869	2.7

Accession	Description	%Cov	#Pept	#PSMs	#AAs	calc.pl	127/126		128/126		129/126		130/126		131/126	
							Ratio	CoV	Ratio	CoV	Ratio	CoV	Ratio	CoV	Ratio	CoV
Q08945	FACT complex subunit SSRP1	14.25%	10	29	709	6.87	0.567	2.6	0.512	10.2	1.012	0.8	0.524	20.1	0.625	7.2
Q09666	Neuroblast differentiation-associated protein AHNAK	37.67%	101	237	5890	6.15	1.098	2.6	0.788	2.1	1.062	0.6	0.674	1.7	0.853	1.2
Q12797	Aspartyl/asparaginyl beta-hydroxylase	30.74%	29	87	758	5.01	0.899	2.6	0.750	7	1.004	1.7	0.853	5.8	1.296	0.1
Q14247	Src substrate cortactin	20.18%	10	21	550	5.4	1.165	2.6	0.839	8.1	0.992	0.7	0.704	3.9	0.837	0.5
Q14847	LIM and SH3 domain protein 1	28.35%	7	18	261	7.05	1.092	2.6	0.867	5.3	1.118	1.9	0.760	12.3	1.146	0.4
Q15645	Pachytene checkpoint protein 2 homolog	14.35%	7	13	432	6.09	0.943	2.6	0.833	1.8	1.000	5.2	0.832	2.4	1.142	1.4
Q4G0N4	NAD kinase domain-containing protein 1, mitochondrial	21.72%	9	20	442	8.18	0.769	2.6	0.959	2.3	0.986	3.2	0.793	8.9	0.879	6.5
Q53HI2	DEAD-box protein abstrakt variant (Fragment)	14.15%	9	16	622	6.84	1.112	2.6	0.777	4.7	0.932	3.4	0.876	4.7	0.898	3.9
Q53S33	BolA-like protein 3	34.58%	5	10	107	9.64	0.751	2.6	0.949	3.4	0.983	6	0.731	15.1	0.637	21.2
Q6PIU2	Neutral cholesterol ester hydrolase 1	13.24%	5	21	408	7.23	0.799	2.6	0.693	2.8	0.979	2.4	0.754	4.3	1.200	0.5
Q7Z7H5	Transmembrane emp24 domain-containing protein 4	31.28%	5	14	227	8.28	0.857	2.6	0.724	6.3	0.987	3.6	0.747	7.2	1.165	0.9
Q86SX6	Glutaredoxin-related protein 5, mitochondrial	21.02%	4	10	157	6.79	0.863	2.6	0.814	15.6	0.943	1.3	0.856	18.1	0.748	2.2
Q86UA8	SMARCA1 protein (Fragment)	12.44%	14	33	965	8.6	0.590	2.6	0.556	10.4	1.052	2.1	0.559	17.7	0.653	1.8
Q8NC56	LEM domain-containing protein 2	14.71%	6	10	503	9	0.558	2.6	0.554	6.2	0.932	1.8	0.601	4.8	1.033	3.4
Q8NHX6	Cervical cancer proto-oncogene 5	13.91%	8	12	676	6.47	0.824	2.6	0.838	5	1.102	4.3	0.825	4.1	1.459	8.5
Q8TB45	DEP domain-containing mTOR-interacting protein	10.76%	4	6	409	8.07	0.889	2.6	0.856	5.2	0.950	6.9	0.821	2.4	1.073	12.3
Q8WVM0	Dimethyladenosine transferase 1, mitochondrial	23.41%	9	27	346	9.26	0.749	2.6	0.781	2.2	1.004	0.9	0.819	18.5	0.764	0.5
Q8WXX5	DnaJ homolog subfamily C member 9	15.77%	4	9	260	5.73	0.646	2.6	0.569	15.3	1.107	5.7	0.637	29.3	0.719	7.4
Q92520	Protein FAM3C	40.53%	9	43	227	8.29	1.089	2.6	0.864	4.3	1.081	2.8	0.830	2	1.129	3.2
Q96EY1	DnaJ homolog subfamily A member 3, mitochondrial	15.46%	5	14	480	7.12	0.961	2.6	0.794	12.3	0.799	7.7	0.598	14.5	0.638	16.6
Q96HE7	ERO1-like protein alpha	10.04%	5	13	468	5.68	0.871	2.6	0.761	1.2	1.017	5.7	0.911	0.5	1.313	0.9
Q96TA1	Niban-like protein 1	27.88%	20	59	746	6.19	1.121	2.6	0.811	1.6	0.943	5.1	0.799	2.4	0.866	5.5
Q9H6F5	Coiled-coil domain-containing protein 86	14.17%	4	5	360	10.33	0.440	2.6	0.396	10.8	1.162	0	0.591	4.3	0.733	8.2

Accession	Description	%Cov	#Pept	#PSMs	#AAs	calc.pl	127/126		128/126		129/126		130/126		131/126	
							Ratio	CoV	Ratio	CoV	Ratio	CoV	Ratio	CoV	Ratio	CoV
Q9HDC9	Adipocyte plasma membrane-associated protein	26.68%	14	50	416	6.16	0.824	2.6	0.781	5	1.001	0.4	0.738	2.8	1.147	2.6
Q9NR12	PDZ and LIM domain protein 7	12.47%	5	9	457	8.41	1.012	2.6	0.676	0.1	0.920	3.2	0.654	14.6	1.025	2.3
Q9P2X0	Dolichol-phosphate mannosyltransferase subunit 3	23.91%	2	8	92	5.94	0.857	2.6	0.841	1	1.109	3.1	0.781	1.1	1.164	10.7
Q9UHD8	Septin-9	16.72%	9	27	586	8.97	0.948	2.6	0.846	2.4	0.976	3.3	0.792	13.7	0.969	3.9
Q9Y230	RuvB-like 2	25.70%	10	16	463	5.64	0.669	2.6	0.716	19.8	1.013	7.6	0.752	5.6	0.999	22.5
Q9Y5B9	FACT complex subunit SPT16	13.09%	16	46	1047	5.66	0.630	2.6	0.608	3.1	1.020	5.4	0.614	3	0.665	1.6
Q9Y6H1	Coiled-coil-helix-coiled-coil-helix domain containing protein 2, mitochondrial	38.41%	4	12	151	9.22	0.568	2.6	0.542	20.2	1.045	0.4	0.370	40.2	0.305	31.1

Table 3.4: Annotation of significantly identified proteins in TMT-based quantitative proteomics profiling. These proteins have shown playing a critical role in oncogenesis, development and progression of cancer, metastasis and/or apoptosis of various human cancers.

#	Protein Name	Gene Symbol	Description	Ref.
1	14-3-3 protein eta	YWHAH	A group of highly conserved phospho-serine/threonine binding proteins that are involved in many vital cellular processes such as metabolism, protein trafficking, signal transduction, apoptosis and cell cycle regulation, and many of these pathways are often becoming dysregulated in disease states such as cancer.	205, 206
	14-3-3 protein theta	YWHAQ		
	14-3-3 protein zeta/delta	YWHAZ		
2	ATP-citrate synthase	ACLY	A transferase that catalyzes the conversion of citrate and coA to acetyl-CoA. It is the key regulator between the high rates of aerobic glycolysis and de novo lipid synthesis exhibited in many types of tumor cells.	207, 208
3	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3	BNIP3	A mitochondrial protein that contains a BH3 domain and acts as a pro-apoptotic factor, interacting with anti-apoptotic proteins including the E1B 19 kDa protein and Bcl2	209, 210

4	Cytochrome c (fragment)	CYCS	A small heme protein that functions as a central component of the electron transport chain in mitochondria and also involved in initiation of apoptosis.	211, 212
5	DEP domain-containing mTOR-interacting protein	DEPTOR	A negative regulator of the mTORC1 and mTORC2 signaling pathways that inhibits the kinase activity of both complexes	213, 214
6	DnaJ homolog subfamily A member 3, mitochondrial	DNAJA3	A member of the DNAJ/Hsp40 protein family, involving in protein folding, degradation, multimeric complex assembly, cell proliferation, survival and apoptotic signal transduction. It also plays a key role in tumor suppression.	215-217
7	Galectin-3-binding protein	LGALS3BP	Belongs to the family of beta-galactoside-binding proteins implicated in modulating cell-cell and cell-matrix interactions. It promotes integrin-mediated cell adhesion, and may stimulate host defense against viruses and tumor cells.	218-220
8	Glucose-6-phosphate isomerase	GPI	A glycolytic enzyme and can function as a tumor-secreted cytokine and an angiogenic factor (AMF) that stimulates endothelial cell motility.	221, 222
9	Growth factor receptor-bound protein 2	GRB2	An adaptor protein that provides a critical link between cell surface growth receptors and the Ras signaling pathway.	223-226

10	Guanine nucleotide-binding protein subunit beta-2-like 1	GNB2L1	It is involved in the recruitment, assembly and/or regulation of a variety of signaling molecules and plays a role in many cellular processes. Relates to ERK signaling and CREB pathway.	227, 228
11	Hepatocyte growth factor receptor	MET	A proto-oncogenic receptor tyrosine kinase and associated with a poor prognosis as it can trigger tumor growth, angiogenesis, and metastasis.	70, 76, 126, 196, 229
12	High mobility group AT-hook 1	HMGA1	A non-histone protein that involved in many cellular processes, e.g. regulation of inducible gene transcription, integration of retroviruses into chromosomes and metastatic progression of cancer cells.	230-232
13	Interferon, gamma-inducible protein 16, isoform CRA_a	IFI16	A member of the HIN-200 family of cytokines that can modulate p53 function, and inhibits cell growth in the Ras/Raf signaling pathway.	233-235
14	Kunitz-type protease inhibitor 2	SPINT2	It is a putative tumor suppressor. It can inhibits HGF activator, as well as plasmin, plasma and tissue kallikrein, and factor Xla.	236-239
15	LIM and SH3 domain protein 1	LASP1	A member of a subfamily of LIM proteins and linked to metastatic breast cancer, hematopoietic tumors and colorectal cancer.	240-243

16	MAGUK p55 subfamily member 6	MPP6	A member of the p55-like MAGUK family that functions in tumor suppression and receptor clustering.	244
17	Metastasis-associated protein MTA2	MTA2	It may be involved in the regulation of gene expression as a repressor and activator. MTA2 is identified as a component of NuRD and closely related to MTA1, a protein that has been correlated with the metastatic potential of certain carcinomas.	245-247
18	NAD(P)H dehydrogenase [quinone] 1	NQO1	A member of the NAD(P)H dehydrogenase (quinone) family. Altered expression of this protein has been seen in many tumors.	248, 249
19	Pachytene checkpoint protein 2 homolog	TRIP13	A protein that interacts with thyroid hormone receptor. It is associated with early-stage non-small cell lung cancer.	250, 251
20	Phosphoglycerate kinase 1	PGK1	A glycolytic enzyme and also a cofactor for polymerase alpha. It is secreted by tumor cells and participates in angiogenesis.	252-254
21	Protein FAM3C	FAM3C	A member of the family with sequence similarity 3 family. It may be involved in retinal laminar formation and promotes epithelial to mesenchymal transition. A change in expression of this protein has been noted in	255-257

pancreatic cancer-derived cells.

22	Pyruvate kinase, M2 splice isoform	PKM2	A protein involved in glycolysis, catalyzing the transfer of a phosphoryl group from phosphoenolpyruvate to ADP, generating ATP and pyruvate. It also involves in the caspase independent cell death of tumor cells.	258-261
23	Ras-related protein Ral-B (Fragment)	RALB	Belongs to the small GTPase superfamily and Ras family of proteins. Involved in a variety of cellular processes including gene expression, cell migration, cell proliferation, oncogenic transformation and membrane trafficking.	262-264
24	Ras-related protein Rap-1b (Fragment)	RAP1B	A member of the RAS-like small GTP-binding protein superfamily. It is related to MAPK signaling pathway and Ras signaling pathway.	265, 266
25	RuvB-like 1 (Fragment)	RUVBL1	A protein with single-stranded DNA-stimulated ATPase and ATP-dependent DNA helicase activity. It plays a critical role in oncogenic transformation by MYC and also modulates transcriptional activation by the LEF1/TCF1-CTNNB1 complex.	267-269

26	RuvB-like 2	RUVBL2	A DNA helicase essential for homologous recombination and DNA double-strand break repair. It plays an essential role in oncogenic transformation by MYC and also modulates transcriptional activation by the LEF1/TCF1-CTNNB1 complex.	270-273
27	Serine/threonine-protein phosphatase PP1-alpha catalytic subunit	PPP1CA	One of the three subunits of protein phosphatase 1 which is essential for cell division, regulation of glycogen metabolism, muscle contractility and protein synthesis.	274-276
28	Signal transducer and activator of transcription 3	STAT3	A member of STAT protein family. It plays a key role in many cellular processes such as cell growth and apoptosis.	277-280
29	SMARCA1 protein (Fragment)	SMARCA1	An ATPase belonged to the SWI/SNF protein family, involving in transcription, DNA damage, growth inhibition and apoptosis of cancer cells.	281
30	Src substrate cortactin	CTTN	It involved in receptor-mediated endocytosis via clathrin-coated pits. Overexpression is found in breast cancer and squamous cell carcinomas of the head and neck.	282-284

31	Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial	SDHB	It is a iron-sulfur protein subunit of SDH in complex II of the respiratory chain in mitochondrial inner membrane. Mutations in SDHB support a link between mitochondrial dysfunction and tumorigenesis.	285, 286
32	Sulfotransferase 1A1 (Fragment)	SULT1A1	It is a sulfotransferase enzyme that catalyzes the sulfate conjugation of many hormones, neurotransmitters, drugs, and xenobiotic compounds.	287, 288
33	Tumor necrosis factor receptor superfamily member 6B	TNFRSF6B	Belongs to the tumor necrosis factor receptor superfamily that can neutralize the cytotoxic ligands TNFSF14/LIGHT, TNFSF15 and TNFSF6/FASL and protect against apoptosis.	289, 290

On the other hand, we compared TMT-based quantitative proteomics results to co-IP data. Proteins involving in catalytic activity, and metabolic and cellular processes were highly enriched in both TMT and co-IP experiments. These proteins showed a significantly decreased expression level when cells were inhibited with 24 h of PHA-665752 and dynasore. We shortlisted the candidates by analyzing proteins that were commonly detected in both TMT-based proteomic profilings and the triplicate runs of mtMET co-IP. A total of 15 proteins were commonly found in both approaches, including the pyruvate kinase M2, tumor necrosis factor receptor superfamily member 6B, ornithine aminotransferase dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, hepatocyte growth factor receptor, X-ray repair cross-complementing protein 6, ephrin type-A receptor 2, Ras-related protein Rab-13, galectin-3-binding protein, neuroblast differentiation-associated protein AHNAK, aspartyl/asparaginyl beta-hydroxylase, high mobility group AT-hock 1, protein FAM3C, ERO-1-like protein alpha and niban-like protein 1. Among the fifteen proteins, five have been revised in Table 3.4 and shown playing a critical role in various human cancers. These five proteins were LGALS3BP (galectin-3-binding protein), HMGA1 (high mobility group AT-hock 1), PKM2 (pyruvate kinase M2), FAM3C (protein FAM3C) and MET (hepatocyte growth factor receptor). Unpaired Student's *t*-test was subsequently performed to examine the statistical significance change of expression level of these protein by comparing ratios of 24-h drug treatment to those of control, and *p* values of < 0.05 were obtained by using online GraphPad QuickCalcs: *t*-test calculator. Thus, besides MET kinase itself, the other four proteins served as the putative substrate of mtMET in SNU5 mitochondria. In current study, two of the four candidate proteins, *i.e.* HMGA1 and PKM2, were selected and focused as the potential substrate of mtMET for further verification and detailed study.

3.4.3 Protein-protein interaction of mtMET and candidate proteins

We performed *in situ* PLA experiments to investigate the protein-protein interaction of mtMET with the three candidate proteins. Compared with MS detection, *in situ* PLA-based detection of protein-protein interactions can be performed using much less materials. There is no need for extensive sample preparation before measurement of PLA signals. Reagents for PLA are commercially available, but upon use for studying biological events, evaluation of antibody performance is highly necessary. In present study, only high quality antibodies, which have been reported and published in top international English language journals, have been selected. The specificity of individual antibody was evaluated using western blotting (data not shown). Prior to PLA assays, SNU5 cells cultured in complete IMDM medium were cytocentrifugated to attach onto the superfrost plus microscope slides. Cells were then fixed, permeabilized, and blocked with BSA. Anti-MET mouse monoclonal antibody (clone 4AT44) were incubated with anti-PKM2 rabbit monoclonal antibody [clone EPR10138(B)], and anti-HMGA1 rabbit monoclonal antibody (clone EPR7839) individually. After secondary probes incubation, ligation and polymerization, and mounting of coverslips, fluorescence staining was visualized using an Axio Observer microscope with AxioVision acquisition software. Anti-TOM20 rabbit polyclonal antibody (clone FL-145) was used as a negative control as in previous immunoblotting experiments described in chapter 2. TOM20 had been shown not interacting with mtMET based on our current study. As shown in Figure 3.10, the green discrete fluorescent spots represented the PLA signals. In the negative control MET-TOM20 experiment, where an irrelevant antibody was used, almost no green spot was detected (the single green spot might be due to non-specific binding). In MET-HMGA1 and MET-PKM2, high intensity of green fluorescent spots was illustrated in the individual merged images (Figure 3.10A). Better quality and high resolution images of the four PLA assays were put in Supplementary Figure 3. Student's *t*-test was performed to show the significance level of experiments. Average PLA signals per cell

for each experiment were plotted in a diagram with bars corresponding to 95 % confidence intervals (Figure 3.10B). Taken together all the results, there is high potential that mtMET interact with HMGA1 and PKM2 in SNU5 mitochondria.

(A)

(B)

Figure 3.10: *In situ* PLA for visualization and quantification of PLA signals. (A) Detection of PLA signals in cytocentrifugation preparations of SNU5 gastric cancer cells using Duolink *in situ* reagents with two primary antibodies. MET-TOM20 served as negative control. PLA signals were shown in green discrete fluorescent spots and the nuclei were in blue (DAPI). Scale bar, 30 μ m. (B) The average number of PLA signals per cell in a diagram for two primary antibodies. Bars represent average PLA signals with 95 % confidence interval. Asterisks represent statistical significance with p value <0.0001.

3.4.4 Putative substrates of mtMET in SNU5 gastric cancer cells

Co-IP experiments coupled with immunoblotting were used to verify our observations in *in situ* PLA experiments. SNU5 cells were cultured in 24 h of 50 nM PHA-665752, 24 h of 80 μ M dynasore and without any treatment (Control). Before pulling down protein complex, a portion from each sample was collected and blotted for TOM20 to ensure equal amount of starting materials for co-IPs. Then, mtMET and associated protein complex were co-immunoprecipitated as described in Materials and Methods. Whole cell lysate from Control sample was used as positive control in order to understand if the bands in IP lanes appeared at the right molecular weight. Protein extracts from each sample were analyzed using western blot to identify the presence of HMGA1 and PKM2. Evidences from western blot showed that both HMGA1 and PKM2 were co-immunoprecipitated with mtMET in mitochondria (Figure 3.11A). When SNU5 were inhibited with 24 h of 50 nM PHA-665752 and 80 μ M dynasore, the abundance of mtMET decreased greatly and so did HMGA1 and PKM2 (Figure 3.11A). To validate the co-IP results, we inversely pull down mtMET using anti-HMGA1 and anti-PKM2 rabbit polyclonal antibody. SNU5 cells were cultured in complete IMDM medium and subjected to co-IP experiments by using normal rabbit IgG (negative control), anti-MET antibody, anti-PKM2 antibody and anti-

HMGA1 antibody. As shown in Figure 3.11B, mtMET was able to be co-immunoprecipitated by using HMGA1 and PKM2 antibody, respectively. In addition, the mitochondrial protein extracts from Control and PHA-665752 treated SNU5 cells were subjected to label-free mass spectrometry analyses. Both candidate proteins were found and the abundance of proteins reduced significantly when mtMET was inhibited (p value < 0.05, Table 3.5). Results from label-free proteomic approach and functional studies revealed that mtMET was present in SNU5 mitochondria and interacting with HMGA1 and PKM2.

(A)

(B)

Figure 3.11: (A) Western blot evidences of protein-protein interaction between mtMET and HMGA1 and PKM2. (B) Anti-PKM2 and anti-HMGA1 antibodies were used to inversely co-immunoprecipitate mtMET from SNU5 mitochondrial lysate. WCL, SNU5 whole cell lysate; PHA, 50 nM PHA-665752; Dyna, 80 μ M dynasore; IgG, normal rabbit IgG (control).

Table 3.5: Label-free quantitative proteomic approach identified the reduced expression level of HMGA1 and PKM2 upon MET inhibition. Ctrl_Score, protein score of Control sample; PHA_Score, protein score of PHA-665752-treated sample; Ctrl_emPAI, normalized emPAI value of Control sample; PHA_emPAI, normalized emPAI value of PHA-665752-treated sample.

#	GS	Name	Mass	Replicate	Ctrl_Score	PHA_Score	Ctrl_emPAI	PHA_emPAI	p value
1	MET	Hepatocyte growth factor receptor	155441	1st	514	743	0.39	0.26	0.0315
				2nd	723	792	0.39	0.3	
2	PKM2	Pyruvate kinase, M2 splice isoform	57900	1st	298	239	0.45	0.25	0.0443
				2nd	348	429	0.49	0.32	
3	HMGA1	High mobility group protein HMG-I/Y	11669	1st	100	63	1.17	0.3	0.0377
				2nd	73	74	0.88	0.3	

3.5 Discussion

Binding of growth factors with their corresponding cell surface receptors rigorously controlled the intracellular signaling network in cells^{291, 292}. Numerous reports have already established and discussed the crucial roles of RTKs in regulating and maintaining the regular functions of various signaling pathways. However, seeing from the other point of view, dysregulation of RTKs is a severe issue and have been implicated in the development and progression of a lot of human cancers. Many of RTKs are actually the products of proto-oncogenes or tumor suppressor genes. A large body of literature implicating specific RTKs in the development, progression and recurrence of human cancers has been reported.

MET, one of the well-studied proto-oncoprotein RTKs, is normally activated upon engagement with HGF ligand and involved in the epithelial cell proliferation and motility under the normal developmental condition. On plasma membrane, upon binding of HGF, MET undergoes homodimerization and trans-phosphorylation on Tyr-1234 and Tyr-1235, followed by the trans-phosphorylation on Tyr-1349 and Tyr-1356 in the carboxy-terminal tail to form a tandem SH2 recognition motif (Y¹³⁴⁹VH¹³⁵⁶VX₃Y¹³⁵⁶VNV)¹²². Various signaling effectors such as GRB2, SHC, PI3K, SRC, STAT3 and GAB1, are recruited to these docking sites, leading to the activation of downstream signal transduction pathways such as ERK/MAPK cascade, PI3K/AKT pathway and STAT pathway. On the other hand, aberrant MET signaling pathways are often identified during oncogenesis, tumor progression, and invasion and metastasis of cancer cells. It has been found that MET is overexpressed in a variety of malignancies and can be constitutively activated through ligand independent mechanisms such as mutations and amplifications. MET inhibition has thus become an attractive target for molecularly targeted

cancer therapy. Indeed, several drugs or SMIs have been developed to inhibit MET activity. Nevertheless, due to the intrinsic or acquired resistance and not complete understanding of altered MET signaling pathway in tumorigenesis, the effectiveness of therapeutic and disease progression is limited.

The unconventional localization of MET in mitochondria has been previously disclosed through quantitative proteomics profiling and other functional assay studies. Yet, the function and substrate of mtMET remain unclear. In previous chapter, we have confirmed that MET can translocalize into mitochondria through clathrin-mediated endocytosis. In this study, we further identified the novel substrates of mtMET in MET TKI sensitive SNU5 gastric cancer cells. To our knowledge, this is the first description of endocytosis and mitochondrial translocation of MET and the first study of its substrates in mitochondria. We adopted co-IP to pull down the associated protein complex from SNU5 mitochondria and analyzed the protein extracts using the high mass resolution Q Exactive instrument. Subsequently, a TMT isobaric tags based quantitative proteomics profiling was performed to study SNU5 mitochondrial proteome under the conditions of MET inhibition and endocytosis inhibition. The experimental approaches described above are extremely reproducible and with high reliability. However, we noticed that SNU5 mitochondrial proteome showed little change when cells were inhibited with a short duration of 50 nM PHA-665752 (PHA665752-12h). We believed that this unexpected outcome was due to the pre-determined concentration of PHA-665752, which is a sublethal concentration that did not significantly impair cell viability of TKI sensitive cancer cells⁸⁴. Thus in the following TMT data analysis we studied and compared the results of PHA665752-24h and Dynasore-24h.

In co-IP analyses, a major fraction of the confidently detected proteins was involved in catalytic activity and metabolic process. Of which, nine proteins have been found connecting with MET through extensive data and text mining in STRING database. These proteins are GAPDH, YWHAZ, NSUN2, ACLY, KPNB1, FASN, POTEF, EZR and PCNA. Similar to co-IP analyses, in TMT study, majority of the proteins whose expression levels were significantly affected after 24-hr MET inhibition (128/126) and endocytosis inhibition (130/131) belonged to the groups of catalytic activity and metabolic processes. Our results strongly suggested that, the potential substrates of mtMET in SNU5 mitochondria associated with these molecular functions. We narrowed down the list of candidates by comparing the TMT-based proteomic profiling data and co-IP pull down protein list. Four proteins were commonly detected in both two datasets and showed a statistically significant change in expression level in TMT data (unpaired Student's *t*-test, *p* value < 0.05). Thus, in current study, two proteins, *i.e.* HMGA1 and PKM2 were selected for downstream study and verification. As expected, the western blotting evidences revealed that protein expression level of HMGA1 and PKM2 in mitochondria decreased greatly when SNU5 cells were treated with 24 h of PHA-665752 and dynasore.

HMGA1 (High Mobility Group AT-Hook 1), *aka.* HMG-I/Y, is originally referred to as a non-histone chromosomal protein that involved in various cellular processes including regulation of transcription, embryogenesis, differentiation, and integration and expression of retroviruses into chromosomes. Recently HMGA1 has been shown to have oncogenic property and overexpression was identified in different cancer types²⁹³⁻²⁹⁷. It has been found that overexpression of HMGA1 led to the interaction with p53 *in vitro* and *in vivo*, inactivating p53-induced apoptosis in thyroid cancer and colorectal adenocarcinoma^{231, 232, 298}. In 2005 dynamic nucleocytoplasmic translocation and mitochondrial internalization of HMGA1 was first-time identified in MCF-7 breast cancer epithelial cells²⁹⁹.

Dement *et al.* and Mao *et al.* further discovered that increased cellular reactive oxygen species (ROS) levels and reduced efficiency of repair of oxidatively damaged mtDNA correlated to the overexpression of HMGA1 in mitochondria, contributing to the increased occurrence of mtDNA mutations in cancer cells^{300, 301}. In our data, expression of HMGA1 showed significant change when cells were inhibited with PHA-665752. Ratio of HMGA1 protein abundance dropped largely ($\text{HMGA1}_{128/126} = 0.387$, p value < 0.05) when compared to control sample (Table 3.3). Moreover, co-IP and PLA assays confidently demonstrated the mitochondrial localization and protein-protein interaction between mtMET and HMGA1 (Figure 3.9 and Supplementary Figure 1). When SNU5 cells were culture in 50nM PHA-665752 for 24 h, the growth rate of SNU5 cells apparently decreased and many dead cells were found in the medium (data not shown). This phenomenon implicates the attenuation of proliferation of SNU5 and cancer cells start to undergo apoptosis. All our results and observations are in accordance to previous reports regarding the roles of HMGA1 in cancer cell mitochondria. The highly selective and specific inhibitor of MET kinase also affects the expression level of HMGA1 in mitochondria while mtMET's activity is suppressed. Therefore we proposed that HMGA1 as the novel substrate of mtMET in TKI sensitive SNU5 gastric cancer cell lines. These findings show that “turn ON” and “turn OFF” of mtMET's activity has a direct influence on HMGA1, leading to the change in downstream signaling pathway and thus the apoptosis of cancer cells.

Besides HMGA1, our study disclosed PKM2 as another novel substrate of mtMET in SNU5 mitochondria. Pyruvate kinase (PK) is normally known as an important glycolytic enzyme catalyzing the transfer of a phosphoryl group from phosphoenolpyruvate (PEP) to ADP to generate ATP and pyruvate³⁰². Its M2 splice isoform PKM2 (*aka.* PKM, PK2 or tumor M2-PK), which was identified in our study, involve in tumor metabolism and energy production. It functions as a key glycolytic enzyme in altered metabolism of

tumor cells, channelling the glucose carbons either into synthetic process or toward glycolytic energy production. PKM2 predominantly exists as a nearly inactive dimeric form and expressed in a wide variety of human cancers including gastrointestinal malignancy^{303, 304}. Recent studies have stated that expression of PKM2 and VEGF can be prognostic factors in patients with advanced gastric cancer and colorectal cancer³⁰⁵⁻³⁰⁷. Dhar *et al.* have shown that high PKM2 expression caused increased cell proliferation of PKM2-transfected HuCCT cells and proposed it as a novel diagnostic tumor marker in human biliary tract cancer³⁰⁸. In our analysis, expression of PKM2 in SNU5 mitochondrial proteome decreased significantly in samples of PHA665752_24h ($\text{PKM2}_{128/126} = 0.789$) and Dynasore_24h ($\text{PKM2}_{130/131} = 0.822$). Coupled with results from co-IP experiments, PLA assay and functional assay study, we showed that the expression of PKM2 correlated with that of mtMET in SNU5 mitochondria. All the evidences indicate a direct protein-protein interaction between mtMET and PKM2. Thus when the activation of mtMET is suppressed, the recruitment of PKM2 to mtMET is reduced as a result.

Our study has identified HMGA1 and PKM2 as two novel substrates of mtMET in SNU5 cells, which is a MET TKI sensitive gastric cancer cell line. When activation and phosphorylation of mtMET is inhibited, the recruitment and thus the expression level of HMGA and PKM2 decrease as well. From our study, metabolic process in SNU5 was seriously affected when cells grew in the condition of MET inhibition or endocytosis inhibition. We believed that, mtMET, which translocalized into SNU5 mitochondria, play a critical role in regulation and alteration of mitochondrial metabolism in gastric cancer cells.

3.6 Conclusions and future work

MET signalling played a significant role in gastric cancer oncogenesis, development and progression of cancer, angiogenesis, invasion and cancer metastasis. In this study, we have applied both label-free and TMT isobaric tags based quantitative approaches coupled with PLA assays to firstly establish two novel substrates of mtMET in mitochondria: HMGA1 and PKM2. Our data revealed a new picture of MET signalling in mitochondria of TKI sensitive gastric cancer cells, shedding light on the unconventional molecular mechanism of RTK translocation and function in human cancers. Based on our experimental observations, many proteins, which are involving in catalytic activities and metabolic processes, have been affected as a result of MET inhibition using PHA-665752. This has implicated that mtMET might be involved in the regulation and alteration of energy metabolism in mitochondria of gastric cancer cells through downstream effectors including HMGA1 and PKM2. In addition, another two potential candidate proteins, *i.e.* galectin-3 binding protein and protein FAM3C, although not covered and studied in current project, may also act as downstream substrates or effectors of mtMET and regulate mitochondrial functions in gastric cancer cells. Other proteins in our list also have potential to be phosphorylated and regulated by mtMET and controlling yet-to-identify functions in mitochondria. For example, FASN and ACLY, which were co-immunoprecipitated with mtMET, are key enzymes of de novo lipogenesis and significantly upregulated and activated in many cancers and portend poor prognosis. Coleman *et al.* has proposed FASN as a novel target for MET-driven prostate cancer^{309, 310}. Moreover, Kaposi *et al.* has identified KPNB1, which is the element of cell proliferation machinery and has been identified in our co-IP data, as one of the key contributors to MET-driven tumor progression in human hepatocellular carcinoma³¹¹. Thus further study and analysis are required for a complete picture of mtMET signalling in SNU5 mitochondria.

Chapter 4

Conclusion and future direction

4.1 Concluding remarks and future perspective

MET receptor tyrosine kinase is a disulphide-linked heterodimer formed by a highly glycosylated extracellular α chain (50 kDa) and a single-pass transmembrane β chain (145 kDa) originating from the proteolytic cleavage of a single chain precursor protein (190 kDa)⁶⁹. Upon binding of HGF, MET becomes activated and its catalytic activity is induced. Two tyrosines (Tyrosine 1234 and 1235) locating in the catalytic site of MET undergo trans-phosphorylation, which are responsible for the regulation of MET activity. The other two tyrosines (Tyrosine 1349 and 1356) locating in the carboxy-terminal tail are subsequently phosphorylated, creating a multifunctional docking site capable of recruiting a wide variety of intracellular signaling effectors and adaptors to transduce the signals triggered by MET-HGF interaction⁶⁹⁻⁷¹. Multiple signaling transduction pathways are induced, including Ras/Raf pathway, PI3k/Akt pathway, STAT pathway, Wnt pathway, Notch pathway, *etc*⁷¹⁻⁷³. Over the past decades, much has been learned about the MET signaling pathway. While normal MET signaling is essential for a broad spectrum of biological processes, dysregulated MET signaling has been strongly implicated in oncogenesis of various human cancers. Aberrant MET signaling has been extensively demonstrated in many different cancerous events, for example, development and progression of cancer, uncontrolled cell proliferation, invasiveness and metastasis, and protection from apoptosis in cancer cells. Yet, the global picture of MET signaling in oncogenesis is still far from complete understanding and extensive study is required in future.

In this research project, we have established suitable biochemical approaches coupled with LC-MS/MS based high throughput proteomic technique to investigate the localization and substrate of MET in mitochondria of gastric cancer cells. Mitochondrial localization of RTKs, such as MET and EGFR, was recently reported by several groups^{84, 158, 165,}

³¹². They have suggested a critical role of these RTKs in regulating the aberrant signalling in mitochondria, contributing to oncogenesis. Using SNU5 as model cell line, a suspension gastric cancer cell line, which has a high amount of mtMET in mitochondria and highly sensitive to MET specific inhibitor, we studied how MET translocalizes into mitochondria. Based on previous mitochondrial proteome profiling of various cancer cell lines, we identified the presence of a vast cohort of components of clathrin-mediated endocytosis. Different kinds of endocytic inhibitors targeting different parts of endocytosis pathway were utilized. In our effort to understand the molecular mechanism underlying mitochondrial localization of MET in MET-dependent SNU5 gastric cancer cells, we uncovered the involvement of clathrin-mediated endocytosis in the translocation of MET into the mitochondria. Inhibition of endocytosis affects not only the abundance, but also the activation and phosphorylation of mtMET. Our data shows the essential role of endocytosis in mtMET activity, which may become one of the direct targets in molecularly targeting gastric cancer therapeutics. On the other hand, evidences from western blot showed the participation of other mechanism in the mitochondrial translocalization of mtMET. A baseline expression level of mtMET was identified even when endocytosis was suppressed for long time. Our findings indicated a significant role of mtMET in mitochondria of gastric cancer. We proposed that, when plasma-membrane-localized MET is activated and endocytosed or after MET is newly synthesized and processed in ER and Golgi, a portion of MET is transported through clathrin-coated vehicle or other way into mitochondria, where they regulate the altered mitochondrial metabolism in gastric cancer.

In order to identify the potential substrate and to deduce the possible function of mtMET in mitochondria, we employed co-immunoprecipitation to pull down the associated protein complex of mtMET and then identified the proteins by LC-MS/MS based proteomic approach and quantify them with emPAI based label-free quantification technique. We identified 342 high

confidence proteins that were uniquely associated with mtMET. A major fraction of the proteins has catalytic activity and involves in metabolic process of SNU5 cells. Strikingly, only 32 % of the co-immunoprecipitated proteins have shown localization on mitochondria based on Gene Ontology subcellular localization information. Moreover, only nine proteins have been found correlating with MET based on STRING database search and text mining. This has indicated that the proteome associated with mtMET is relatively unexplored and poorly understood. There is a huge reserve of biological information waiting to be discovered.

Mitochondria are responsible for cellular ATP production through oxidative phosphorylation. “Warburg effect” was previously proposed by Otto Warburg³¹³. He observed that, despite the presence of oxygen, most cancer cells predominantly produce energy through a high rate of “aerobic glycolysis” followed by lactic acid fermentation in cytosol, rather than by a comparatively low rate of glycolysis followed by oxidation of pyruvate in mitochondria as in normal cells³¹³. However, nowadays it is clear that mutations in oncogenes and tumor suppressor genes are the main reasons of malignant transformation, and Warburg effect is proposed to be the result rather than a cause of cancers³¹⁴⁻³¹⁶. Several studies clearly demonstrated that cancer cells utilize both glycolysis and oxidative phosphorylation to satisfy their metabolic needs and to support tumor growth^{317, 318}. Different mitochondrial proteins, such as p32 and TFAM, as well as cytosolic proteins, such as STAT3, have been demonstrated *in vivo* and *in vitro* necessary for regulation of mitochondrial activities to sustain tumorigenicity in human cancers³¹⁹⁻³²¹. Here we applied proteomic method to study the mtMET associated proteome to elucidate the function of mtMET and to identify the key regulator potential drug target in oncogenesis for future cancer treatment. TMT isobaric tags based quantitative approach was adopted to study the mitochondrial proteome during mtMET inhibition and endocytosis inhibition as described in Chapter

3. In accordance to the co-IP results in Chapter 2 and previous study, metabolic processes in SNU5 mitochondria were seriously affected. In sample PHA665752-24h and Dynasore_24h, expression levels of proteins involved in metabolic processes reduced significantly. Besides, our data firstly revealed two novel substrates of mtMET in mitochondria: HMGA1 and PKM2. Our experimental observations and evidences reflected that, mtMET, together with its substrates, involved in maintenance of abnormal mitochondrial functions in gastric oncogenesis. Depletion of mtMET abundance as well as activation has a profound ravage on the energy metabolism of mitochondria and drives the cells towards apoptosis. In addition to these two proteins, many other proteins in our list have potential to be phosphorylated and regulated by mtMET and thus controlling yet-to-identify functions in mitochondria. Further study and analysis are thus required for a complete picture of mtMET signaling in SNU5 mitochondria.

This research project has disclosed a new paradigm of MET signaling in mitochondria of TKI sensitive gastric cancer cells, shedding light on the unconventional molecular mechanism of RTK translocation and function in human cancers. To further establishment of the importance of mtMET in gastric oncogenesis, we need to evaluate the tumorigenesis property of mtMET-depleted phenotype in both in vitro and in vivo models based experiment which might strengthen our findings and proven its potentiality as a therapeutic target.

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Appendix A

Publications

1. Anna, M., Yair, A., Sailaja, B. S., Edupuganti, V. R. R., Arigela, H., Mallm, J. P., **Sim, K. H.**, Malka, N. R., Emmanuelle, S., Prim, B. S., Sze, S. K., Gasser, S. M., Rippe, K., and Meshorer, E. (2015) Heterochromatin Protein 1 β (HP1 β) has distinct functions and distinct nuclear distribution in pluripotent versus differentiated cells. *Genome Biology* (In preparation)
2. Dutta, B., Ren, Y., Hao, P., **Sim, K. H.**, Cheow, E., Adav, S., Tam, J. P., and Sze, S. K. (2014) Profiling of the chromatin-associated proteome identifies HP1BP3 as a novel regulator of cell cycle progression. *Molecular & Cellular Proteomics* 13, 2183-2197
3. Chung, H. H., Sze, S. K., Woo, A. R. E., Sun, Y., **Sim, K. H.**, Xue, M. D., and Lin, V. C. L. (2014) Lysine methylation of progesterone receptor at activation function 1 regulates both ligand-independent activity and ligand sensitivity of the receptor. *The Journal of Biological Chemistry* 289.9, 5704-5722
4. Ren, Y., Hao, P., Dutta B., Cheow, E. S. H., **Sim, K. H.**, Gan, C. S., Lim, S. K., and Sze, S. K. (2013) Hypoxia modulates A431 cellular pathways association to tumor radioresistance and enhanced migration revealed by comprehensive proteomic and functional studies. *Molecular & Cellular Proteomics* 12, 485-498
5. Hao, P., Qian, J., Dutta, B., Cheow, E. S. H., **Sim, K. H.**, Meng, W., Adav, S., Alpert, A., and Sze, S. K. (2012) Enhanced separation and characterization of deamidated peptides with RP-ERLIC-based multidimensional chromatography coupled with tandem mass spectrometry. *Journal of Proteomics Research* 11, 1804-1811

Appendix B

Conference Presentation

1. Conference: 62nd ASMS conference on Mass Spectrometry and Allied Topics 2014, from June 15th – 19th 2014.

Venue: Baltimore Convention Center, One West Pratt Street, Baltimore, Maryland 21201.

Title: Elucidating the Role of Mitochondrial-localized Hepatocyte Growth Factor Receptor in Gastric Oncogenesis

2. Conference: 2nd SOCRATES Scientific Meeting 2014, Singapore, from 3rd – 4th November 2014

Venue: A*STAR Singapore, 30 Biopolis Street, Matrix, Singapore 138671.

Title: Elucidating the Role of Mitochondrial-localized Hepatocyte Growth Factor Receptor in Gastric Oncogenesis

3. Natural Products and Health 2013, Singapore, from 5th – 7th September 2013.

Venue: School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive, Singapore 637551.

Title: Elucidating the Role of Mitochondrial-localized Hepatocyte Growth Factor Receptor in Gastric Oncogenesis

Supplementary Data

Supplementary Figure 1: Western blot results showing no contamination from other organelles. Calnexin (endoplasmic reticulum-associated protein), GM130 (one of the golgins localized to the Golgi) and GAPDH (cytosolic protein) were solely detected in SNU5 whole cell lysate but not in mitochondrial fractions pull down by two-step mitochondria isolation approach. WCL, whole cell lysate; Mito., mitochondrial lysate.

>TOM20_HUMAN Mitochondrial import receptor subunit TOM20 homolog
MVGRNS**AIAAGVCGALFIGYCIYFDRKRRSDPNFKNRLRERRKK**

>BCL-XL_HUMAN Bcl-2-like protein 1
ESRKGQERFNR**WFLTGMTVAGVLLGS**LFSRK

>MET_HUMAN Hepatocyte growth factor receptor
GKVIVQPDQNFT**GLIAGVVSISTALLLLGFFLWL**KKRKQIKDLGSELVR

>EGFR_HUMAN Epidermal growth factor receptor
PKIPSI**ATGMVGALLLLLVVALGIGLFM**RRRHIVRKRTLRR

>PGFRB_HUMAN Platelet-derived growth factor receptor beta
LPFK**VVVISAILALVVLTIISLIILIMLWQKKPRYEIRWKVIE**

>VGFR2_HUMAN Vascular endothelial growth factor receptor 2
EKTNL**EIIILVGTAVIAMFFWLLLVIL**RTVKRANGGELKTG

Supplementary Figure 2: RTKs and mitochondrial proteins TOM20 and Bcl-xL sharing sequence which is rich in amino acids (arginine and lysine) of high hydrophobicity and basicity. In *in silico* study, RTKs, including MET, EGFR, PGFRB and VGFR2, were found contain an amino acid sequence consisting of highly hydrophobic and basic amino acid-rich sequences in their transmembrane domains and juxtamembrane regions. Yellow box indicated the transmembrane domains; R, arginine residue; K, lysine residue.

(A)

(B)

(C)

Supplementary Figure 3: Detection of PLA signals in cytocentrifugation preparations of SNU5 gastric cancer cells using Duolink *in situ* reagents with two primary antibodies (large images). MET-TOM20 served as negative control. PLA signals were shown in green discrete fluorescent spots and the nuclei were in blue (DAPI). Scale bar, 30 μ m

Supplementary table 1: Original co-IP data for 342 significantly identified protein in triplicate MET-colPs. Proteins uniquely identified in MET-colP and found commonly in triplicate runs are listed. All proteins have at least one unique peptide detected with ion score higher than 20. # > 20, number of peptide with ion score higher than 20; #Total, total number of peptide hit.

Accession	GS	Name	Mass	Protein Score	emPAI value	# > 20	#Total
A2A274	ACO2	Aconitate hydratase, mitochondrial	88563	103; 135; 126	0.12; 0.24; 0.24	10; 11; 8	11; 12; 10
A5A3E0	POTEF	POTE ankyrin domain family member F	123020	233; 205; 243	0.05; 0.05; 0.05	9; 10; 8	11; 13; 14
A6NNI4	CD9	CD9 antigen	18265	41; 35; 28	0.18; 0.18; 0.18	1; 1; 2	1; 1; 2
A6NP24	CRYZ	Quinone oxidoreductase (Fragment)	26137	35; 45; 39	0.27; 0.13; 0.13	2; 1; 1	2; 2; 1
A8K854	TUBB3	HCG1983504, isoform CRA_f	42804	211; 243; 222	0.35; 0.25; 0.16	10; 9; 9	10; 9; 9
A8MUD9	RPL7	60S ribosomal protein L7	24474	51; 45; 52	0.67; 0.47; 0.47	12; 11; 12	13; 13; 14

B1AKY9	ATP1A2	Sodium/potassium-transporting ATPase subunit alpha-2	112046	429; 462; 416	0.06; 0.09; 0.09	7; 10; 10	8; 13; 11
B3KQ95	FDFT1	Squalene synthase	35421	51; 42; 69	0.09; 0.09; 0.09	1; 1; 2	2; 1; 2
B3KW46	SCARB1	Scavenger receptor class B member 1	57531	43; 45; 37	0.06; 0.06; 0.06	3; 1; 2	3; 1; 2
B4DEM7	CCT8	T-complex protein 1 subunit theta	58179	294; 314; 294	0.32; 0.25; 0.32	10; 11; 14	11; 12; 15
B4DER1	LARS	Leucine--tRNA ligase, cytoplasmic	132410	70; 48; 46	0.05; 0.02; 0.02	3; 3; 3	3; 3; 5
B4DGQ0	LASP1	LIM and SH3 domain protein 1	23167	30; 33; 28	0.14; 0.14; 0.14	2; 2; 1	3; 3; 1
B4DJE7	ACADM	Medium-chain-specific acyl-CoA dehydrogenase, mitochondrial	25704	50; 32; 63	0.13; 0.28; 0.44	2; 1; 3	3; 3; 4

B4DN45	MAT2A	S-adenosylmethionine synthase	33172	85; 51; 56	0.21; 0.1; 0.21	3; 2; 2	3; 2; 2
B4DP52	DDX39B	HCG2005638, isoform CRA_c	40893	75; 42; 52	0.08; 0.17; 0.26	4; 2; 5	5; 4; 5
B4DQJ8	PGD	6-phosphogluconate dehydrogenase, decarboxylating	52352	185; 176; 241	0.13; 0.13; 0.2	6; 7; 10	7; 9; 10
B4DT31	FUBP1	Far upstream element-binding protein 1	69993	41; 39; 65	0.15; 0.15; 0.15	3; 5; 9	6; 10; 11
B4DUC5	CSE1L	Exportin-2	86045	33; 148; 43	0.04; 0.08; 0.04	2; 5; 2	2; 6; 2
B4DUW4	SUMO3	Small ubiquitin-related modifier 3	15929	28; 40; 48	0.21; 0.21; 0.21	1; 1; 2	2; 1; 2
B4DVB8	ELAVL1	ELAV-like protein 1	39200	86; 88; 110	0.18; 0.18; 0.18	3; 3; 3	3; 3; 3
B4DXI8	PSMD7	26S proteasome non-	28337	76; 69; 54	0.12; 0.12; 0.12	1; 1; 1	1; 2; 1

ATPase regulatory subunit 7

B4DY49	FRK	Tyrosine-protein kinase FRK	42238	84; 69; 78	0.08; 0.08; 0.08	12; 12; 9	16; 16; 13
B4DYH1	HSPH1	Heat shock 105kDa/110kDa protein 1, isoform CRA_b	98196	85; 93; 62	0.1; 0.1; 0.1	5; 8; 5	7; 9; 7
B4E022	TKT	Transketolase	63410	103; 143; 84	0.22; 0.16; 0.16	5; 4; 6	7; 5; 6
B4E1K7	STOML2	Stomatin-like protein 2, mitochondrial	33317	50; 43; 29	0.21; 0.1; 0.1	2; 2; 2	2; 3; 3
B4E2W0	HADHB	3-ketoacyl-CoA thiolase	49076	63; 80; 71	0.07; 0.22; 0.22	2; 3; 3	2; 5; 4
B4E363	FARSA	Phenylalanine--tRNA ligase alpha subunit	54181	35; 27; 40	0.06; 0.06; 0.06	2; 1; 2	2; 1; 2
B4E3P0	ACLY	ATP-citrate synthase	91782	182; 122; 178	0.11; 0.11; 0.15	8; 5; 7	10; 7; 8
B5MCQ5	PDIA6	Protein disulfide-isomerase A6	53627	429; 263; 307	0.27; 0.27; 0.27	12; 8; 8	12; 8; 10

B7Z1L3	PGRMC1	Membrane-associated progesterone receptor component 1	15926	116; 158; 110	0.21; 0.21; 0.21	2; 3; 2	4; 4; 3
B7Z2F4	CCT4	T-complex protein 1 subunit delta	42729	24; 42; 39	0.08; 0.08; 0.16	3; 1; 2	4; 3; 3
B7Z6B8	DECR1	2,4-dienoyl-CoA reductase, mitochondrial	35200	43; 47; 53	0.2; 0.31; 0.31	4; 4; 4	4; 4; 5
B7ZA04	DYNC112	Cytoplasmic dynein 1 intermediate chain 2	70897	72; 117; 127	0.05; 0.05; 0.05	1; 4; 2	1; 5; 2
B8ZZ51	MDH1	Malate dehydrogenase, cytoplasmic	18735	34; 35; 30	0.18; 0.18; 0.18	2; 1; 2	3; 2; 2
C9J830	PRKAR2A	cAMP-dependent protein kinase type II-alpha regulatory subunit (Fragment)	12751	35; 54; 35	0.27; 0.27; 0.27	2; 2; 1	2; 2; 1

C9J9K3	RPSA	40S ribosomal protein SA (Fragment)	29601	147; 346; 175	0.38; 0.53; 0.53	5; 10; 7	5; 10; 7
C9JDR0	NSDHL	Sterol-4-alpha-carboxylate 3-dehydrogenase, decarboxylating (Fragment)	28356	61; 28; 44	0.12; 0.12; 0.12	3; 1; 1	4; 1; 1
C9JG87	MRPL39	39S ribosomal protein L39, mitochondrial (Fragment)	34410	45; 39; 54	0.1; 0.1; 0.1	2; 1; 2	2; 3; 3
C9JJ19	MRPS34	28S ribosomal protein S34, mitochondrial	26373	47; 38; 42	0.13; 0.13; 0.13	1; 1; 1	1; 1; 1
C9JNW5	RPL24	60S ribosomal protein L24	17646	36; 43; 39	0.19; 0.19; 0.19	1; 1; 1	1; 1; 1
D6R9P3	HNRNPAB	Heterogeneous nuclear ribonucleoprotein A/B	30398	50; 55; 29	0.23; 0.23; 0.11	4; 5; 4	5; 5; 4
D6RAN4	RPL9	60S ribosomal protein L9 (Fragment)	20918	109; 83; 87	0.16; 0.16; 0.16	3; 2; 2	3; 2; 2

E1P506	C6orf163	Chromosome 6 open reading frame 163, isoform CRA_a	23780	35; 33; 41	0.14; 0.06; 0.14	9; 6; 12	12; 6; 14
E5RFT8	MRPS28	28S ribosomal protein S35, mitochondrial	9422	35; 29; 32	0.37; 0.37; 0.37	1; 1; 1	1; 1; 1
E5RHW4	ERLIN2	Erlin-2 (Fragment)	37929	241; 328; 234	0.29; 0.29; 0.4	5; 8; 9	6; 9; 9
E7EPB3	RPL14	60S ribosomal protein L14	14663	96; 100; 135	0.23; 0.23; 0.23	3; 2; 3	3; 2; 3
E7EPT4	NDUFV2	NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial	28231	101; 126; 50	0.25; 0.25; 0.25	5; 7; 3	5; 8; 3
E7EQG2	EIF4A2	Eukaryotic initiation factor 4A-II	41492	105; 28; 26	0.08; 0.08; 0.08	3; 1; 3	4; 1; 4
E7EQR4	EZR	Ezrin	65653	63; 48; 44	0.22; 0.16; 0.1	6; 4; 3	8; 4; 4
E7ESK7	YWHAZ	14-3-3 protein zeta/delta	15801	169; 231; 161	0.22; 0.22; 0.22	6; 6; 8	7; 7; 8

(Fragment)

E7EUU4	EIF4G1	Eukaryotic translation initiation factor 4 gamma 1	172277	52; 24; 50	0.06; 0.02; 0.04	3; 2; 4	4; 2; 6
E7EUY0	PRKDC	DNA-dependent protein kinase catalytic subunit	470050	31; 61; 62	0.01; 0.01; 0.01	2; 5; 7	13; 10; 14
E9PET8	KIF5C	Kinesin heavy chain isoform 5C	99336	53; 40; 34	0.03; 0.03; 0.03	1; 2; 1	2; 2; 3
E9PFN5	GSTK1	Glutathione S-transferase kappa 1	21807	62; 90; 111	0.33; 0.33; 0.33	2; 2; 4	2; 2; 4
E9PG15	YWHAQ	14-3-3 protein theta (Fragment)	17209	256; 209; 181	0.2; 0.43; 0.2	5; 5; 4	5; 5; 4
E9PH29	PRDX3	Thioredoxin-dependent peroxide reductase, mitochondrial	26107	45; 66; 76	0.44; 0.44; 0.62	4; 4; 5	4; 4; 5

E9PKH2	SERPINH1	Serpin H1	23077	34; 72; 71	0.31; 0.31; 0.31	2; 2; 2	2; 2; 2
F5H037	PPP1CA	Serine/threonine-protein phosphatase PP1-alpha catalytic subunit (Fragment)	19629	75; 56; 83	0.17; 0.17; 0.17	3; 2; 2	5; 3; 4
F5H0T1	STIP1	Stress-induced-phosphoprotein 1	60367	40; 35; 29	0.05; 0.05; 0.05	2; 1; 3	3; 2; 3
F5H157	RAB35	Ras-related protein Rab-35 (Fragment)	21486	35; 70; 65	0.16; 0.16; 0.16	2; 5; 3	3; 5; 4
F5H335	EIF3A	Eukaryotic translation initiation factor 3 subunit A	162936	119; 70; 79	0.04; 0.04; 0.06	6; 5; 4	9; 5; 6
F5H5A9	SUGT1	Suppressor of G2 allele of SKP1 homolog	31769	53; 66; 66	0.1; 0.1; 0.1	1; 1; 1	2; 1; 2
F5H6X6	GANAB	Neutral alpha-glucosidase AB	96441	100; 111; 121	0.14; 0.14; 0.14	9; 18; 13	10; 20; 18

F5H740	VDAC3	Voltage-dependent anion-selective channel protein 3	31080	206; 52; 77	0.11; 0.11; 0.11	5; 3; 3	7; 4; 3
F5H7M3	DLAT	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial	44919	227; 247; 281	0.24; 0.24; 0.15	14; 9; 14	17; 13; 18
F5H801	OGDH	2-oxoglutarate dehydrogenase, mitochondrial	111681	76; 68; 117	0.12; 0.09; 0.16	5; 8; 13	9; 8; 14
F6SBX2	IARS2	Isoleucine--tRNA ligase, mitochondrial	106884	41; 78; 46	0.03; 0.06; 0.09	3; 4; 7	5; 5; 8
F6VDH7	ST13	Hsc70-interacting protein (Fragment)	18131	90; 100; 74	0.41; 0.41; 0.41	3; 3; 3	3; 3; 3
F8VVM2	SLC25A3	Phosphate carrier protein, mitochondrial	36537	56; 41; 37	0.19; 0.09; 0.19	2; 1; 2	2; 2; 2

F8W8J4	MYOF	Myoferlin	236041	147; 254; 146	0.07; 0.09; 0.07	11; 10; 11	15; 11; 12
F8W914	RTN4	Reticulon	37178	57; 27; 64	0.09; 0.09; 0.09	4; 4; 6	5; 5; 7
F8W930	IGF2BP2	Insulin-like growth factor 2 mRNA-binding protein 2	66859	61; 69; 55	0.1; 0.1; 0.1	3; 2; 2	4; 4; 3
G3V153	CAPRIN1	Caprin-1	70481	47; 55; 70	0.05; 0.05; 0.05	3; 2; 2	3; 2; 2
G3V1K3	PON2	Paraoxonase 2, isoform CRA_a	41672	52; 40; 101	0.08; 0.08; 0.08	1; 2; 5	1; 2; 5
G3V1R4	NSUN2	NOL1/NOP2/Sun domain family, member 2, isoform CRA_b	59916	83; 55; 46	0.05; 0.05; 0.05	2; 1; 2	3; 1; 2
G3V295	PSMA6	Proteasome subunit alpha type	23226	132; 127; 95	0.5; 0.31; 0.31	5; 3; 3	5; 3; 3
G3V2B8	MTHFD1	C-1-tetrahydrofolate synthase, cytoplasmic	102152	109; 161; 92	0.13; 0.17; 0.17	5; 7; 7	6; 7; 10

G3V325	ATP5J2-PTCD1	Pentatricopeptide repeat-containing protein 1, mitochondrial	84684	55; 106; 61	0.04; 0.04; 0.04	3; 4; 3	3; 4; 3
G5E9V5	MRPS22	28S ribosomal protein S22, mitochondrial	41354	43; 75; 65	0.08; 0.17; 0.08	3; 3; 3	4; 4; 3
G5EA24	MCOLN2	Mucolipin 2, isoform CRA_a	29581	56; 90; 57	0.11; 0.11; 0.11	2; 2; 2	3; 2; 2
G5EA52	PDIA3	Protein disulfide isomerase family A, member 3, isoform CRA_b	55328	132; 147; 130	0.41; 0.34; 0.34	13; 8; 9	15; 10; 14
H0Y2W2	ATAD3A	ATPase family AAA domain-containing protein 3A (Fragment)	64600	36; 47; 51	0.05; 0.1; 0.05	2; 2; 2	3; 3; 3
H0Y3T6	SDF4	45 kDa calcium-binding protein (Fragment)	28152	56; 59; 55	0.12; 0.12; 0.12	1; 1; 1	1; 2; 3
H0Y6T7	NCSTN	Nicastrin (Fragment)	30195	31; 55; 68	0.11; 0.23; 0.37	2; 3; 5	3; 3; 5

H0Y852	SUCLG2	Succinyl-CoA ligase [GDP-forming] subunit beta, mitochondrial (Fragment)	20408	75; 95; 110	0.36; 0.16; 0.16	4; 6; 6	9; 12; 10
H0Y8C6	IPO5	Importin-5 (Fragment)	125319	182; 122; 76	0.05; 0.05; 0.08	4; 4; 4	4; 4; 4
H0Y8E6	MCM2	DNA replication licensing factor MCM2 (Fragment)	94792	74; 71; 102	0.03; 0.03; 0.11	2; 2; 5	3; 2; 7
H0Y8G5	HNRNPD	Heterogeneous nuclear ribonucleoprotein D0 (Fragment)	29877	62; 97; 98	0.24; 0.24; 0.24	2; 4; 3	2; 4; 3
H0Y8N7	MRPL1	39S ribosomal protein L1, mitochondrial (Fragment)	26708	63; 66; 121	0.12; 0.26; 0.26	1; 2; 2	1; 3; 2
H0Y8W2	GNB2L1	Guanine nucleotide-binding protein subunit beta-2-like 1 (Fragment)	30871	80; 163; 160	0.11; 0.36; 0.36	1; 5; 5	1; 5; 5
H0Y9Q1	LAP3	Cytosol aminopeptidase	22641	166; 102; 123	0.51; 0.32; 0.32	7; 3; 6	7; 4; 7

(Fragment)

H0YAS9	EMC2	ER membrane protein complex subunit 2 (Fragment)	16089	56; 54; 49	0.21; 0.21; 0.21	2; 1; 1	2; 1; 2
H0YCY6	DAK	FAD-AMP lyase (cyclizing) (Fragment)	55026	69; 61; 65	0.06; 0.06; 0.06	1; 1; 2	1; 1; 3
H0YH36	SLC3A2	4F2 cell-surface antigen heavy chain (Fragment)	2623	44; 35; 47	1.46; 1.46; 1.46	1; 1; 2	1; 1; 2
H0YKE7	CHP1	Calcineurin B homologous protein 1	14261	90; 69; 67	0.91; 0.54; 0.54	5; 2; 2	5; 2; 2
H0YKS4	ANXA2	Annexin (Fragment)	19632	1012; 771; 822	8.15; 6.81; 4.69	41; 37; 35	41; 41; 41
H0YM70	PSME2	Proteasome activator complex subunit 2	26166	50; 113; 107	0.13; 0.43; 0.27	2; 5; 3	2; 5; 3
H3BMH2	RAB11A	Ras-related protein Rab-11A (Fragment)	17657	48; 60; 62	0.19; 0.19; 0.19	2; 2; 2	2; 2; 2

H3BNX8	COX5A	Cytochrome c oxidase subunit 5A, mitochondrial	17452	89; 59; 59	0.19; 0.19; 0.19	2; 1; 1	3; 1; 2
H3BPJ9	NDUFB10	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 10	19531	44; 31; 42	0.17; 0.17; 0.17	1; 1; 1	1; 1; 1
H3BQN4	ALDOA	Fructose-bisphosphate aldolase	39772	471; 591; 489	0.62; 0.9; 0.75	16; 25; 20	17; 27; 24
H3BS70	ECI1	Enoyl-CoA delta isomerase 1, mitochondrial (Fragment)	24974	101; 103; 83	0.29; 0.13; 0.13	2; 2; 1	2; 2; 1
H3BUX2	CYB5B	Cytochrome b5 type B	15878	132; 151; 163	0.47; 0.47; 0.79	10; 10; 10	11; 11; 11
H7BZJ3	PDIA3	Thioredoxin (Fragment)	13739	72; 121; 91	0.25; 0.25; 0.25	1; 3; 2	2; 3; 3
H7C0A3	ARPC4-TTLL3	Protein ARPC4-TTLL3 (Fragment)	19558	49; 31; 24	0.17; 0.17; 0.17	1; 1; 1	2; 1; 1
H7C2V1	PLOD3	Procollagen-lysine,2- oxoglutarate 5-dioxygenase	31449	39; 26; 28	0.11; 0.11; 0.11	2; 3; 2	2; 3; 4

3 (Fragment)

H9KV70	LCN2	Neutrophil gelatinase-associated lipocalin	23059	53; 143; 72	0.15; 0.31; 0.15	1; 3; 2	1; 3; 3
I3L2C7	GEMIN4	Gem-associated protein 4	120456	36; 35; 35	0.03; 0.03; 0.03	2; 2; 1	3; 3; 3
J3KN47	TF	Serotransferrin	65318	236; 183; 139	0.1; 0.1; 0.1	11; 7; 6	11; 8; 7
J3KN69	NCEH1	Arylacetamide deacetylase-like 1	49431	236; 354; 294	0.21; 0.21; 0.21	13; 18; 13	13; 18; 13
J3KNA1	KIF5A	Kinesin heavy chain isoform 5A	107985	53; 40; 34	0.03; 0.03; 0.03	1; 1; 1	3; 2; 5
J3KP06	LMO7	LIM domain only protein 7	191723	65; 89; 85	0.02; 0.02; 0.02	3; 2; 2	3; 5; 3
J3KPD9	NME2	Nucleoside diphosphate kinase B	22522	48; 50; 30	0.15; 0.32; 0.32	3; 3; 4	3; 6; 5
J3KPF3	SLC3A2	4F2 cell-surface antigen heavy chain	68230	706; 618; 435	0.6; 0.6; 0.53	23; 26; 24	25; 28; 29

J3KQ48	PTRH2	Peptidyl-tRNA hydrolase 2, mitochondrial	19597	50; 42; 53	0.17; 0.17; 0.17	3; 1; 1	3; 1; 1
J3KQ58	TTC34	Tetratricopeptide repeat protein 34	40214	38; 54; 38	0.08; 0.08; 0.19	2; 2; 1	3; 3; 2
J3KQY1	MRPL22	39S ribosomal protein L22, mitochondrial	26686	52; 49; 31	0.13; 0.13; 0.13	2; 1; 2	2; 1; 2
J3KR24	IARS	Isoleucine--tRNA ligase, cytoplasmic	132820	98; 102; 200	0.05; 0.05; 0.05	3; 3; 5	3; 4; 7
J3KSV6	ALDOC	Fructose-bisphosphate aldolase C (Fragment)	19298	255; 263; 323	0.62; 0.38; 0.38	6; 6; 7	7; 6; 8
J3KTA4	DDX5	Probable ATP-dependent RNA helicase DDX5	69557	49; 35; 27	0.05; 0.05; 0.05	1; 1; 1	2; 1; 2
J3QRD1	ALDH3A2	Fatty aldehyde dehydrogenase	45019	105; 123; 123	0.15; 0.15; 0.15	4; 3; 3	4; 4; 3

J3QRM1	CDK5RAP3	CDK5 regulatory subunit-associated protein 3 (Fragment)	30299	78; 74; 91	0.11; 0.11; 0.11	2; 1; 2	2; 1; 2
K7ELL7	PRKCSH	Glucosidase 2 subunit beta	61124	74; 162; 121	0.05; 0.11; 0.05	1; 4; 3	2; 4; 4
K7ELW0	PARK7	Protein DJ-1	18070	101; 93; 107	0.41; 0.19; 0.67	2; 2; 3	2; 2; 3
K7EM13	PRKAR1A	cAMP-dependent protein kinase type I-alpha regulatory subunit (Fragment)	17504	51; 73; 56	0.19; 0.19; 0.19	2; 2; 1	2; 3; 2
M0QXB5	ETHE1	Persulfide dioxygenase ETHE1, mitochondrial	28883	132; 75; 68	0.12; 0.12; 0.12	4; 3; 2	4; 4; 3
M0R210	RPS16	40S ribosomal protein S16	14524	88; 90; 60	0.52; 0.52; 0.23	4; 3; 2	4; 4; 3
O00299	CLIC1	Chloride intracellular channel protein 1	27248	94; 91; 69	0.12; 0.12; 0.12	2; 3; 2	2; 3; 3

O00425	IGF2BP3	Insulin-like growth factor 2 mRNA-binding protein 3	64008	97; 213; 138	0.16; 0.28; 0.28	3; 8; 7	6; 9; 7
O14828	SCAMP3	Secretory carrier-associated membrane protein 3	38661	129; 73; 175	0.18; 0.09; 0.18	5; 1; 4	5; 1; 4
O15120	AGPAT2	1-acyl-sn-glycerol-3- phosphate acyltransferase beta	31293	34; 29; 33	0.11; 0.11; 0.22	3; 4; 7	4; 6; 8
O15173	PGRMC2	Membrane-associated progesterone receptor component 2	23861	161; 113; 121	0.3; 0.14; 0.3	4; 2; 3	4; 3; 3
O15427	SLC16A3	Monocarboxylate transporter 4	50064	85; 51; 49	0.14; 0.14; 0.14	3; 2; 2	4; 2; 2
O43399	TPD52L2	Tumor protein D54	22281	135; 82; 83	0.32; 0.32; 0.32	3; 2; 3	3; 2; 4
O60271	SPAG9	C-Jun-amino-terminal kinase-interacting protein 4	146913	39; 30; 32	0.02; 0.02; 0.02	2; 1; 1	5; 1; 2

O60506	SYNCRIP	Heterogeneous nuclear ribonucleoprotein Q	69788	125; 262; 212	0.05; 0.2; 0.05	7; 12; 8	8; 14; 10
O60664	PLIN3	Perilipin-3	47217	259; 297; 360	0.22; 0.31; 0.5	9; 9; 13	9; 10; 13
O60701	UGDH	UDP-glucose 6-dehydrogenase	55674	108; 75; 76	0.12; 0.06; 0.19	2; 1; 3	5; 3; 5
O60812	HNRNPCL1	Heterogeneous nuclear ribonucleoprotein C-like 1	32180	98; 121; 134	0.34; 0.1; 0.22	6; 7; 6	9; 9; 12
O75306	NDUFS2	NADH dehydrogenase [ubiquinone] iron-sulfur protein 2, mitochondrial	52911	74; 85; 94	0.2; 0.27; 0.27	9; 7; 8	10; 11; 9
O75369	FLNB	Filamin-B	280157	496; 727; 547	0.18; 0.22; 0.16	28; 34; 26	32; 36; 33
O75489	NDUFS3	NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, mitochondrial	30337	115; 110; 137	0.23; 0.23; 0.23	3; 4; 7	4; 5; 8

O94826	TOMM70A	Mitochondrial import receptor subunit TOM70	68096	55; 47; 43	0.1; 0.05; 0.05	2; 1; 1	3; 1; 1
O94874	UFL1	E3 UFM1-protein ligase 1	89996	47; 38; 73	0.04; 0.04; 0.04	2; 1; 2	2; 1; 2
O94925	GLS	Glutaminase kidney isoform, mitochondrial	74269	23; 58; 63	0.04; 0.04; 0.04	2; 1; 2	3; 2; 5
O95202	LETM1	LETM1 and EF-hand domain-containing protein 1, mitochondrial	83986	261; 176; 180	0.12; 0.21; 0.21	10; 10; 7	12; 12; 8
O95292	VAPB	Vesicle-associated membrane protein- associated protein B/C	27439	157; 115; 160	0.41; 0.41; 0.41	6; 5; 7	10; 6; 8
O95407	TNFRSF6B	Tumor necrosis factor receptor superfamily member 6B	33856	46; 38; 48	0.1; 0.1; 0.1	2; 1; 1	2; 1; 1
P00403	MT-CO2	Cytochrome c oxidase	25719	47; 57; 78	0.28; 0.28; 0.28	3; 3; 5	5; 3; 5

subunit 2

P01009	SERPINA1	Alpha-1-antitrypsin	46878	457; 773; 603	0.31; 0.31; 0.31	16; 27; 21	18; 31; 23
P01023	A2M	Alpha-2-macroglobulin	164613	114; 77; 87	0.06; 0.02; 0.06	6; 5; 7	8; 5; 7
P01024	C3	Complement C3	188569	181; 444; 147	0.05; 0.09; 0.09	7; 15; 8	12; 20; 8
P02647	APOA1	Apolipoprotein A-I	30759	47; 80; 75	0.11; 0.23; 0.11	1; 3; 2	2; 3; 2
P02671	FGA	Fibrinogen alpha chain	95656	60; 31; 53	0.03; 0.03; 0.03	2; 1; 1	2; 1; 1
P04083	ANXA1	Annexin A1	38918	960; 1003; 1194	1.89; 1.45; 1.89	40; 34; 39	43; 36; 40
P04181	OAT	Ornithine aminotransferase, mitochondrial	48846	45; 42; 58	0.07; 0.07; 0.07	1; 1; 1	1; 1; 1
P04406	GAPDH	Glyceraldehyde-3- phosphate dehydrogenase	36201	330; 326; 299	0.42; 0.3; 0.3	10; 12; 13	12; 14; 13
P04792	HSPB1	Heat shock protein beta-1	22826	67; 113; 108	0.31; 0.31; 0.51	5; 5; 6	6; 8; 7

P04843	RPN1	Dolichyl- diphosphooligosaccharide-- protein glycosyltransferase subunit 1	68641	41; 57; 66	0.05; 0.15; 0.05	3; 3; 2	3; 5; 3
P05023	ATP1A1	Sodium/potassium- transporting ATPase subunit alpha-1	114135	727; 715; 750	0.18; 0.22; 0.25	22; 21; 25	23; 23; 25
P05198	EIF2S1	Eukaryotic translation initiation factor 2 subunit 1	36374	118; 101; 153	0.09; 0.09; 0.09	3; 3; 4	4; 5; 6
P05388	RPLP0	60S acidic ribosomal protein P0	34423	175; 272; 321	0.32; 0.58; 0.58	8; 11; 11	9; 11; 13
P05455	SSB	Lupus La protein	46979	69; 36; 36	0.15; 0.07; 0.07	3; 1; 2	4; 1; 3
P06576	ATP5B	ATP synthase subunit beta, mitochondrial	56525	593; 851; 793	0.57; 0.66; 0.49	31; 37; 36	33; 43; 38
P06733	ENO1	Alpha-enolase	47481	150; 221; 352	0.4; 0.5; 0.4	17; 16; 16	19; 16; 17

P07900	HSP90AA1	Heat shock protein HSP 90-alpha	85006	637; 761; 629	0.52; 0.52; 0.64	36; 53; 42	39; 55; 45
P08107	HSPA1A	Heat shock 70 kDa protein 1A/1B	70294	556; 522; 616	0.58; 0.44; 0.58	20; 17; 19	22; 19; 20
P08574	CYC1	Cytochrome c1, heme protein, mitochondrial	35741	117; 105; 65	0.19; 0.19; 0.19	4; 3; 2	4; 3; 2
P08581	MET	Hepatocyte growth factor receptor	157779	765; 932; 782	0.23; 0.25; 0.28	54; 49; 46	62; 58; 54
P09874	PARP1	Poly [ADP-ribose] polymerase 1	113811	210; 269; 125	0.09; 0.09; 0.06	5; 6; 2	12; 9; 3
P0C0S8	HIST1H2AG	Histone H2A type 1	14083	20; 50; 70	0.24; 0.54; 0.54	1; 2; 3	2; 3; 3
P0CG38	POTEI	POTE ankyrin domain family member I	122858	139; 146; 116	0.08; 0.08; 0.08	10; 11; 7	13; 15; 16
P10412	HIST1H1E	Histone H1.4	21852	28; 52; 23	0.15; 0.15; 0.15	1; 2; 1	6; 7; 5

P10599	TXN	Thioredoxin	12015	59; 70; 55	0.29; 0.29; 0.29	1; 2; 1	1; 2; 1
P11021	HSPA5	78 kDa glucose-regulated protein	72402	554; 607; 660	0.7; 0.56; 0.63	30; 27; 27	33; 34; 33
P11166	SLC2A1	Solute carrier family 2, facilitated glucose transporter member 1	54391	62; 40; 78	0.12; 0.06; 0.12	4; 2; 3	7; 2; 4
P11717	IGF2R	Cation-independent mannose-6-phosphate receptor	281155	55; 57; 85	0.01; 0.01; 0.02	2; 2; 5	2; 2; 7
P11940	PABPC1	Polyadenylate-binding protein 1	70854	149; 109; 104	0.44; 0.31; 0.25	13; 15; 11	13; 20; 12
P12004	PCNA	Proliferating cell nuclear antigen	29092	38; 53; 51	0.11; 0.24; 0.24	2; 2; 2	4; 3; 2
P12109	COL6A1	Collagen alpha-1(VI) chain	109602	136; 59; 67	0.03; 0.03; 0.03	4; 1; 1	4; 1; 1

P12532	CKMT1A	Creatine kinase U-type, mitochondrial	47406	87; 127; 241	0.07; 0.07; 0.07	2; 2; 4	2; 2; 4
P12956	XRCC6	X-ray repair cross- complementing protein 6	70084	156; 74; 85	0.1; 0.1; 0.1	6; 4; 4	7; 6; 5
P13073	COX4I1	Cytochrome c oxidase subunit 4 isoform 1, mitochondrial	19621	59; 45; 39	0.17; 0.17; 0.17	2; 1; 1	2; 1; 1
P13639	EEF2	Elongation factor 2	96246	222; 237; 199	0.18; 0.18; 0.18	13; 16; 15	16; 17; 15
P13667	PDIA4	Protein disulfide-isomerase A4	73229	132; 104; 140	0.14; 0.14; 0.14	3; 5; 4	3; 7; 5
P14618	PKM	Pyruvate kinase PKM	58470	1331; 1452; 1175	0.55; 0.55; 0.55	32; 36; 32	33; 38; 35
P16401	HIST1H1B	Histone H1.5	22566	38; 40; 35	0.15; 0.15; 0.15	1; 1; 1	2; 2; 1
P16402	HIST1H1D	Histone H1.3	22336	28; 52; 23	0.15; 0.15; 0.15	2; 2; 3	7; 9; 7

P16435	POR	NADPH--cytochrome P450 reductase	77097	59; 86; 68	0.13; 0.09; 0.13	4; 7; 4	4; 7; 4
P17096	HMGA1	High mobility group protein HMG-I/HMG-Y	11669	33; 35; 42	0.3; 0.3; 0.3	1; 1; 2	1; 1; 2
P18206	VCL	Vinculin	124292	171; 104; 115	0.11; 0.08; 0.11	8; 5; 6	16; 10; 9
P19338	NCL	Nucleolin	76625	198; 470; 275	0.46; 0.4; 0.59	16; 19; 15	20; 20; 18
P20648	ATP4A	Potassium-transporting ATPase alpha chain 1	115756	98; 128; 163	0.03; 0.06; 0.06	3; 5; 4	4; 7; 8
P21796	VDAC1	Voltage-dependent anion-selective channel protein 1	30868	550; 371; 436	1.27; 1.27; 1.27	16; 14; 14	19; 16; 15
P22307	SCP2	Non-specific lipid-transfer protein	59640	42; 42; 57	0.06; 0.06; 0.11	1; 1; 2	1; 2; 3
P22314	UBA1	Ubiquitin-like modifier-activating enzyme 1	118858	325; 434; 420	0.21; 0.18; 0.18	14; 16; 16	19; 17; 17

P22626	HNRNPA2B1	Heterogeneous nuclear ribonucleoproteins A2/B1	37464	443; 387; 530	0.4; 0.29; 0.66	17; 11; 20	21; 13; 22
P23246	SFPQ	Splicing factor, proline- and glutamine-rich	76216	56; 62; 97	0.09; 0.04; 0.09	3; 2; 4	3; 4; 5
P23284	PPIB	Peptidyl-prolyl cis-trans isomerase B	23785	209; 130; 165	0.93; 0.69; 0.69	9; 5; 5	9; 6; 6
P23526	AHCY	Adenosylhomocysteinase	48255	35; 147; 142	0.14; 0.22; 0.22	2; 7; 5	3; 7; 6
P24752	ACAT1	Acetyl-CoA acetyltransferase, mitochondrial	45456	203; 183; 207	0.07; 0.23; 0.07	4; 7; 7	5; 8; 7
P25788	PSMA3	Proteasome subunit alpha type-3	28643	74; 45; 42	0.12; 0.12; 0.12	1; 1; 1	1; 1; 1
P26599	PTBP1	Polypyrimidine tract-binding protein 1	57357	288; 288; 303	0.25; 0.18; 0.18	9; 9; 9	10; 10; 10

P28072	PSMB6	Proteasome subunit beta type-6	25570	37; 39; 48	0.13; 0.13; 0.13	1; 1; 1	1; 1; 2
P29317	EPHA2	Ephrin type-A receptor 2	109679	139; 87; 137	0.12; 0.12; 0.16	9; 7; 8	11; 9; 9
P29966	MARCKS	Myristoylated alanine-rich C-kinase substrate	31707	121; 69; 84	0.35; 0.22; 0.35	5; 2; 4	5; 3; 4
P30044	PRDX5	Peroxiredoxin-5, mitochondrial	22301	111; 93; 90	0.75; 0.15; 0.32	8; 5; 6	8; 5; 6
P30050	RPL12	60S ribosomal protein L12	17979	51; 43; 51	0.19; 0.19; 0.19	2; 1; 1	2; 1; 1
P30084	ECHS1	Enoyl-CoA hydratase, mitochondrial	31823	110; 198; 133	0.1; 0.22; 0.35	2; 4; 3	3; 6; 5
P31930	UQCRC1	Cytochrome b-c1 complex subunit 1, mitochondrial	53297	57; 60; 59	0.06; 0.13; 0.06	2; 4; 4	2; 5; 4
P31937	HIBADH	3-hydroxyisobutyrate dehydrogenase,	35705	119; 83; 164	0.3; 0.19; 0.3	4; 2; 4	5; 3; 4

mitochondrial

P32119	PRDX2	Peroxiredoxin-2	22049	141; 91; 69	0.33; 0.33; 0.15	4; 3; 3	4; 3; 3
P33176	KIF5B	Kinesin-1 heavy chain	110358	64; 70; 55	0.06; 0.06; 0.09	2; 2; 3	2; 2; 5
P33993	MCM7	DNA replication licensing factor MCM7	81884	74; 74; 40	0.12; 0.08; 0.08	4; 3; 3	4; 3; 4
P34931	HSPA1L	Heat shock 70 kDa protein 1-like	70730	437; 358; 428	0.37; 0.31; 0.37	19; 15; 16	21; 15; 18
P35241	RDX	Radixin	68635	49; 34; 28	0.1; 0.05; 0.05	3; 2; 1	5; 2; 2
P35270	SPR	Sepiapterin reductase	28316	146; 182; 154	0.25; 0.25; 0.25	2; 3; 3	3; 3; 3
P35613	BSG	Basigin	42573	113; 70; 135	0.16; 0.16; 0.16	5; 3; 6	5; 4; 7
P36578	RPL4	60S ribosomal protein L4	47953	35; 36; 72	0.07; 0.14; 0.14	1; 3; 6	3; 3; 11
P39019	RPS19	40S ribosomal protein S19	16051	52; 65; 52	0.47; 0.21; 0.21	2; 2; 2	3; 3; 2

P40926	MDH2	Malate dehydrogenase, mitochondrial	35937	1249; 1463; 1033	1.02; 1.02; 1.41	45; 57; 42	51; 62; 43
P40939	HADHA	Trifunctional enzyme subunit alpha, mitochondrial	83688	418; 393; 391	0.31; 0.31; 0.21	24; 24; 16	28; 26; 19
P41250	GARS	Glycine--tRNA ligase	83854	98; 80; 110	0.12; 0.12; 0.08	8; 7; 6	9; 7; 9
P42765	ACAA2	3-ketoacyl-CoA thiolase, mitochondrial	42354	79; 76; 139	0.08; 0.16; 0.08	3; 5; 5	4; 6; 8
P43490	NAMPT	Nicotinamide phosphoribosyltransferase	55772	35; 35; 19	0.12; 0.12; 0.06	4; 2; 4	6; 3; 5
P46940	IQGAP1	Ras GTPase-activating-like protein IQGAP1	189761	36; 125; 111	0.05; 0.05; 0.05	8; 4; 8	14; 10; 12
P47914	RPL29	60S ribosomal protein L29	17798	115; 118; 107	0.19; 0.19; 0.19	4; 3; 3	5; 3; 3
P47985	UQCRCF1	Cytochrome b-c1 complex subunit Rieske, mitochondrial	29934	71; 77; 71	0.11; 0.11; 0.23	1; 2; 2	2; 2; 2

P48047	ATP5O	ATP synthase subunit O, mitochondrial	23377	243; 205; 179	0.49; 0.71; 0.71	10; 7; 7	10; 8; 8
P49327	FASN	Fatty acid synthase	275877	505; 439; 569	0.21; 0.18; 0.21	32; 27; 29	36; 32; 33
P49368	CCT3	T-complex protein 1 subunit gamma	61066	122; 85; 139	0.17; 0.17; 0.17	6; 4; 6	8; 9; 8
P49411	TUFM	Elongation factor Tu, mitochondrial	49852	73; 137; 145	0.14; 0.21; 0.21	4; 8; 8	4; 9; 10
P49721	PSMB2	Proteasome subunit beta type-2	22993	39; 34; 55	0.15; 0.15; 0.15	1; 1; 2	1; 1; 2
P49748	ACADVL	Very long-chain specific acyl-CoA dehydrogenase, mitochondrial	70745	267; 243; 198	0.25; 0.31; 0.31	13; 9; 8	15; 9; 10
P50416	CPT1A	Carnitine O- palmitoyltransferase 1, liver isoform	88995	72; 62; 111	0.04; 0.04; 0.04	1; 2; 5	1; 3; 6

P51148	RAB5C	Ras-related protein Rab-5C	23696	155; 184; 184	0.3; 0.3; 0.3	7; 6; 6	7; 7; 6
P51149	RAB7A	Ras-related protein Rab-7a	23760	201; 126; 98	0.48; 0.48; 0.69	6; 7; 5	7; 7; 6
P51153	RAB13	Ras-related protein Rab-13	22988	35; 70; 65	0.15; 0.15; 0.15	2; 5; 4	5; 7; 4
P51659	HSD17B4	Peroxisomal multifunctional enzyme type 2	80092	287; 597; 641	0.27; 0.27; 0.32	13; 22; 20	14; 22; 21
P52292	KPNA2	Importin subunit alpha-1	58168	40; 71; 40	0.06; 0.06; 0.06	1; 3; 1	2; 4; 2
P52597	HNRNPF	Heterogeneous nuclear ribonucleoprotein F	45985	289; 130; 210	0.15; 0.07; 0.07	7; 2; 4	7; 2; 4
P53621	COPA	Coatomer subunit alpha	139797	85; 70; 64	0.07; 0.07; 0.1	4; 3; 5	9; 4; 7
P54886	ALDH18A1	Delta-1-pyrroline-5-carboxylate synthase	87989	211; 179; 179	0.12; 0.08; 0.08	8; 5; 7	11; 7; 12
P55884	EIF3B	Eukaryotic translation initiation factor 3 subunit B	92823	81; 77; 85	0.04; 0.04; 0.04	3; 4; 4	5; 5; 5

P61019	RAB2A	Ras-related protein Rab-2A	23702	238; 346; 241	0.3; 0.3; 0.3	5; 6; 4	5; 6; 4
P61026	RAB10	Ras-related protein Rab-10	22755	53; 92; 112	0.32; 0.32; 0.32	3; 7; 6	4; 7; 6
P61106	RAB14	Ras-related protein Rab-14	24110	97; 78; 69	0.68; 0.3; 0.14	5; 5; 2	5; 5; 4
P61289	PSME3	Proteasome activator complex subunit 3	29602	63; 119; 124	0.24; 0.24; 0.24	2; 4; 4	5; 4; 4
P61981	YWHAG	14-3-3 protein gamma	28456	72; 111; 137	0.12; 0.12; 0.12	1; 2; 3	1; 2; 3
P62277	RPS13	40S ribosomal protein S13	17212	35; 51; 33	0.43; 0.71; 0.2	3; 3; 3	3; 6; 3
P62701	RPS4X	40S ribosomal protein S4, X isoform	29807	57; 40; 47	0.11; 0.11; 0.11	2; 1; 1	2; 1; 1
P62851	RPS25	40S ribosomal protein S25	13791	54; 43; 79	0.94; 0.56; 0.94	3; 4; 5	4; 5; 5
P67809	YBX1	Nuclease-sensitive element-binding protein 1	35903	202; 168; 194	0.3; 0.42; 0.3	11; 10; 11	13; 12; 15

Q00839	HNRNPU	Heterogeneous nuclear ribonucleoprotein U	91269	281; 414; 319	0.15; 0.19; 0.24	14; 19; 17	16; 20; 17
Q01105	SET	Protein SET	33469	57; 50; 53	0.1; 0.1; 0.1	2; 1; 1	2; 1; 1
Q01518	CAP1	Adenylyl cyclase-associated protein 1	52325	42; 32; 47	0.13; 0.06; 0.13	4; 4; 5	4; 6; 6
Q02878	RPL6	60S ribosomal protein L6	32765	109; 149; 135	0.21; 0.21; 0.21	4; 4; 6	5; 4; 8
Q02952	AKAP12	A-kinase anchor protein 12	191937	47; 60; 63	0.02; 0.02; 0.02	2; 3; 2	3; 3; 4
Q06323-2	PSME1	Isoform 2 of Proteasome activator complex subunit 1	28755	178; 142; 188	0.93; 0.93; 1.15	11; 8; 10	11; 9; 12
Q07021	C1QBP	Complement component 1 Q subcomponent-binding protein, mitochondrial	31742	167; 149; 153	0.49; 0.35; 0.22	6; 5; 4	6; 6; 6
Q08380	LGALS3BP	Galectin-3-binding protein	66202	217; 148; 145	0.21; 0.16; 0.16	10; 9; 6	11; 9; 6

Q09666	AHNAK	Neuroblast differentiation-associated protein AHNAK	629213	354; 292; 341	0.08; 0.08; 0.06	27; 27; 33	33; 39; 45
Q12797	ASPH	Aspartyl/asparaginyl beta-hydroxylase	86266	106; 204; 123	0.12; 0.08; 0.12	5; 6; 8	5; 6; 9
Q12905	ILF2	Interleukin enhancer-binding factor 2	43263	78; 90; 106	0.08; 0.16; 0.16	2; 2; 3	2; 2; 3
Q12907	LMAN2	Vesicular integral-membrane protein VIP36	40545	74; 43; 47	0.37; 0.17; 0.17	4; 3; 4	4; 3; 4
Q13011	ECH1	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial	36136	184; 130; 232	0.19; 0.19; 0.3	5; 3; 5	5; 5; 7
Q13200	PSMD2	26S proteasome non-ATPase regulatory subunit 2	100877	44; 109; 103	0.03; 0.14; 0.14	3; 8; 8	4; 10; 10
Q13423	NNT	NAD(P) transhydrogenase, mitochondrial	114564	63; 134; 58	0.03; 0.06; 0.03	6; 3; 1	6; 4; 3

Q14566	MCM6	DNA replication licensing factor MCM6	93801	70; 45; 40	0.03; 0.03; 0.03	2; 2; 3	2; 2; 3
Q14764	MVP	Major vault protein	99551	216; 177; 219	0.25; 0.25; 0.25	12; 16; 16	19; 18; 17
Q14974	KPNB1	Importin subunit beta-1	98420	225; 202; 247	0.1; 0.1; 0.07	10; 8; 9	11; 10; 10
Q15046	KARS	Lysine--tRNA ligase	68461	54; 49; 47	0.05; 0.1; 0.05	2; 2; 2	2; 2; 4
Q15365	PCBP1	Poly(rC)-binding protein 1	37987	33; 43; 42	0.09; 0.09; 0.09	1; 1; 1	1; 1; 1
Q15526	SURF1	Surfeit locus protein 1	33481	38; 45; 59	0.1; 0.1; 0.1	1; 2; 1	1; 2; 2
Q15758	SLC1A5	Neutral amino acid transporter B(0)	57018	155; 157; 71	0.12; 0.12; 0.18	4; 8; 7	5; 10; 7
Q16795	NDUFA9	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9, mitochondrial	42654	130; 142; 113	0.45; 0.25; 0.25	10; 8; 7	11; 12; 8

Q16822	PCK2	Phosphoenolpyruvate carboxykinase [GTP], mitochondrial	71483	230; 250; 222	0.25; 0.2; 0.2	8; 8; 8	9; 8; 11
Q16850	CYP51A1	Lanosterol 14-alpha demethylase	57169	27; 40; 20	0.06; 0.06; 0.06	2; 2; 3	3; 4; 3
Q32MZ4	LRRFIP1	Leucine-rich repeat flightless-interacting protein 1	89826	94; 84; 94	0.11; 0.04; 0.07	5; 2; 3	9; 4; 6
Q3KQZ2	SYNGR2	SYNGR2 protein	30926	67; 31; 51	0.23; 0.11; 0.23	2; 2; 2	2; 4; 3
Q3ZCQ8-2	TIMM50	Isoform 2 of Mitochondrial import inner membrane translocase subunit TIM50	50946	89; 170; 102	0.13; 0.21; 0.13	4; 6; 4	5; 8; 5
Q5H9R2	DKFZp781K1356	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 5	13611	61; 48; 66	0.25; 0.25; 0.25	2; 1; 1	2; 1; 1
Q5HY54	FLNA	Filamin-A	279115	48; 70; 50	0.01; 0.02; 0.01	2; 3; 1	5; 3; 4

Q5JR95	RPS8	40S ribosomal protein S8	22094	74; 131; 88	0.15; 0.15; 0.15	2; 6; 4	2; 6; 6
Q5QNZ2	ATP5F1	ATP synthase F(0) complex subunit B1, mitochondrial	22318	39; 52; 49	0.15; 0.15; 0.15	1; 1; 2	2; 1; 3
Q5T0D2	CMPK1	UMP-CMP kinase	19195	50; 49; 28	0.18; 0.18; 0.18	1; 1; 1	1; 1; 1
Q5T6W5	HNRNPK	Heterogeneous nuclear ribonucleoprotein K	47756	570; 484; 457	0.95; 0.82; 0.71	26; 16; 17	27; 17; 19
Q5T8U3	RPL7A	60S ribosomal protein L7a (Fragment)	21702	175; 265; 179	0.77; 1.05; 0.77	8; 11; 8	8; 12; 11
Q5TCU6	TLN1	Talin-1	260030	65; 21; 26	0.01; 0.01; 0.01	1; 1; 1	1; 6; 4
Q5VVC8	RPL11	60S ribosomal protein L11 (Fragment)	20167	61; 71; 119	0.17; 0.36; 0.17	1; 2; 4	2; 2; 4
Q6P3R8	NEK5	Serine/threonine-protein kinase Nek5	82363	46; 44; 45	0.04; 0.04; 0.04	14; 9; 9	17; 13; 13

Q6P587	FAHD1	Acylpyruvase FAHD1, mitochondrial	25112	113; 94; 149	0.13; 0.13; 0.13	2; 2; 3	2; 2; 3
Q6PI48	DARS2	Aspartate--tRNA ligase, mitochondrial	74086	65; 29; 55	0.04; 0.04; 0.04	3; 2; 3	3; 2; 3
Q6S8J3	POTEE	POTE ankyrin domain family member E	122882	247; 240; 265	0.11; 0.11; 0.11	13; 16; 11	16; 18; 17
Q6UYC3	LMNA	Prelamin-A/C	69492	110; 74; 94	0.1; 0.1; 0.1	2; 3; 3	2; 5; 5
Q6ZV30	ACSS1	Acetyl-coenzyme A synthetase 2-like, mitochondrial	67393	121; 123; 70	0.05; 0.1; 0.05	2; 4; 2	2; 4; 3
Q86VP6	CAND1	Cullin-associated NEDD8- dissociated protein 1	137999	23; 35; 59	0.02; 0.07; 0.02	1; 3; 3	2; 4; 3
Q86VV8	RTTN	Rotatin	252290	36; 35; 35	0.01; 0.01; 0.01	3; 1; 1	5; 6; 5
Q8NFV4	ABHD11	Alpha/beta hydrolase	34725	46; 32; 41	0.31; 0.1; 0.1	4; 3; 3	5; 4; 4

domain-containing protein 11

Q92520	FAM3C	Protein FAM3C	24950	245; 120; 109	0.13; 0.13; 0.13	3; 1; 1	3; 1; 1
Q92945	KHSRP	Far upstream element-binding protein 2	73355	246; 303; 226	0.3; 0.42; 0.3	8; 10; 9	8; 11; 9
Q96AG4	LRRC59	Leucine-rich repeat-containing protein 59	35308	171; 129; 203	0.43; 0.43; 0.57	7; 8; 7	11; 9; 8
Q96ER9	CCDC51	Coiled-coil domain-containing protein 51	46011	36; 48; 40	0.07; 0.07; 0.07	1; 1; 1	3; 1; 2
Q96EY7	PTCD3	Pentatricopeptide repeat domain-containing protein 3, mitochondrial	79184	145; 92; 289	0.13; 0.13; 0.13	4; 4; 6	4; 4; 6
Q96HE7	ERO1L	ERO1-like protein alpha	55213	46; 72; 82	0.06; 0.12; 0.06	1; 2; 2	2; 2; 3
Q96HY6	DDRGK1	DDRGK domain-containing protein 1	35589	49; 75; 33	0.09; 0.19; 0.19	2; 4; 3	3; 4; 3

Q96I99	SUCLG2	Succinyl-CoA ligase [GDP-forming] subunit beta, mitochondrial	46824	204; 154; 234	0.31; 0.23; 0.23	6; 6; 6	7; 7; 7
Q96S66	CLCC1	Chloride channel CLIC-like protein 1	62667	94; 107; 52	0.11; 0.17; 0.05	6; 7; 5	7; 7; 5
Q96TA1	FAM129B	Niban-like protein 1	84598	75; 77; 97	0.12; 0.12; 0.12	8; 7; 8	9; 7; 8
Q99714	HSD17B10	3-hydroxyacyl-CoA dehydrogenase type-2	27134	368; 444; 478	0.42; 0.79; 0.79	12; 11; 14	12; 12; 14
Q99735	MGST2	Microsomal glutathione S-transferase 2	16781	44; 32; 37	0.2; 0.2; 0.2	5; 2; 2	5; 2; 3
Q9BSE5	AGMAT	Agmatinase, mitochondrial	38206	96; 151; 136	0.18; 0.28; 0.18	3; 5; 4	3; 5; 4
Q9BSH4	TACO1	Translational activator of cytochrome c oxidase 1	32913	36; 43; 43	0.1; 0.1; 0.1	2; 2; 1	2; 2; 1
Q9BSJ8	ESYT1	Extended synaptotagmin-1	123293	30; 80; 45	0.05; 0.08; 0.05	2; 5; 3	5; 7; 3

Q9BTU6	PI4K2A	Phosphatidylinositol 4-kinase type 2-alpha	54388	50; 54; 103	0.06; 0.12; 0.12	1; 2; 3	1; 2; 3
Q9BVC6	TMEM109	Transmembrane protein 109	26194	50; 67; 54	0.13; 0.13; 0.27	1; 1; 2	2; 1; 3
Q9BW60	ELOVL1	Elongation of very long chain fatty acids protein 1	32755	32; 34; 45	0.21; 0.1; 0.1	2; 2; 2	3; 2; 2
Q9H009	NACA2	Nascent polypeptide-associated complex subunit alpha-2	23209	105; 84; 86	0.14; 0.14; 0.14	3; 1; 2	3; 2; 2
Q9H2G4	TSPYL2	Testis-specific Y-encoded-like protein 2	79615	61; 43; 47	0.04; 0.04; 0.04	3; 4; 3	5; 5; 4
Q9H2W6	MRPL46	39S ribosomal protein L46, mitochondrial	31799	69; 87; 61	0.22; 0.1; 0.1	2; 2; 1	4; 3; 1
Q9H3N1	TMX1	Thioredoxin-related transmembrane protein 1	32170	76; 58; 52	0.34; 0.22; 0.1	3; 2; 3	3; 2; 3

Q9H7Z7	PTGES2	Prostaglandin E synthase 2	42088	53; 41; 47	0.08; 0.08; 0.08	1; 1; 1	1; 1; 1
Q9H9B4	SFXN1	Sideroflexin-1	35881	273; 375; 220	0.42; 0.56; 0.3	12; 17; 11	16; 19; 14
Q9H9J2	MRPL44	39S ribosomal protein L44, mitochondrial	37854	122; 223; 147	0.09; 0.09; 0.18	3; 3; 4	3; 3; 4
Q9HAV7	GRPEL1	GrpE protein homolog 1, mitochondrial	24492	60; 82; 76	0.47; 0.47; 0.47	3; 3; 3	4; 3; 3
Q9HB71	CACYBP	Calcyclin-binding protein	26308	44; 41; 41	0.13; 0.13; 0.13	1; 1; 1	1; 1; 1
Q9NQ39	RPS10P5	Putative 40S ribosomal protein S10-like	20279	68; 73; 72	0.17; 0.36; 0.17	1; 2; 2	2; 2; 2
Q9NQH7	XPNPEP3	Probable Xaa-Pro aminopeptidase 3	57624	38; 37; 37	0.06; 0.06; 0.06	4; 4; 3	6; 6; 3
Q9NSD9	FARSB	Phenylalanine--tRNA ligase beta subunit	66701	115; 116; 84	0.1; 0.1; 0.16	4; 4; 7	7; 4; 8

Q9P0L0	VAPA	Vesicle-associated membrane protein- associated protein A	28103	91; 59; 71	0.4; 0.25; 0.12	6; 3; 2	6; 3; 3
Q9UBF2	COPG2	Coatomer subunit gamma-2	98700	78; 69; 67	0.03; 0.03; 0.03	2; 1; 2	2; 2; 4
Q9UHD9	UBQLN2	Ubiquilin-2	65655	89; 84; 78	0.05; 0.1; 0.05	4; 2; 5	6; 2; 5
Q9UJS0	SLC25A13	Calcium-binding mitochondrial carrier protein Aralar2	74528	855; 1071; 947	0.83; 0.91; 0.68	37; 44; 37	41; 46; 43
Q9UQ80	PA2G4	Proliferation-associated protein 2G4	44101	103; 107; 83	0.24; 0.16; 0.07	4; 2; 2	5; 4; 2
Q9Y265	RUVBL1	RuvB-like 1	50538	79; 130; 113	0.29; 0.21; 0.21	5; 4; 4	5; 5; 6
Q9Y2K7-3	KDM2A	Isoform 3 of Lysine-specific demethylase 2A	91543	45; 44; 47	0.04; 0.04; 0.04	7; 7; 8	12; 10; 11
Q9Y3D6	FIS1	Mitochondrial fission 1	16984	30; 28; 28	0.2; 0.2; 0.2	2; 3; 2	3; 4; 4

protein

Q9Y4W6	AFG3L2	AFG3-like protein 2	88984	43; 55; 31	0.04; 0.07; 0.04	1; 2; 1	2; 2; 1
Q9Y5K6	CD2AP	CD2-associated protein	71635	26; 34; 79	0.09; 0.09; 0.09	1; 3; 4	6; 9; 9
Q9Y5M8	SRPRB	Signal recognition particle receptor subunit beta	29912	81; 99; 135	0.37; 0.24; 0.37	3; 4; 5	5; 4; 7
S4R3N1	MOB4	MOB-like protein phocein	30231	65; 64; 50	0.11; 0.11; 0.11	1; 1; 1	1; 2; 1
U3KPR7	CAPNS1	Calpain small subunit 1 (Fragment)	11506	35; 28; 40	0.3; 0.3; 0.3	1; 1; 1	2; 1; 2

Supplementary table 1: Original co-IP data for 342 significantly identified protein in triplicate MET-colPs. Proteins, which are uniquely identified in MET-colP and found commonly in triplicate runs, are listed. All identified proteins have at least one unique peptide detected with ion score higher than 20. # > 20, number of peptide with ion score higher than 20; #Total, total number of peptide hit.