Functional study of popx2 phosphatase in breast cancer metastasis

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NANYANG
TECHNOLOGICAL
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**FUNCTIONAL STUDY OF POPX2 PHOSPHATASE IN
BREAST CANCER METASTASIS**

ZHANG SONGJING

SCHOOL OF BIOLOGICAL SCIENCES

2014

FUNCTIONAL STUDY OF POPX2 PHOSPHATASE IN BREAST CANCER METASTASIS

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Publications

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Abbreviations

Ang	Angiopoietin
AQUA	Absolute quantification of proteins
ATF2	Activating transcription factor 2
C/EBP	CCAAT/Enhancer-binding protein
CAM	Chick chorioallantoic membrane
CaMKII	Calmodulin-dependent protein kinase II
CREB	cAMP responsive element-binding protein
CSDA	Cysteine sulfinic acid decarboxylase
CTTN	Cortactin
DAVID	Database for Annotation, Visualization and Integrated Discovery
DEL-1	Developmental endothelial locus-1
DNM1	Dynamin1
DNM2	Dynamin2
ECM	Extracellular matrix
EHS	Engelbreth-Holm-Swarm
ELK-1	ETS domain-containing protein
EMT	Epithelial and mesenchymal transition
ER	Estrogen receptor
ERLIC	Electrostatic repulsion hydrophilic interaction chromatography
FGF	Fibroblast growth factor
FOXO3	Forkhead box O3
GFP	Green fluorescence protein

GR	Glucocorticoid receptor
GSK3	Glycogen synthase kinase 3
HIF	Hypoxia-inducing factor
HILIC	Hydrophilic interaction liquid chromatography
HSF1	Heat shock factor 1
ICAT	Isotope coded affinity tag
IL	Interleukin
IMAC	Immobilized metal affinity chromatography
IQGAP1	Ras GTPase-activating-like protein
iTRAQ	Isobaric tags for relative and absolute quantification
KEGG	Kyoto Encyclopedia of Gene and Genomes
LIMK	LIM domain kinase
MAPK	Mitogen-activated protein kinase
MARCKS	Myristoylated alanine-rich C-kinase substrate
mDia	Diaphanous-related formin-1
MLC	Myosin light chain
MMP	Matrix metalloproteinase
MTOC	Microtubule organizing center
PAK	p21-activated kinase 1
PDGF	Platelet-derived growth factor
PHDs	Proline hydroxylases
PTM	Post-translational modification
PXN	Paxillin
RhoGAP	RhoGTPase activating proteins

RhoGDI	RhoGTPase guanine nucleotide dissociation inhibitor
RhoGEF	RhoGTPase guanine nucleotide exchange factor
ROCK	Rho-associated protein kinase
SAX	Strong anion exchange
SCX	Strong cation exchange
SILAC	Stable Isotope Labeling of Amino acid in Cell culture
SRE	Serum response element
SREBP1	Sterol regulatory element-binding protein 1
SRF	Serum response factor
STMN1	Stathmin
TMHMM	Transmembrane prediction using hidden markov models
VEGF	Vascular endothelial growth factor
VHL	Von hippel-lindau tumor suppressor protein

Abstract

POPX2, a serine/threonine phosphatase belonging to the PP2C family, is involved in the regulation of actin cytoskeleton, mDia-SRF (serum response factor) mediated transcription and breast cancer cell motility and invasiveness. In this project, we propose to use three different approaches to explore the biological functions of POPX2 in breast cancer. Firstly, we used an integrative transcriptome and proteome approach to decipher the roles of POPX2 in cancer motility and related signaling. Our study suggests that POPX2 is involved in cancer cell migration through modulating MAPK1/3-stathmin-mediated microtubule dynamics, in collaboration with other possible signaling molecules to modulate actin cytoskeleton and focal adhesions. POPX2 is also implicated in transcriptome regulation possibly through modulating MAPK1/3 and GSK3 β as well as the downstream transcription factors. To further explore the role of POPX2 in angiogenesis, we also initiated a combined phosphoproteome and secretome study. Our phosphoproteome data implicate the participation of POPX2 in CDK1-mediated cell cycle progression. Secretome analysis combined with extensive biological validation verified that there is exosome enrichment and accelerated EMT progression in response to POPX2 silencing, which could further contribute to angiogenesis.

Apart from the –omics approaches to understand the signaling network regulated by POPX2, we have also taken a candidate approach to specifically study candidate proteins which might be regulated by POPX2. RhoGDIs, the candidate proteins related to POPX2-regulated signaling, are also examined as we have earlier found that the levels of RhoGDI are affected by POPX2 knockdown¹. The effects of RhoGDIs on actin cytoskeleton are studied. Overexpression of RhoGDI1 and RhoGDI2 result in cell rounding with concomitant loss of stress fiber and focal adhesions to different extent. RhoGDI1 appears to exert a more prominent effect compared to that of RhoGDI2. This might be due to their differential binding affinities to RhoGTPases, one of the major regulators of the actin cytoskeleton. Further characterization of RhoGDIs' interaction with the different RhoGTPases reveals two conserved leucine residues, which are critical to RhoGTPases binding. We also verify other mutants, which affect RhoGDI binding affinity to various RhoGTPases. These mutants help to elucidate that RhoGDIs affect the actin cytoskeleton via RhoGTPases.

Chapter 1 Introduction

1.1 Breast cancer

Breast cancer is one of the most prevalent cancer in women². In 2008, up to 23% of all cancers were diagnosed as breast cancer and 458,000 patients died from breast cancer³. The prevention and treatment for breast cancer show good progress as the cancer biomarker research field has greatly expanded in these few years. Understanding the molecular mechanism of breast cancer will help the development of target-specific drugs. Several different breast cancer cell lines have been established as model system for research. Many of them have been well characterized and reported to be useful to study the pathophysiology of breast cancer⁴. Since breast cancer is a complex and heterogeneous disease, using cell lines as model system does have disadvantages⁵. In our earlier study, the breast cancer cell lines used to examine POPX2 (our protein of interest) levels are MDA-MB-231, BT-549, BT-20, MCF7, MDA-MB-361, MDA-MB-468, T-47D, Sk-Br3¹. In our current study, we have chosen to use MDA-MB-231 as the model. One of the main reasons is that MDA-MB-231 cells are found to have highest levels of POPX2 among all the breast cancer cell lines examined¹.

1.2 Mechanism of cancer metastasis

Metastasis is the dissemination of tumor cells from their original sites to a new microenvironment⁶. The spread of tumor cells to certain organs depends on specific primary cancer cell type. Breast cancer cells primarily spread to bone, lung, brain and liver. Prostate cancer cells most probably distribute to bone. Pancreatic cancer cells preferentially metastasize to liver⁷. So far, two critical cancer metastasis mechanisms have been proposed. One is the “Seed and soil” hypothesis which was proposed by Stephen Paget in 1889⁸ suggesting that certain cancer cell type could only metastasize to specific organ site. The other one is the “mechanical factors” theory, which was suggested by James Ewing in 1929⁹,¹⁰. The main view of this theory is that the tumor cells will be arrested at a distant organ by mechanical factors. Current studies provide support that both theories contribute to metastasis ultimately¹¹.

A cascade of events is involved in the successive metastatic process (Figure 1)¹²⁻¹⁴: (a) Metastatic tumor cells originate from primary tumor; (b) they invade locally through the degradation of the extracellular matrix membrane; (c) EMT (epithelial-mesenchymal transition) and angiogenesis follow leading to (d) intravasation which involves tumor cells entry into the bloodstream. Once the tumor cells can successfully penetrate and circulate in the bloodstream, they will survive and attach to the blood vessel wall in a target organ site (e). Upon arriving at the new microenvironment, the tumor cells extravasate through the blood vessel wall (f) and form a new micrometastatic tumor (g). Angiogenesis also occurs in this new metastatic tumor site to provide nutrients and oxygen for rapid proliferation. Metastasis has long been considered as an inefficient process. The earlier steps, from the invasion to extravasation, perform efficiently. But the subsequent proliferation of the tumor cells at a new organ site and the formation of new micrometastatic tumor are difficult and inefficient⁹. Understanding the individual step in the metastasis process is critical for the disruption of metastasis. Our research focuses on three of the complex and successive metastatic processes, namely tumor cell migration, angiogenesis, and epithelial- mesenchymal transition.

Figure 1. Tumor metastasis mechanism (Figure adapted from Zetter, B.R., 1998¹⁵). (a) Metastatic tumor cells originated from the primary organ. (b) In order to metastasize, primary tumor cells need to invade through the primary organ and (c) angiogenesis occurs at the primary organ to promote the primary tumor expansion. (d) Primary tumor cells intravasate through the blood vessel and (e) adhere

to the blood vessel in a secondary organ. (f) Tumor cells extravasate through the blood vessel and (g) form a new micrometastatic tumor at the distant organ.

1.3 Tumor cell migration

Cell migration is essential for cancer metastasis¹⁶. Therefore, understanding the fundamental cell movement mechanisms is important for designing cancer treatment.

1.3.1 The cell migration cycle

Generally, several main migratory steps are involved in the migration cycle (Figure 2)^{17, 18}. Cell migration begins with cell polarization and protrusion formation towards the migratory direction in response to the environmental cues (Figure 2a). Two types of protrusions have earlier been recorded in cell migration, and they are called lamellipodia and filopodia (Figure 2a-b). Lamellipodia are sheet-like protrusions filled with branch-like actin bundles, pushing the plasma membrane forward^{19, 20}. Filopodia are finger-like structures extended from lamellipodia at the leading edge. They function as sensors to probe the front environmental cues and lead the movement²¹. Another protrusion structure called invadopodia is for metastatic cancer cell movement and invasion²². Invadopodia are finger-like structures mainly involved in extracellular matrix degradation to facilitate cancer cell invasion²³. These protrusions are induced by actin polymerization at the cortex under the regulation of RhoGTPases, which will be further discussed in the following part.

Cell-substratum adhesions are essential for mediating the attachment of cell protrusion to the extracellular matrix (Figure 2b-c). Several types of cell-matrix adhesions have been documented, including focal complex, focal adhesion and fibrillar adhesion^{24, 25}. Focal complexes are small and highly dynamic structures, residing at the leading lamellipodia. Focal adhesions are big and mature adhesions, evenly distributing throughout the cells. Fibrillar adhesions are elongated cell-matrix adhesion structures and found to bind specifically to fibronectin via $\alpha 5 \beta 1$ -integrin^{26, 27}. In highly motile cells, focal complexes play a principle role in maintaining the connection between the cell leading edge and the

substratum²⁸. All classes of adhesion formation are regulated by actin dynamics and RhoGTPases²⁹.

Figure 2. The cell migration cycle²¹. (a) Cell motility is initiated by protrusions formation at the leading edge. Two types of protrusions have earlier been recorded in cell migration, and they are called lamellipodia and filopodia. (b) New adhesion formation is essential for cell movement. (c) The cell body is translocating forward via the retraction force. (d) The retraction fibres pull the cell body forward and the adhesions at the cell rear disassemble.

The last step in the migration cycle is rear retraction and detachment of adhesions (Figure 2c-d). RhoA and myosin promote the generation of retraction force³⁰. The release and disassembly of focal adhesions at the cell rear is reported to be regulated by several mechanisms. RhoA and its downstream effector, ROCK, are involved in rear adhesion disassembly to facilitate tail retraction³¹. Microtubules targeting to adhesion sites promote adhesion relaxation at the cell rear³². In addition, endocytosis of integrin at the contact sites mediated by clathrin contributes to focal adhesion disassembly in migrating cells³³. Calpain-mediated proteolysis of adhesion components (talin and FAK) is crucial for the disassembly of focal adhesions and retraction of the rear^{34, 35}.

1.3.2 Diversity in tumor cell migration strategies

In general, tumor cells can either migrate individually or collectively with differential integrins, proteases as well as cadherins expression levels³⁶. Based on the morphology variants, single-cell can migrate either as amoeboid or mesenchymal modes (Figure 3). During collective migration, cells still maintain their cell-cell adhesions, which are almost absent in the single cell movement. Two main collective movements have been described. They either move in cohorts (clusters) or sheets (strands) (Figure 3)³⁷.

Single-cell migration

Amoeboid movement is the most effective migration mode, with highly dynamic morphological expansion and contraction. The expressions of integrins, proteases as well as cadherins are relatively low³⁶. Protrusion and retraction formation for amoeboid movement are independent of stress fiber and focal adhesions³⁸. RhoA and ROCK are responsible for generating cortical rigidity and maintaining the roundish shape³¹. Mesenchymal cells adopt a spindle-like and elongated morphology. The cells are found with relatively high levels of integrins and proteases, but with low levels of cadherins. Accordingly, mesenchymal migration exhibits cell-extracellular matrix interaction along with extracellular matrix proteolysis³⁹. Mesenchymal cell movement strictly follows the three-step cell migration cycle, occupying a predominant role in cancer cell metastasis³⁷.

Collective-cell migration

Collective-cell migration requires integrins, proteases and cadherins to maintain cell-substratum and cell-cell interactions⁴⁰. Collective-cell migration exhibits three major characteristics. Firstly, collective-cell migration is a coordinated movement among the cells. Thus stable links between cells, which are maintained by cadherins, are indispensable⁴¹. Secondly, similar to single-cell movement, the three-step migration mechanism is still retained in collective-cell migration. Nevertheless, the differences are the coordination of multiple cell protrusions and retractions at the cell leading edge and rear⁴². Thirdly, ECM degradation is necessary to generate a wider path for the clusters or sheets of cells to pass through⁴³. Collective-cell migration is much more complicated compared with single-cell migration and a variety of signaling mechanisms are involved, including cell-cell and cell-substratum adhesions, ECM degradation, and cell-cell coordination.

Figure 3. Diversity in cell migration strategies³⁷. Tumor cells can move either individually (top) or collectively (bottom). Single cell can move either as amoeboid or mesenchymal mode. Collective cells either move in cohorts (clusters) or sheets (strands). Strong cell-cell adhesion and cell-ECM interaction are maintained in the collective cell movement but weaker in single cell movement.

1.3.3 Coordination of actin and microtubule during cell migration

RhoGTPases and actin cytoskeleton regulate cell migration

RhoGTPases are the members of the Ras superfamily. They act like molecular switches alternating between the inactive GDP-bound state and active GTP-bound state. Three main groups of Rho protein regulators have been documented: RhoGTPase guanine nucleotide exchange factor (RhoGEF), RhoGTPase activating proteins (RhoGAP), and RhoGTPase guanine nucleotide dissociation inhibitor (RhoGDI)⁴⁴. The GDP-bound Rho protein associates with RhoGDI in cytosol and translocates to plasma membrane for activation. RhoGEF catalyzed nucleotide exchange to activate RhoGTPases, following by downstream effector activation at the plasma membrane. RhoGAP stimulates the hydrolysis of the GTPase, and the inactive Rho proteins return to the cytosol through binding to RhoGDIs (Figure 4)⁴⁵.

Figure 4. The RhoGTPases cycle (Figure adapted from Etienne-Manneville, S. and Hall, A., 2002⁴⁵). Rho proteins cycle between the inactive (GDP-bound) state and the active (GTP-bound) state. This cycle is strictly regulated by three main Rho proteins regulators, RhoGAPs, RhoGEFs, and RhoGDIs.

RhoGDIs associate with the inactive GDP-bound Rho proteins and translocate them to the plasma membrane for activation. In the active state, RhoGTPs interact with the downstream effectors.

Among the Rho family, RhoA, Cdc42 and Rac1 are the well-studied. They are involved in multiple cellular activities, such as actin cytoskeleton organization, cell migration, cell adhesion formation and apoptosis⁴⁶. In addition, RhoGTPases also regulate SRF-mediated transcription⁴⁷. Some of the effectors and cellular activities regulated by the RhoGTPases and their effectors are summarized below⁴⁸ (Figure 5). In migrating cell, high levels of active Cdc42 and Rac1 are localized to the cell front to induce filopodia and lamellipodia through WASP and WAVE, respectively. WASP and WAVE family proteins contain VCA domain at the C-terminal, which bind and activate the Arp2/3 complex, stimulating rapid actin nucleation and polymerization at the cell cortex. These actin structures facilitate protrusive forces generation^{19, 49}. PAK is another common downstream effector of Cdc42 and Rac1. PAK phosphorylates and activates LIMK, which results in cofilin inactivation^{50, 51}. Cofilin binds to the actin filaments and causes depolymerization of the actin filament. This filament severing ability is essential for providing free barbed ends for actin polymerization at the migrating front⁵². On the other hand, active RhoA promotes focal adhesion assembly and retraction force generation at the cell rear through ROCK and mDia⁵³. Active ROCK enhances the phosphorylation level of MLC of myosinII through inhibiting MLC phosphatase or by direct phosphorylation of MLC⁵⁴. Active myosinII promotes actomyosin filament assembly and contraction force generation⁵⁵. ROCK also regulates the LIMK-cofilin pathway⁵⁶. mDia is another effector of RhoA which binds to profilin via its FH1 domain. In addition, mDia is also able to polymerize actin⁵⁷. Thus mDia principally contributes to stress fiber formation in collaboration with ROCK⁵⁸.

Figure 5. RhoGTPases and their respective effectors regulate actin cytoskeleton reorganization. The signaling pathways leading by RhoA, Rac1 and Cdc42 contribute to actin stabilization, lamellipodia and filopodia formation at the cell leading edge.

Microtubule dynamics in migration cells

Microtubules are made up of α and β tubulin heterodimers, switching between the growth phase (alternatively named as rescue) and the shrinkage phase (alternatively named as catastrophe). This highly dynamic process is characterized as microtubule instability, also called microtubule dynamics⁵⁹. Based on the modification of microtubules, two classes have been categorized: one is unmodified which is highly dynamic; the other one is post-translational modified which represents the stable microtubules. Two types of post-translational modification have been documented: acetylation and detyrosination⁶⁰.

One of the microtubule regulatory proteins related to our research is stathmin. Stathmin exerts two distinct effects on microtubule dynamics. Stathmin associates with free tubulin, preventing their incorporation into the polymerized microtubules. On the other hand, stathmin also binds to the polymerized microtubules and promotes depolymerization⁶¹. The microtubule-destabilizing activity of stathmin is turned off by phosphorylation⁶².

Recent studies have shown that RhoGTPases can also play a central role in regulating microtubule dynamics during cell migration. In turn, microtubule dynamics also affect RhoGTPases' activities^{17, 63}. Active mDia, a RhoA effector, is reported to stabilize microtubules⁶⁴. At the leading edge of the migrating cell, PAK which is activated by Rac1 and Cdc42 could stabilize microtubule through phosphorylation and inhibition of stathmin⁶⁵. Microtubule stability at the cell front is reported to be essential for cell motility^{66, 67}.

1.4 Tumor Angiogenesis

Tumor angiogenesis refers to new blood vessel outgrowth from the existing blood vessels in response to rapid tumor expansion. Angiogenesis nourishes the tumor cells with oxygen and nutrients to facilitate their rapid growth. Besides, it also provides an exit route for tumor cells entering the blood stream to another organ site¹⁵. Understanding the mechanism related to tumor-induced angiogenesis and signaling could facilitate the identification of anti-tumor strategies.

1.4.1 Mechanisms of tumor-induced angiogenesis

Blood vessel extension formed by a process called sprouting is involved in angiogenesis. Several well-characterized stages in sprouting angiogenesis are shown in Figure 6. Endothelial cells stay in a quiescent state under normal conditions. They form a monolayer along the basement membrane, interconnected mainly by VE-cadherin. Pericytes wrap around the endothelial cells of capillaries, protecting the endothelial cells from insults and cell death (Figure 6a). When pro-angiogenic factors secreted from tumor cells, such as VEGF (vascular endothelial growth factor), Ang (angiopoietin) and FGF (fibroblast growth factor), bind to their respective endothelial cell membrane receptors, pericytes become detached from the basement membrane. Subsequently, the VE-cadherin-mediated endothelial cell-cell adhesions are progressively weakened. The vessels then dilate to facilitate endothelial cell invasion (Figure 6b). Active endothelial cells release proteases, such as the MMPs, to degrade the basement membrane and to pave way for their escape to outside space (Figure 6c). In order to prevent the random endothelial cell migration, a tip cell is selected to lead the direction towards the stimulus. The neighboring cells continue to divide and elongate the

vessels (Figure 6d). Finally, cell-cell adhesions are re-established and the sprouting vessels fuse with other sprouts under unknown mechanisms⁶⁸⁻⁷⁰.

Tumor-induced angiogenesis exhibits unique features, including irregular and unstable shapes, variations in diameter, lacking of pericytes and complete basement membrane, leaky as well as loosely associated endothelial cells^{71, 72}. Besides, the tumor cells also get incorporated into the tubes⁷³. Two distinct models have been suggested. The first model hypothesizes that secretion of pro-angiogenic factors attract blood vessels elongation towards the tumor. The second model proposes that tumor grows on the site of pre-existing blood streams, inducing blood vessels elongation around the tumor⁷⁴.

Figure 6. Mechanism of sprouting angiogenesis. The consecutive steps of blood vessel extension are shown. (a) The quiescent state of blood vessel is shown under normal conditions. Endothelial cells form monolayer, and the capillaries are wrapped by pericytes. (b) With the stimulation of pro-angiogenic factors, the pericytes detach from the capillaries. (c) The quiescent blood vessels dilate, and endothelial cells secrete proteases to degrade the basement membrane. (d) During the endothelial cells invasion, a tip cell is selected to ensure the branch formation.

1.4.2 Tumor angiogenesis signaling

Tumor angiogenesis is mainly induced by rapid tumor expansion. Rapidly growing tumor reduces intracellular oxygen level⁷⁵. HIFs (hypoxia-inducible factors) are transcription factors that respond to decreased intracellular oxygen concentration, specifically, to hypoxia⁷⁶.

Up to six HIF members have been reported, among which HIF1 is the key element in angiogenesis signaling. HIF1 is reported to be a heterodimer consisting of HIF1 α and HIF1 β ⁷⁷. Under normoxia, HIF1 α is firstly hydroxylated by PHDs (proline hydroxylases), directing its binding to VHL (von hippel-lindau tumor suppressor protein). The binding of VHL to HIF1 α targets the complex to VHL-mediated ubiquitin degradation^{78, 79}. Under hypoxia, the hydroxylation of HIF1 α by PHD is inhibited and HIF1 α is translocated into nucleus, forming heterodimer with HIF1 β . The p300/CBP coactivator is then recruited to this DNA-protein complex to induce downstream targets expression^{76, 80}. The target products contain growth factors like VEGF, FGF, and TGF (transforming growth factor)⁸¹. A summary of angiogenetic factors are listed in Table 1. These pro-angiogenic factors bind to their respective receptors on the endothelial cells surface and initiate subsequent signaling to promote endothelial cell survival, migration and proliferation and vascular permeability (Figure 7)⁸².

Table1. Pro-angiogenic factors that regulate tumor angiogenesis.

Factor	Roles in tumor angiogenesis
VEGF	Significantly increased in many cancer types ⁸³ Enhance endothelial cells permeability ⁸⁴ Stimulate endothelial cells proliferation and migration ⁸⁵
Ang	Enhance endothelial cells survivin, branching and migration ^{86, 87} Render newly formed blood vessels unstable and plastic ⁸⁸
FGF	Inhibition of FGF activity suppresses tumor growth ⁸⁹
IL-8	Enhance endothelial cells proliferation and survivin ⁹⁰
MMP-2	Inhibition of MMP-2 directly disrupts angiogenesis and tumor growth ⁹¹

Figure 7. Angiogenesis signaling. <http://www.cellsignal.com/reference/pathway/Angiogenesis.html>.

Rapid tumor growth induces intracellular hypoxia, resulting in the activation of HIF1 α , a hypoxia marker. Translocation of HIF1 α into nucleus facilitates the association between HIF1 α and hypoxia response element, leading to the target genes' expression. The target genes include (1) tumor cell-released ECM protease, involving in extracellular matrix remodeling; (2) tumor cell-released pro-angiogenic factors, involving in endothelial cells survival, vascular permeability, migration and proliferation via activating endothelial cell surface receptors and downstream signaling; (3) ECM, extracellular matrix.

1.5 Epithelial and mesenchymal transition in cancer metastasis

EMT describes the transition from epithelial cells to mesenchymal cells, with the loss of tight junction, adherens junction and desmosome. In addition, the cells also acquire highly motile as well as invasive characteristics, and lose sensitivity to anoikis (Figure 8)⁹². The reversible process of EMT is called MET (mesenchymal and epithelial transition), which is also critical for cancer metastasis. The activation of EMT program is essential during cancer metastasis to a secondary organ. EMT is initiated during the invasion through basement membrane, intravasation and extravasation⁹³.

Epithelial and mesenchymal cells are totally different cell types with distinct morphological and functional characteristics. Epithelial cells are extremely densely packed with one another through sequential assembly of cell-cell contacts, such as adherens junctions, gap junctions, desmosomes, and tight junctions. They lack motility and invasiveness. Epithelial cells play crucial roles in secretion, protection, transcellular transport and some other important biological activities. Conversely, mesenchymal cells are loosely connected, adopting a spindle-shaped, fibroblast-like morphology. Mesenchymal cells are highly motile in *in vitro* experimental tests. Mesenchymal cells are able to form lymphatic and circulatory tissues, as well as connective tissues^{94, 95}.

A number of EMT markers are documented. E-cadherin, β -catenin, Claudins, Occludins, Desmoplakin, Cytokeratin and Mucin-1 are classified as epithelial markers, whereas Vimentin, N-cadherin, Fibronectin, Vitronectin, FSP-1, Smooth-muscle actin are grouped into mesenchymal markers (Figure 8).

Figure 8. Epithelial-mesenchymal transition process. The figure depicts the consecutive events during the epithelial-mesenchymal transition, including tight junction disassociation, adhere junction disassociation and loss of desmosome. The reported epithelial markers and mesenchymal markers are listed.

Among these EMT markers, E-cadherin and Vimentin are critical for EMT process^{93, 95}. E-cadherin is required to maintain stable adherens junctions in the epithelial cells and its expression is repressed during EMT process. E-cadherin itself is considered to be a tumor suppressor based on two findings: the expression of E-cadherin is considerably down-regulated in various tumor types and silencing of E-cadherin also promotes tumor invasion and metastasis *in vitro*⁹⁶. E-cadherin transcriptional repression is resulted from the activation of some transcription repressors, e.g. Snail, Slug, Sip1 and Ets, which are regulated by MAPK and GSK3 β ^{96, 97}. Vimentin is a type III intermediate filament protein, which is highly up-regulated in mesenchymal cells, and is proposed to be a significant mesenchymal cell marker. A positive correlation between Vimentin and breast cancer progression has been consistently observed recently. Vimentin is suggested to be involved in EMT process from three aspects: (1) Vimentin plays an indirect role in EMT proteins transcriptional regulation. (2) Vimentin interacts with Scribble and stabilizes it to support the cell polarity during cell migration. Vimentin also binds to plectin isoform 1f and integrin $\alpha 2\beta 1$ to stabilize the focal adhesion. (3) Phosphorylation of Vimentin by AKT1 protects it from degradation and in turn promotes cell migration and cancer metastasis⁹⁸.

1.6 Proteomic study towards the understanding of cancer biology

Gene and protein expression profiling through DNA microarray and mass spectrometry-based proteomics pave new ways for the study of cancer signaling and discovery of biomarkers. Global study of protein post-translational modifications, such as phosphorylation, glycosylation and degradation, further contributes to the understanding of cancer biology. Body fluids, like secretome and exosome, provide another rich source of materials for investigating the underlying mechanisms leading to cancer.

1.6.1 SILAC (Stable Isotope Labeling by Amino acid in Cell culture)-based proteomic study

Several strategies have been employed for quantitative proteomics, and they are grouped into four categories, including metabolic labeling, chemical labeling, enzymatic labeling as well as absolute quantification by spiked synthetic internal peptide standards. In addition, label-free method is also applicable for quantitative proteomic study^{99, 100}. Metabolic labeling allows label incorporation during cell replication and combination of different compared states at a very early stage, among which SILAC is the mostly well-developed strategy. Labeling can also be done by chemical reactions, such as ICAT (Isotope Coded Affinity Tag) and iTRAQ (isobaric Tags for Relative and Absolute Quantification). Enzymatic labeling includes the use of trypsin- or Glu-C-catalyzed incorporation of ^{18}O in protein digestion. Absolute quantification is achieved by using a known quantity of internal standards, such as AQUA (Absolute Quantification of Proteins).

The SILAC strategy

SILAC is firstly described by Matthias Mann's group in 2002, and has been widely adopted in relative quantitative proteome analyses, especially for phosphoproteome and secretome quantifications¹⁰¹⁻¹⁰³. In SILAC-based cell culturing, two cellular states are compared. One cell type or experimental condition is maintained in heavy medium containing heavy amino acids (e.g. $^{13}\text{C}_6$ -arginine and $^{13}\text{C}_6$ -lysine), and the other is grown in light medium containing normal amino acids (Figure 9). All other culture conditions and environmental factors are kept the same for these two compared states. During cell replication, cells grown in heavy

medium incorporate heavy amino acids into their proteins. At least five cell doubling times are required to allow the full incorporation of heavy amino acids into the proteome. Protein are harvested and combined at a 1:1 ratio, followed by trypsin digestion and peptides fractionation. Peptides were identified and quantified by LC-MS/MS. Incorporation of heavy amino acids results in a known mass shift (6Da for $^{13}\text{C}_6$ -arginine, for instance) between the light and heavy peptide pairs.

Advantages and disadvantages of SILAC

Usage of SILAC in quantitative proteomics does have several advantages. (1) Usually, the amount of starting material from biological samples is limited and valuable, thus it is necessary to reduce sample preparation steps in order to minimize sample loss. No *in vitro* peptide labeling steps are required in the SILAC method. Label incorporation into the peptides or proteins is already accomplished during the cell doubling phase. (2) The extend of label incorporation reaches up to 100% within at least five cell doubling times, which diminishes sample differences and increases results accuracy. (3) Usually, each peptide contains labeled arginine and lysine, which enable the comparison between several peptide pairs from each protein, ensuring the final changes on the protein level. (4) In contrast to other quantitative method, such as ICAT, SILAC makes it possible to quantify every protein even those proteins without cysteine. (5) Another benefit is the labeling of ^{13}C instead of deuterium, which will cause the well-known difference in elution time. (6) The mass differences between heavy and light forms of peptides are known, which simplifies the peptide pair's identification and quantification. (7) Earlier incorporation of labeling minimizes sample errors during each sample preparation step and mass spectrometry procedure¹⁰⁴.

Nevertheless, the SILAC method has its own limitations. (1) The maximum compared cellular states are limited by the heavy amino acids available. Currently, up to five cellular states can be compared. (2) SILAC can only be applied in cell culture-based quantification where the cells are metabolically active. It is not applicable for tissue sample quantification. (3) Compared to other labeling methods, SILAC is more expensive¹⁰⁵.

Figure 9. Work-flow of the SILAC strategy. This flow chart describes the principle and work flow of the SILAC strategy. In this example, two populations of cells are used. One population is grown in the “light medium” which contains normal amino acids. The other one is grown in “heavy medium” which contains heavy amino acids. The two populations of cells are lysed and mixed in 1:1 ratio at very early stage, so that subsequent sample preparation steps will not introduce any discrepancy between the two samples. Incorporation of the heavy amino acids into a peptide leads to a known mass shift compared to the same peptide containing normal amino acids.

1.6.2 Phosphoproteomics

Protein kinases and phosphatases are effective biomarkers for cancer drug targeting. Advances in the cancer phosphoproteome uncover thousands of phosphorylation sites, involved in multiple cancer signaling pathways^{106, 107}.

Phosphoproteome analysis

Most proteins are regulated at post-translational level, especially by phosphorylation. Phosphorylation and its reverse process, dephosphorylation, play key roles in regulating protein stability, translocation and protein-protein interaction¹⁰⁸. Sensitive and high-throughput approaches to identify the large amount of phosphorylation sites and investigate the complicated phosphorylation functions are essential. Mass-spectrometry based phosphoproteome study is rapidly developing and proving to be helpful for the study of protein phosphorylation^{109, 110}.

Phosphopeptides enrichment

One of the major challenges of phosphoproteome study is the enrichment of phosphopeptides. In general, phosphopeptide enrichment strategies are classified into four categories (Table 2): immunoprecipitation, affinity chromatography, ion exchange chromatography, hydrophilic interaction chromatography^{111, 112}. Immunoprecipitation is widely used for tyrosine-phosphopeptide enrichment using phospho-tyrosine antibodies. It is highly specific and easily manipulated. But it is not applicable for the enrichment of serine- and threonine-phosphopeptides due to the antibody specificity. IMAC (Immobilized Metal Affinity Chromatography) and TiO₂ are two affinity chromatography approaches with similar principles. The IMAC method relies on the binding of negatively charged phosphopeptides to positively charged metal ions, like Fe³⁺, Al³⁺, Ga³⁺, Co³⁺, followed by elution with an alkaline solution. Similarly, negatively charged phosphopeptides are also enriched by TiO₂ beads. However, non-phosphorylated peptides with multiple acidic amino acid residues will also associate with the IMAC column, which will interfere with signals from the phosphopeptides in mass spectrometry analysis. Several improvements have been proposed, such as (1) using low pH loading buffer for IMAC to reduce the non-specific binding; (2)

reducing sample complexity through fractionation prior to IMAC, and (3) derivatizing the carboxylic groups on acidic amino acid residues by O-methyl esterification¹¹¹. Similarly, non-specific binding of acidic amino acids also occurs in the TiO₂ approach. Better results could be obtained through using substituted organic acids, e.g. 2,5-dihydroxybenzoic acid (DHB), phthalic acid or glycolic acid¹¹³. SAX (Strong Anion Exchange) and SCX (Strong Cation Exchange) are two typical ion exchange chromatography approaches to fractionate and enrich phosphopeptides. For SCX, tryptic digested peptides are firstly acidified in low pH buffer to obtain positive charges at arginines and lysines. Phosphopeptides carry additional negatively charged phospho-groups will reduce the total positive charge. Accordingly, phosphopeptides display weaker binding affinity to the negatively charged SCX column and elute before non-phosphorylated peptides. In contrast, phosphopeptides are strongly retained in the SAX column and eluted after non-phosphorylated peptides¹¹². HILIC (Hydrophilic Interaction Liquid Chromatography) is based on polarity. The principle is very straightforward. Peptides in organic solution bind to the hydrophilic column via hydrogen bonds and elute by decreasing the organic phase in the elution buffer. Phosphopeptides with polar phosphate groups will be strongly retained in the stationary hydrophilic phase and collected in the latter fractions. ERLIC (Electrostatic repulsion hydrophilic interaction chromatography) was introduced in 2008¹¹⁴. It is based on HILIC with an additional ion exchange included at the stationary hydrophilic phase, which could reduce the retention time of those strong polar peptides.

Each method has its own strength and weakness. None of these approaches offer 100% efficiency in phosphopeptide enrichment. Therefore, various combinations of two to three strategies are necessary to improve efficiency. In our study, ERLIC method is firstly applied to fractionate the whole tryptic digested peptides, followed by IMAC as well as TiO₂ to extensively enrich phosphopeptides.

Table 2. Classification of different phosphopeptides enrichment approaches.

Category	Strategy
Immunoprecipitation	Phospho-tyrosine-antibody pull down
Affinity chromatography	IMAC (Immobilized Metal Affinity Chromatography) TiO ₂ (Titanium Dioxide)
Ion exchange chromatography	SAX (Strong Anion Exchange) SCX (Strong Cation Exchange)
Hydrophilic interaction chromatography	HILIC (Hydrophilic Interaction Liquid Chromatography) ERLIC (Electrostatic repulsion hydrophilic interaction chromatography)

1.6.3 Secretomics

The term “secretome” was firstly introduced in 2000, to describe a sub-set of proteins released from cells, tissues or organisms¹¹⁵. Secreted proteins are involved in a variety of cancer signaling pathways, particularly in angiogenesis, extracellular matrix remodeling, and cancer cells invasion. Secretomics has thus been proposed to be a promising strategy for cancer biomarker discovery and investigation.

Protein secretion pathways and prediction tools

Before we look into the secretome analysis, we first take an overview on how proteins are released to the extracellular environment (Figure 10). The majority of proteins are secreted in highly evolved classical secretory pathway¹¹⁶. Secreted proteins are firstly synthesized in ribosomes with N-terminal signal peptides targeting them to endoplasmic reticulum (ER). They are further glycosylated and folded through molecular chaperones in ER. The vesicles containing the correctively folded proteins are translocated to the Golgi apparatus and subsequently to the extracellular space, fused with plasma membrane, and secreted to the extracellular space. This class of secreted proteins can be predicted by SignalP¹¹⁷. Moreover, protein secretion independent of ER and Golgi is also documented and classified as the non-

classical secretory pathway. The non-classical secretory pathway is also N-terminal signal peptide independent. Four distinct non-classical secretory pathways are described in recent years¹¹⁸⁻¹²⁰.

Figure10. Protein secretion pathways and prediction tools (Figure adapted from Karagiannis, G. S., et al, 2010¹¹⁹). The majority of proteins are secreted through the classical ER/Golgi-dependent pathway and require the N-terminal signal peptide for translocation. SignalP is the tool used to predict the presence of signal peptide. Another class of secreted proteins is ER/Golgi-independent and signal peptide-independent, and is classified as non-classical secretory pathway. Four distinct non-classical secretory pathways are described. They are endosome recycling, protein transport, flip-flop mechanism and membrane internalization. These classes of secreted proteins can be predicted using SecretomeP and TMHMM. Recently, a database called ExoCarta has been established to include all the exosome-associated secreted proteins.

Interleukin-1 β , a cytokine, is secreted through a process known as endosomal recycling. It is imported into intracellular endosomal compartments and released to the outside

microenvironment through fusion of endosomes with plasma membrane. Other proteins, such as the pro-angiogenic factor FGF, are released to the outside through direct translocation across plasma membrane using distinct transport systems. Another remarkable but less studied non-classical secretory pathway is called the flip-flop mechanism. Proteins secreted through this pathway are required to be membrane anchored and dual acetylated at the N-terminus, such as HASPB (Hydrophilic Acylated Surface Protein B). HASPB is translocated to the plasma membrane and inserted into the inner side of the membrane. But how it is translocated to the outside still remains to be investigated. All these three typical non-classical secretory pathways can be predicted by SecretomeP¹²¹. Moreover, secreted proteins could be shed membrane receptors, which are predicted by TMHMM (Trans-Membrane prediction using Hidden Markov Models)¹²². Last but not the least, proteins can be secreted through exosome, e.g. the well-known exosome markers CD81, CD9, CD63, and Alix. These proteins are transported through early endosome, followed by MVB (Multi-Vesicular Body) formation. The included vesicles are termed as ILV (Intra-Lumenal Vesicles). Finally, the MVBs fuse with the plasma membrane and the exosomes are released to trigger subsequent signaling pathways. The exosome associated proteins can be identified using ExoCarta database¹²³.

Challenges of secretomic study

A basic protocol for secretome sample collection and preparation is described here. Firstly, cells are serum-starved for an optimal time period. The longer the cells are incubated in serum-starved condition, the more secretome will be produced. However, one of the challenges for secretome collection is the contamination of intracellular proteins derived from cell death during long-time serum starvation. Therefore, the optimal serum-starved period refers to the safe time period which is long enough to generate adequate secretome with minimal cell death. Different cell types have different optimal serum-starved period for secretome collection. As reported, the optimal serum-starved period for MDA-MB-231 is 24hrs to obtain a biological informative secretome¹²⁴. Secondly, the conditioned medium (also named as cell secretome) is collected, and filtered to remove cell debris. Medium concentration is required to minimize sample volume before LC-MS/MS. The challenges of secretome analysis are due to the following technical problems: (1) contamination from the cytoplasmic proteins released by dead cells; (2) contamination from FBS (fetal bovine serum),

normally supplemented in cell culture medium; (3) highly diluted body fluids or culture medium and low secreted protein concentration¹²⁵.

1.7 POPX2 phosphatase

The main proteins, which we have studied extensively in this project, are described in detail in the following sections.

POPX1 (partner of PIX) and POPX2, belonging to PP2C phosphatase family, are isolated as the interacting partner of both α PIX and β PIX in a yeast two-hybrid screen¹²⁶. POPX1 and POPX2 comprise of 757 and 454 amino acids respectively. They share 66% homology over their core phosphatase domains (Figure 11). POPX1 is highly expressed in the brain and testis, while POPX2 is ubiquitously expressed, including lung, thymus, spleen, brain, uterus and pancreas¹²⁷.

Figure 11. Schematic representation of POPX1 and POPX2 (Figure adapted from Koh, C. G., et al, 2002¹²⁶). The brown bar represents the PP2C phosphatase domain. The dark green bar plus dark orange bar represents that 66% similarity is shared between POPX1 and POPX2. The blue bar represents the regions showing no similarity.

Two well-characterized substrates of POPX2, PAK1 and CaMKII (calmodulin-dependent protein kinase II) have been reported earlier^{126, 128}. PAK1 and POPX2 bind to different

regions of PIX and these proteins are able to form trimetric complex. POPX2 dephosphorylates PAK1 on T422 and blocks the phenotypic effects of PAK1¹²⁶. POPX2 is also reported to be the protein phosphatase specific for CaMKII and dephosphorylates CaMKII on T286. Inhibitory effect of POPX2 on CaMKII results in a decreased phosphorylation of Vimentin, a CaMKII known substrate^{128, 129}. Knocking down the POPX2 using morpholino-based antisense oligonucleotide in Zebrafish leads to inhibition of CaMKI activity and morphological defects such as increased round-shaped embryos with smaller mid- and hind- brain when compared to normal embryos. There are also increased apoptotic cells in whole body¹³⁰. These observations suggested that POPX2 is required for the normal development of zebrafish.

POPX2 is also reported to be involved in SRF (serum response factor)-mediated transcriptional regulation via its N-terminal non-catalytic domain. mDia1 (Diaphanous-related formin-1), the downstream effector of RhoA, is found to be associated with POPX2. The binding affinity between mDia and POPX2 could be enhanced in the presence of active RhoA. Binding of POPX2 to mDia1 appears to inhibit RhoA-mDia-SRF mediated transcription and MAL nuclear translocation independent of its phosphatases activity. Accordingly, POPX2 is not only linked to Cdc42/Rac1 signaling via PAK1, but also associated with RhoA signaling via mDia. POPX2 is suggested to be significant in linking various Rho signaling pathways that play essential roles in multiple biological activities, such as actin cytoskeleton and microtubule dynamics, cell migration, transcription regulation, as well as cell cycle progression¹³¹.

The role of POPX2 in cancer cell motility and invasion has also been reported recently. Over-expression of POPX2 in NIH3T3 enhances cell motility in wound healing assays¹³². Consistently, silencing of POPX2 in MDA-MB-231 cells results in lower cell motility and invasiveness, suggesting positive correlation between POPX2 levels and cell motility. POPX2 knockdown also reduces stress fibers and focal adhesions in MCF-7 cells. Reduced RhoGDI1 and β 1-integrin levels, as well as reduced cell attachment to the extracellular matrix. These observations imply that POPX2 could affect cancer cell motility and invasiveness through modulating actin cytoskeleton and cell attachment. Furthermore,

elevated POPX2 expression levels in various breast cancer cell types provide insights into its potential effect on breast cancer cellular signalings¹.

1.8 RhoGDIs

RhoGDIs, Rho guanine nucleotide dissociation inhibitors, act as one of the crucial regulators of Rho family proteins. So far, three isoforms have been identified. RhoGDI1 (ARHGDIA), also called RhoGDI α , is ubiquitously expressed and found mainly in the cytosol¹³³. RhoGDI2 (ARHGDIB) is also called RhoGDI β , D4-GDI, or Ly-GDI¹³⁴. RhoGDI2 is predominantly expressed in hematopoietic tissue, and is found in the cytosol. Overexpression of both RhoGDI1 and RhoGDI2 resulted in rounded cells with loss of stress fiber and focal adhesions, which resembled phenotypes of inactivation of Rho family proteins¹³⁵. RhoGDI3 (ARHGDIG), also called RhoGDI γ , is first identified as a non-cytosolic GDI. It is preferentially expressed in the brain¹³⁶.

1.8.1 Structures and functions

As shown in Figure 12, all the RhoGDI proteins contain two domains, the N-terminal flexible domain and the C-terminal folded domain¹³⁷⁻¹³⁹. The N-terminal domain adopts a helix-loop-helix conformation and C-terminal is an ordered β -sandwich immunoglobulin-like domain. The N-terminal flexible domain interacts with the nucleotide-binding loop of the Rho protein, preventing dissociation of the nucleotide. The C-terminal immunoglobulin-liked cavity of RhoGDI interacts with the isoprenyl group of Rho protein to extract the RhoGTPases from the membrane to cytosol. RhoGDI3 has an additional alpha helix extension¹³⁶. It has been reported that the N-terminal alpha helix extension of RhoGDI3 targets it to the Golgi apparatus¹⁴⁰.

RhoGDIs have three distinct functions. The first function is to inhibit the dissociation of GDP from the Rho proteins. The second function is to inhibit GTP hydrolysis. The last function is to extract the Rho proteins from the membrane to the cytosol. A recently published paper suggests a novel role of RhoGDI in the regulation of Rho proteins. It reports that RhoGDI regulates the stability of Rho and protects them from degradation¹⁴¹.

Figure 12. The domain architectures of RhoGDIs. RhoGDIs have three isoforms, RhoGDI1, RhoGDI2 and RhoGDI3. All three isoforms have an N-terminal flexible domain, 1-69 (RhoGDI1), 1-55(RhoGDI2), 25-79(RhoGDI3) and a C-terminal folded domain, 70-204(RhoGDI1), 56-201(RhoGDI2), 80-225(RhoGDI3). RhoGDI3 has an alpha helix extension from residue1 to 25 for targeting to Golgi.

Figure 13. Structure of the Cdc42/GDI Complex¹³⁹. Ribbon representation of GDI (blue) bound to Cdc42 (yellow). The switch I and switch II regions are highlighted in red and gold, respectively. The geranyl group of Cdc42 is highlighted in magenta. The detailed information for this structure is able to be obtained from Protein Data Bank (accession code: 1DOA).

Collectively, RhoGDIs' three main activities could be understood from the structure of RhoGDI-Cdc42 complex (Figure 13). The N-terminal domain of GDI interacts with the switch-I and switch-II regions of the Cdc42, thus inhibiting GDP dissociation and GTP hydrolysis. For the third function of RhoGDI, a "two step" model¹⁴² links GDI structure-function together. The first step is the fast process: the N-terminal domain rapidly binds to switch-I and switch-II regions of Cdc42, followed by a slower isomerization of the Cdc42-RhoGDI complex. The second step is the rate-limiting step: geranyl group of Cdc42 switching from the membrane into the hydrophobic binding pocket in the C-terminal of

RhoGDI¹³⁹. Another structural analysis on RhoGDI mutants suggests a modification to this “two step” model. In the fast step, formation of Rho/GDI complexes occurs not only at the N-terminal domain of RhoGDI but also at the C-terminal domain. The formation of N-terminal, helix-loop-helix^{GDI}/switch-I^{Rho} interface is able to detect the nucleotide state. On the other hand, the formation of C-terminal, β -sandwich^{GDI}/switch-II^{Rho} interface coordinates RhoGDI facing to membrane and to the Rho isoprenyl tail. When two conserved amino acids (D45^{RhoGDI1} and D185^{RhoGDI1}) are mutated in these two interaction interfaces, the inhibitory activity on Rho proteins is lost¹⁴³.

1.8.2 Complexes formation and regulation with RhoGTPases family proteins

RhoGDI1 is able to form complexes with RhoA, RhoB, RhoC, Cdc42, Rac1 and Rac2¹⁴⁰ both *in vivo* and *in vitro*. RhoGDI2 is preferentially associated with RhoA, Cdc42 and Rac1, Rac2 *in vitro* but not *in vivo*^{144, 145}. RhoGDI3 only binds to RhoG and RhoB¹³⁶. RhoGDI1 is able to interact with both the GDP and GTP bound forms and extracts both forms from membrane. Rho proteins targeting to differential cellular compartments are regulated by RhoGDI in the case of RhoA, Cdc42, Rac1 and Rac2 but not RhoB and TC10¹⁴⁶.

Although both RhoGDI1 and RhoGDI2 interact with Rho proteins, RhoGDI2 exhibits much lower affinities to Rho proteins. In addition, the functions of RhoGDI2 in inhibiting GDP dissociation and GTP hydrolysis are much weaker than RhoGDI1. Site-directed mutagenesis shows that the change of residue on RhoGDI2 (N174) to the corresponding residue on RhoGDI1 (I177) is able to accomplish up to 70% of RhoGDI activity on inhibition of GDP dissociation from Cdc42^{136, 147}. Structure analysis shows that a specific region (48-58) in the N-terminal domain of RhoGDI1 makes dramatic contribution to RhoGDI1 binding to Rac1¹⁴⁸. Another structural analysis shows that two leucine residues in this region (L55 and L56) stabilize the RhoGDI1-Rac1 complex¹⁴⁹. However, there is no report on the critical region which contributes to the RhoGDI-RhoA complexes formation so far.

1.8.3 Phosphorylation and its implication in function

RhoGDIs are phosphorylated by both serine/threonine kinases (PAK1, PAK2, PKC α) and tyrosine kinases (Src, Fer). PAK1 binds RhoGDI1 through its C-terminal domain, which is different from the one binding to the Rho proteins. In addition, PAK1 phosphorylates RhoGDI both *in vivo* and *in vitro* at S101 and S174. PAK1 phosphorylation results in dissociation of Rac1 from the Rac1-RhoGDI complex, but not RhoA and Cdc42. Because PAK1 is one of the downstream effectors of Rac1, PAK1 phosphorylation-mediated Rac1 release and activation may work as a positive loop. Active Rac1 activates PAK1, which in turn phosphorylates RhoGDI1. Phosphorylated RhoGDI1 leads to more Rac1 released from the RhoGDI complex and further activation of Rac1 and its downstream effectors¹⁵⁰. PAK2 phosphorylates RhoGDI1 at S34 and S101 which results in the release of Cdc42 and Rac1 from the RhoGDI complex in PC12 cells¹⁵¹. PKC α phosphorylates RhoGDI1 on S34 and facilitates the release of RhoA specifically¹⁵². It has been shown that Src kinase binds and phosphorylates RhoGDI1 and RhoGDI2 both *in vivo* and *in vitro* at Y156 and Y153¹⁵³. However, Src phosphorylation on RhoGDI2 releases Rac1 from RhoGDI2 and phosphorylated RhoGDI2 is retained at membrane. In addition, Src phosphorylation of RhoGDI2 enhances its metastasis suppressor role in bladder cancer cells¹⁵⁴. Another tyrosine kinase Fer is also able to bind and phosphorylates RhoGDI1 and prevents Rac1 binding to RhoGDI2¹⁵⁵. Not much is known about the phosphatases which catalyzed the dephosphorylation of RhoGDI. It is of interest to study the coordination of phosphorylation events regulated by kinases and phosphatases in the regulation of RhoGDIs.

1.8.4 Roles of RhoGDI in cancer and actin cytoskeleton regulation

RhoGDI2 was first discovered as a metastasis suppressor in bladder cancer cell¹⁵⁶. However, RhoGDI2 acts as metastasis promoter in breast cancer cell. Both the protein and transcript levels are enhanced in the invasive MDA-MB-231 cell. Silencing RhoGDI2 results in reduced cell motility and invasiveness^{157, 158}. Proteomic analysis shows that RhoGDI1 level is unregulated in colon cancer cells, and is proposed as a colon cancer promoter¹⁵⁹.

All the three isoforms of RhoGDI have been reported to disrupt the actin cytoskeleton, leading to cells rounding with loss of stress fiber and focal adhesions. It may be due to the

inactivation of Rho proteins, but the exact mechanism still remains to be determined. The RhoGDI phenotype is prevented by co-microinjection of RhoGDI with GTP-bound form of RhoA, but not with the GTP-bound form of Cdc42 or Rac1. This suggests that RhoGDI regulates the actin cytoskeleton through the RhoA pathway¹⁶⁰. When RhoGDI3 is overexpressed in baby hamster kidney cells, the cells become rounded up or spindle shaped with loss of stress fiber. Co-staining with Hoechst 33342 for nuclear DNA shows fragmentation of the nuclei in some of the rounded cells, suggesting a possible link between RhoGDI3 expression and apoptosis¹⁶¹. However, in, MDA-MB-231 breast cancer cells, overexpression of RhoGDI1 has no effect on cell morphology¹⁶² suggesting that effects of RhoGDIs on actin cytoskeleton might be cell types dependent.

1.9 Objectives of this study

Earlier work from our laboratory has suggested that POPX2 can regulate cell motility. POPX2 overexpression leads to increased cell motility¹³² and silencing of POPX2 results in lower motility and invasiveness in breast cancer cells¹. It is not likely that the regulation of cell motility is moderated via regulating PAK and CaMK. Hence, we embarked on proteomic and phospho-proteomic approaches to decipher the signaling network regulated by POPX2 with particular hope to find possible substrates of POPX2 and/or signaling pathways affected by POPX2 dephosphorylation of its targets.

In addition, we have also reported that POPX2 can regulate serum response factor mediated transcription, which is downstream of active RhoA and mDia¹³¹. A study of the transcriptome of POPX2 knockdown cells compared with control cells will be helpful to determine what other genes are regulated by POPX2 at transcript levels. Since we also found that RhoGDI levels are downregulated in POPX2 knockdown cells¹, a candidate approach to determine the functional relationship between POPX2 and RhoGDI will help to define the roles of POPX2 in the regulation of cell motility.

The specific aims of this project are summarized below:

- (1) To explore the role of POPX2 in cancer cell migration and in transcription regulation using integrative transcriptome and proteome strategy.
- (2) To identify targets of this phosphatase using phosphoproteomics, and investigate phosphorylation based signaling networks regulated by POPX2.
- (3) To uncover the role of POPX2 in angiogenesis and tumor growth through secretome study.
- (4) To elucidate the mechanism of POPX2 in regulating breast cancer cell migration and invasion by functional study of candidate genes.

Chapter 2 Materials and methods

2.1 Materials

2.1.1 Chemicals

ABB (Ammonium Bicarbonate)	Sigma
Acrylamide	Bio-Rad
Acetic acid	Merck
Agarose	Bio-Rad
Ampicillin	USB
APS (Ammonium persulfate)	Bio-Rad
Bromphenol blue	Sigma
BSA (bovine serum albumin)	Sigma
Coomassie G250	Serva
Collagen	BD
Complete EDTA free Protease Inhibitor Cocktail Tablet	Roche
Deoxynucleotides (dG/A/T/CTP)	Roche
DTT (Dithiothreitol)	Sigma
DMSO (Dimethyl Sulphoxide)	Sigma
Ethidium bromide	Bio-Rad
Ethanol	Merck
Fibronectin	Sigma
Glycerol	Sigma
Glycine	1 st BASE
HEPES	Sigma

IAA (Iodoacetic acid)	Sigma
IPTG (Isopropyl β -D-1-thiogalactopyranoside)	Gibco
Isopropanol	Merck
1KB DNA ladder	NEB
Methanol	Merck
β -Mercaptoethanol	Merck
Paraformaldehyde	Merck
Poly-L-lysine	Sigma
PhosStop	Roche
Poly-L-Lysine	Sigma
Potassium Chloride	Sigma
Ponceau S	Sigma
Protease Inhibitor Cocktail	Sigma
Protein assay	Bio-Rad
RNase A	Qiagen
SDS (Sodium dodecyl sulfate)	USB
Sodium Chloride	BDH
Sodium Hydrogen Carbonate	Sigma
Sodium Thiosulphate	Merck
TEMED (N,N,N',N'-Tetramethylethylenediamine)	Bio-Rad
Triton X-100	Bio-Rad
Tris Base	Promega
Tween 20	Bio-Rad
Urea	USB

2.1.2 Mammalian cell lines and tissue culture materials

COS-7	ATCC-CRL-1651
HeLa	ATCC-CCL-2
MDA-MB-231	HTB-26
MDA-MB-231 POPX2 Ctrl	
MDA-MB-231 POPX2 KD1	
MDA-MB-231 POPX2 KD2	
MCF-7	HTB-22
MCF-7 POPX2 Ctrl	
MCF-7 POPX2 KD1	
MCF-7 POPX2 KD2	
PC-3	CRL-1435
DU145	HTB-81
LNCaP	CRL-1740
SW620	CCL-227
SW480	CCL-228
HCT116	CCL-247
HCT15	CCL-225
Caco-2	HTB-37
COLO 320HSR	CCL-220.1
DLD1	CCL-221
DMEM (Dulbecco's Modified Eagle's Medium) 1000	Sigma
DMEM (Dulbecco's Modified Eagle's Medium) 4500	Sigma

Fetal Bovine Serum (FBS)	PAA
L-15 Media	Invitrogen
L-Glutamine	Sigma
MEM (Minimum Essential Medium Eagle Modified)	Sigma
Penicillin/Streptomycin	Invitrogen
RPMI (Roswell Park Memorial Institute medium) 1640	Sigma
Sodium bicarbonate	Merck
Trypsin	Sigma
Tissue culture dish	Corning/Nunc

2.1.3 SILAC-culture materials

RPMI 1640 lacking arginine, lysine, methionine	Biowest
$^{13}\text{C}_6$ -Arginine	Cambridge Isotope Laboratories
$^{13}\text{C}_6$ -Lysine	Cambridge Isotope Laboratories
$^{12}\text{C}_6$ -Arginine	Sigma
$^{12}\text{C}_6$ -Lysine	Sigma
$^{12}\text{C}_6$ -Methionine	Sigma
Dialyzed fetal bovine serum	Invitrogen

2.1.4 Bacterial strains and culture materials

DH5 α	Invitrogen
Agar	BD
Sodium Chloride	BDH

Tryptone	BD
Yeast extract	BD

2.1.5 Cloning enzymes

Alkaline Phosphatase (CIP)	NEB
Expand High Fidelity Polymerase	Roche
Expand Long Template Polymerase	Roche
Ex Tag TM Polymerase	Takara
Restriction endonucleases	NEB
T4 DNA ligase	NEB

2.1.6 Commercial Kits

QIAquick [®] Gel Extraction Kit	Qiagen
QIAquick [®] PCR Purification Kit	Qiagen
QIAquick [®] Spin Maxiprep Kit	Qiagen
QIAquick [®] Spin Miniprep Kit	Qiagen
QuikChange [®] Site-Directed Mutagenesis Kit	Stratagene
ECL Western blotting detection reagents and analysis system	Amersham
RNeasy Mini Kit	Qiagen
SuperScript [®] VILO cDNA Synthesis Kit	Invitrogen

2.1.7 Mammalian cells transfection reagents

LipofectAMINE [®] 2000	Invitrogen
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2.1.8 Other materials

Anti-FLAG M2 agarose beads	Sigma
Nitrocellulose membrane	Bio-Rad
Clear Blue X-Ray film	Thermo Scientific
Sterile filter 0.22 µm, cellulose acetate	Sartorius
Sterile filter 0.45 µm, cellulose acetate	Sartorius

2.1.9 Buffers

DNA loading buffer	10ml TE buffer 10ml Glycerol
DMEM 1000 cell culture medium	10g DMEM1000 3.7g NaHCO ₃ Top up to 1000ml with water Adjust pH to 7.2
DMEM 4500 cell culture medium	13.44g DMEM4500 3.7g NaHCO ₃ Top up to 1000ml with water Adjust pH to 7.2
MEM cell culture medium	9.4g MEM 2.2g NaHCO ₃ Top up to 1000ml with water

	Adjust pH to 7.2
Mammalian cell lysis buffer	25mM HEPES, pH7.5 0.3M NaCl 1mM MgCl ₂ 1mM EGTA 1mM sodium vanadate 20mM β-glycerol phosphate 10mM NaF 5% Glycerol 0.5% Triton X-100 50× Protease inhibitor cocktail tablet 10× Phosphatase inhibitor cocktail tablet 200× DTT
10× Nitrocellulose transfer buffer	30.3 g Tris base 144g Glycine 1000ml water
1× Nitrocellulose transfer buffer	100ml 10× Nitrocellulose transfer buffer 100ml Methanol 800ml water
10× Phosphate Buffered Saline (PBS)	13.7mM NaCl

	2.7mM KCl
	80.9mM Na ₂ HPO ₄
	1.5mM KH ₂ PO ₄
	Top up to 1000ml with water
	Adjust pH to 7.4
Phosphopeptides enrichment buffer A	72% acetonitrile
	0.5% formic acid
Phosphopeptides enrichment buffer B	10% acetonitrile
	1.0% formic acid
Peptides fractionation Buffer A	85% acetonitrile
	1.0% formic acid
	10mM ammonium acetate
Peptides fractionation Buffer B	30% acetonitrile
	0.1% formic acid
Protein lysis buffer	20mM HEPES
	8M Urea
	50× Protease inhibitor cocktail tablet
	10× Phosphatase inhibitor cocktail tablet

RPMI 1640 cell culture medium	10.4g RPMI1640 2g NaHCO ₃ Top up to 1000ml with water Adjust pH to 7.2
SILAC culture medium (Heavy)	450ml RPMI 1640 lacking arginine, lysine, methionine 50mg/L ¹³ C ₆ -Arginine 50mg/L ¹³ C ₆ -Lysine 15mg/L ¹² C ₆ -Methionine 10% FBS 1% Penicillin/Streptomycin
SILAC culture medium (Light)	450ml RPMI 1640 lacking arginine, lysine, methionine 50mg/L ¹² C ₆ -Arginine 50mg/L ¹² C ₆ -Lysine 15mg/L ¹² C ₆ -Methionine 10% FBS 1% Penicillin/Streptomycin
6× SDS sample buffer	7ml 4× Tris-Cl/SDS pH6.8 3ml Glycerol 1g SDS 0.93g DTT

	1.5mg bromophenol blue
10× SDS-PAGE electrophoresis buffer	30.2g Tris base 144g Glycine 10g SDS Top up to 1000ml with water
1× SDS-PAGE electrophoresis buffer	100ml 10× SDS-PAGE electrophoresis buffer 900ml water
10× TBS buffer	24.2g Tris base 80g NaCl Top up to 1000ml with water Adjust pH to 7.6
1× TBST buffer	100ml 10×TBS 900ml water 500μl Triton
Trypsin stock solution	0.2% (w/v) trypsin 1mM EDTA

2.1.10 Constructs

pXJ-GFP-POPX2	POPX2 full length
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pXJ-GFP-flag-POPX2	POPX2 full length
pXJ-GFP-POPX2R	POPX2 resistance to X2-siRNA
pEGFP-RhoGDI1 ^{FL}	RhoGDI1 full length
pXJ-GFP-flag-RhoGDI1 ^{FL}	RhoGDI1 full length
pXJ-GFP-flag-RhoGDI1 ^{NT}	RhoGDI1 N-terminal domain (1-69)
pXJ-GFP-flag-RhoGDI1 ^{CT}	RhoGDI1 C-terminal domain (70-204)
pXJ-GFP-flag-RhoGDI1 ^{D45A}	RhoGDI1 full length mutant
pXJ-GFP-flag-RhoGDI1 ^{D185A}	RhoGDI1 full length mutant
pXJ-GFP-flag-RhoGDI1 ^{I177N}	RhoGDI1 full length mutant
pXJ-GFP-flag-RhoGDI1 ^{L55S, L56S}	RhoGDI1 full length mutant
pEGFP-RhoGDI2 ^{FL}	RhoGDI2 full length
pXJ-GFP-flag-RhoGDI2 ^{FL}	RhoGDI2 full length
pXJ-GFP-flag-RhoGDI2 ^{NT}	RhoGDI2 N-terminal domain (1-55)
pXJ-GFP-flag-RhoGDI2 ^{CT}	RhoGDI2 C-terminal domain (56-201)
pXJ-GFP-flag-RhoGDI2 ^{N174I}	RhoGDI2 full length mutant
pXJ-GFP-flag-RhoGDI2 ^{L52S, L53S}	RhoGDI2 full length mutant
pXJ-HA-RhoA ^{WT}	RhoA full length
pXJ-HA-RhoA ^{V14}	RhoA full length dominant active mutant
pXJ-HA-RhoA ^{N19}	RhoA full length dominant negative mutant
pXJ-HA-RhoA ^{L63}	RhoA full length dominant active mutant
pXJ-HA-Cdc42 ^{WT}	Cdc42 full length
pXJ-HA-Cdc42 ^{V12}	Cdc42 full length dominant active mutant
pXJ-HA-Cdc42 ^{N17}	Cdc42 full length dominant negative mutant
pXJ-HA-Cdc42 ^{L61}	Cdc42 full length dominant active mutant

pXJ-HA-Rac1 ^{WT}	Rac1 full length
pXJ-HA-Rac1 ^{V12}	Rac1 full length dominant active mutant
pXJ-HA-Rac1 ^{N17}	Rac1 full length dominant negative mutant
pXJ-HA-Rac1 ^{L61}	Rac1 full length dominant active mutant

2.1.11 siRNA

siRNA against POPX2 Sense 5' CCU ACU UUG CUG UGU UUGA 3'
 Antisense 5' UCAA ACA CAG CAA AGU AGG 3'

siRNA against RhoGDI1

siRNA1 Sense 5' CAG AAG AGC AUC CAG GAG AUC CAGG 3'
 Antisense 5' CCUG GAU CUC CUG GAU GCU CUU CUG 3'

siRNA 2 Sense 5' CAG GAA AGG CGU CAA GAU UGA CAAG 3'
 Antisense 5' CUUG UCA AUC UUG ACG CCU UUC CUG 3'

siRNA against RhoGDI2

siRNA1 Sense 5' CAA AGA UGA UGA GAG UCU AAU UAAG 3'
 Antisense 5' CUUA AUU AGA CUC UCA UCA UCU UUG 3'

siRNA 2 Sense 5' GAA AGU GGA UAA AGC AAC AUU UAUG 3'
 Antisense 5' CAUA AAU GUU GCU UUA UCC ACU UUC 3'

2.1.12 Primers

pEGFP-RhoGDI1^{FL} For 5' GCGAATTCTATGGCTGAGCAGGAG 3'
 Rev 5'ACGGATCCTCAGTCCTTCCAGTCC 3'

pXJ-GFP-flag-RhoGDI1 ^{FL}	For 5' TACTCGAGATGGCTGAGCAGGAG 3' Rev 5' CCTAGATCTTCAGTCCTTCCAGTCCT 3'
pXJ-GFP-flag-RhoGDI1 ^{NT}	For 5' TACTCGAGATGGCTGAGCAGGAG 3' Rev 5' CCTAGATCTTCAGTTGGGGACGTTGGG 3'
pXJ-GFP-flag-RhoGDI1 ^{CT}	For 5' TACTCGAGATGGTCGTGGTGACTGGC 3' Rev 5' CCTAGATCTTCAGTCCTTCCAGTCCT 3'
pXJ-GFP-flag-RhoGDI1 ^{D45A}	For 5' CTGGACAAGGACGCCGAGAGCCTGCGA 3' Rev 5' TCGCAGGCTCTCGGCGTCCTTGTCCAG 3'
pXJ-GFP-flag-RhoGDI1 ^{D185A}	For 5' CTTCACAGACGACGCCAAGAC CGACCACC 3' Rev 5' GGTGGTCGGTCTTGGCGTCGT CTGTGAAG 3'
pXJ-GFP-flag-RhoGDI1 ^{I177N}	For 5' CGGGGCAGCTACAGCGACAAGTCC CGCTTCAC 3' Rev 5' GTGAAGCGGGACTTGTCGCTGTAG CTGCCCCG 3'
pXJ-GFP-flag-RhoGDI1 ^{L55S, L56S}	For 5' GAAAGTACAAGGAGGCCTCGTCG GGCCGCGTGGCCGTTT 3' Rev 5' AAACGGCCACGCGGCCCGACGAG GCCTCCTTGTACTTTC 3'

pEGFP-RhoGDI2 ^{FL}	For 5' CGGAATTCTATGACTGAAAAAGCCCC 3' Rev 5' ACGGATCCTCATTCTGTCCACTCCTT 3'
pXJ-GFP-flag-RhoGDI2 ^{FL}	For 5' ACAAAGCTTATGACTGAAAAAGCCCCA 3' Rev 5' TTCTCGAGTCATTCTGTCCACTCCTTCT 3'
pXJ-GFP-flag-RhoGDI2 ^{NT}	For 5' ACAAAGCTTATGACTGAAAAAGCCCCA 3' Rev 5' TTCTCGAGTCAATCTCCCAGCAGCGTT 3'
pXJ-GFP-flag-RhoGDI2 ^{CT}	For 5' ACAAAGCTTATGGGTCCTGTGGTGACA 3' Rev 5' TTCTCGAGTCATTCTGTCCACTCCTTCT 3'
pXJ-GFP-flag-RhoGDI2 ^{N174I}	For 5' CGAGGCACGTACCACATCAAGTCCT TCTTCACC 3' Rev 5' GGTGAAGAAGGACTTGATGTGGTAC GTGCCTCG 3'
pXJ-GFP-flag-RhoGDI2 ^{L52S, L53S}	For 5' AGTCTAATTAAGTACAAGAAAACGTCGT CGGGAGATGGTCCTGTGGTGACAG 3' Rev 5' CTGTCACCACAGGACCATCTCCCGACGA CGTTTTCTTGTACTTAATTAGACT 3'

2.1.13 Antibodies

Primary antibodies

Actin	mouse	Sigma
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Alexa-Fluor 546 phalloidin		Invitrogen
β -catenin	rabbit	Sigma
α -tubulin	mouse	Sigma
α -acetylated-tubulin	mouse	Sigma
β -tubulin	Mouse	Sigma
Cdc42	mouse	Santa Cruz
CD9	rabbit	Santa Cruz
CD81	mouse	Santa Cruz
E-cadherin	mouse	BD transduction
FAK	mouse	BD transduction
Flag	mouse	Sigma
GFP	rabbit	Invitrogen
GSK3 α	rabbit	Cell signaling
p-GSK3 α	rabbit	Cell signaling
GSK3 β	rabbit	Cell signaling
p-GSK3 β	rabbit	Cell signaling
HA	rabbit	Invitrogen
Integrin β 1	goat	Santa Cruz
MAPK1/3	rabbit	Cell signaling
p-MAPK1/3	rabbit	Cell signaling
N-cadherin	mouse	BD transduction
Paxillin	mouse	Upstate
POPX2	rabbit	Self-raised
Rac1	mouse	Upstate

Stathmin	rabbit	Cell signaling
p-stathmin	rabbit	Cell signaling
Vimentin	mouse	BD transduction
Vinculin	mouse	Chemicon

Secondary antibodies

Mouse IgG, HRP conjugated	goat	Dako
Rabbit IgG, HRP conjugated	goat	Dako
Mouse Alexa Fluor 488	goat	Invitrogen
Mouse Alexa Fluor 546	goat	Invitrogen
Rabbit Alexa Fluor 488	goat	Invitrogen
Rabbit Alexa Fluor 546	goat	Invitrogen

2.2 Methods

2.2.1 Cell culture

Cells were grown in a humidified 95% air, 5% CO₂ incubator at 37°C. HeLa cell were cultured in Minimum Essential Medium Eagle (MEM) supplemented with 0.292g/L L-glutamine, 10% FBS. The colon cancer cell line Caco-2 were also maintained in MEM supplemented with 20% FBS. MDA-MB-231 cells were cultured in low glucose Dulbecco's modified Eagle medium (DMEM) supplemented with 10%FBS. COS-7, MCF-7, SW620, SW480, HCT116 were maintained in high glucose Dulbecco's modified Eagle medium (DMEM) supplemented with 10%FBS. The prostate cancer cell lines PC-3, DU145, LNCaP and some of these colon cancer cell lines, HCT-15, DLD-1, COLO 320HSR were maintained in Roswell Park Memorial Institute medium (RPMI) supplemented with 10%FBS.

2.2.2 SILAC (stable isotope labeling by amino acids in cell culture) and cell culture

A custom RPMI1640 medium lacking arginine, lysine and methionine was purchased from Biowest (Maimi, FL, USA). $^{13}\text{C}_6$ -arginine (heavy) and $^{13}\text{C}_6$ -lysine (heavy) were purchased from Cambridge Isotopes Laboratories, Inc. (Andover, MA, USA). $^{12}\text{C}_6$ -arginine (light), $^{12}\text{C}_6$ -lysine (light) and $^{12}\text{C}_6$ -methionine (light) were purchased from Sigma-Aldrich. Dialyzed fetal bovine serum was purchased from Invitrogen. The control and POPX2 knockdown (X2-shRNA1/2) MDA-MB-231 stable cells were described previously¹. The X2-shRNA1 cells were cultured in RPMI1640 medium containing heavy arginine, heavy lysine and light methionine, along with 10% (v/v) dialyzed fetal bovine serum and 100U penicillin, 100 μg streptomycin per ml (Invitrogen, CA, USA). The control cells were maintained in RPMI1640 medium containing light arginine, light lysine and light methionine, along with 10% (v/v) dialyzed fetal bovine serum and 100U penicillin, 100 μg streptomycin per ml (Invitrogen, CA, USA). All cells were grown in 37°C humidified incubator supplemented with 5% CO_2 . The cells used in the experiments were grown up to seven passages to ensure full incorporation of heavy and light amino acids.

2.2.3 Protein extraction, digestion and fractionation

Cells harvested from heavy and light media were washed with 1 $\times$ PBS five times and lysed in protein lysis buffer containing 8M urea (Sigma-Aldrich), 20mM HEPES (Merck), complete protease inhibitor cocktail (Roche) and phosphostop phosphatase inhibitor cocktail (Roche). Protein concentration was determined by Bicinchoninic Acid (BCA) protein assay. Equal amounts of protein (15 mg each) from heavy and light samples were mixed at a 1:1 ratio and stored at -80°C for the following experiments. Proteins were reduced with 10mM dithiothreitol at 33°C for 1hr, followed by alkylation with 55mM iodoacetamide at room temperature for 45min. After diluting 8 times with 20mM HEPES (Merck), mixed extracts were digested in-solution by trypsin (Sigma-Aldrich) at 1:100 (m/m). Peptide samples were adjusted to pH2 using 10% formic acid and desalted using SEP-PAK C18 cartridges (Waters, Milford, MA), and vacuum dried.

The resulting peptides were fractionated using ERLIC¹¹⁴. Briefly, the peptides were dissolved in buffer A (72% acetonitrile + 0.5% formic acid) and injected into a PolyLC PolyWAX LP

column ($4.6 \times 200\text{mm}$, $5\mu\text{m}$ particle size, $300\text{\AA}$ pore size) mounted on a Shimadzu Prominence UFLC unit (Shimadzu Corporation, Kyoto, Japan). The 40 min gradient was generated using a combination of buffer A (72% acetonitrile + 0.5% formic acid) and buffer B (10% acetonitrile + 1.0% formic acid). The gradient was composed of 5 min 100% A, followed by 30 min gradient from 0% to 100% B, and ended with 5 min 100% B. The first five collections were combined into one tube. The subsequent collections, containing phospho-peptides, were combined into 15 fractions for phospho-peptide analysis. The combined first five fractions were further fractionated into 20 fractions for proteome analysis. For the proteome fractionation, a different 40 min gradient was generated using a combination of buffer A (85% acetonitrile + 10mM ammonium acetate + 1% formic acid) and buffer B (30% acetonitrile + 0.1% formic acid). The gradient was composed of 5 min 100% A, followed by 30 min gradient from 0% to 100% B, and ended with 5 min 100% B. All 35 fractions were desalted using SEP-PAK C18 cartridges (Waters, Milford, MA), vacuum dried and stored at $-80\text{ }^{\circ}\text{C}$ prior to LC-MS/MS analysis.

2.2.4 Phosphopeptides enrichment

ERLIC

The phosphopeptide was firstly enriched using ERLIC, followed by IMAC and TiO_2 . Briefly, the peptide was injected to a PolyLC PolyWAX LP column ($4.6 \times 200\text{mm}$, $5\mu\text{m}$ particle size, $300\text{\AA}$ pore size) mounted on a Shimadzu Prominence UFLC unit (Shimadzu Corporation). The 40min gradient was the combination of a BufferA (72% acetonitrile + 0.5% formic acid) and a bufferB (10% acetonitrile + 1.0% formic acid). The first five fractions were combined and vacuum-dried for IMAC. The following collections were combined into 22 fractions and vacuum dried for LC-MS/MS analysis.

IMAC

Briefly, peptide sample was resuspended in IMAC loading buffer (40% acetonitrile + 25mM formic acid) and subsequently added into IMAC slurry (Pho-Select, Sigma), shaking at room temperature for 1 hr. After centrifuge, supernatant was collected and vacuum dried for TiO_2 . Phosphopeptides were washed with IMAC loading buffer for three times and eluted with

elution buffer (50mM K_2HPO_4) three times. All the eluted samples were pH-adjusted to 2 using 10% formic acid. Phosphopeptide was desalted using SEP-PAK C18 cartridges (Waters) and vacuum dried for LC-MS/MS analysis.

TiO₂

For TiO₂ enrichment, firstly, TiO₂ beads (GL Science) were dissolved in bufferA (50% acetonitrile + 0.1% trifluoroacetic acid). Peptide sample was dissolved in TiO₂ loading buffer (50% acetonitrile + 0.1% trifluoroacetic acid + 35mg/ml 2,5-dihydroxy- benzoic acid). Equal amount of beads were added into each sample, shaking at room temperature for 1hr. After centrifuge, supernatant was collected and vacuum dried, store at -80°C. Phosphopeptides were washed with TiO₂ loading buffer for three times and eluted with elution buffer (Concentrated NH_4OH) three times. All the eluted samples were pH-adjusted to 2 using 10% formic acid. Phosphopeptide was desalted using SEP-PAK C18 cartridges (Waters) and vacuum dried for LC-MS/MS analysis.

2.2.5 Conditioned medium collection and concentration

The X2-shRNA1 cells were cultured in RPMI1640 medium containing light arginine, light lysine and light methionine, along with 10% (v/v) dialyzed fetal bovine serum and 100U penicillin, 100µg streptomycin per ml (Invitrogen, CA, USA). The control cells were maintained in RPMI1640 medium containing heavy arginine, heavy lysine and light methionine, along with 10% (v/v) dialyzed fetal bovine serum and 100U penicillin, 100µg streptomycin per ml (Invitrogen, CA, USA). All cells were grown in 37°C humidified incubator supplemented with 5% CO₂. The cells used in the experiments were grown up to seven passages to ensure full incorporation of heavy and light amino acids. When cells were at 70% confluence, all cells were washed with 1×PBS for four times and serum free RPMI 1640 medium once, and then exposed to serum free medium for 6hrs to reduce the amount of serum proteins for subsequent work. The culture media was replaced with fresh serum free medium for another 24hrs, which was then used as the conditioned medium for secretome analysis.

The conditioned medium was collected (400ml each), adding protease inhibitor accordingly. The conditioned medium was then centrifuged at 1000 rpm for 10min at 4°C and filtered (0.22µm pore size) to remove cell debris, followed by concentration using 10 KDa 15ml ultra filter (Millipore) with 4000rpm at 4°C until the volume was reduced to 200-300µl. The concentrated conditioned medium was collected into a sterile 1.5ml tube and stored at -80°C until use.

2.2.6 SDS-PAGE

The conditioned medium was resuspended in 10% SDS, along with complete protease inhibitor cocktail (Roche) and phosphostop phosphatase inhibitor cocktail (Roche) and dithiothreitol (Sigma). Protein concentration was determined by Bicinchoninic Acid (BCA) protein assay. Equal amounts of protein (50µg each) from heavy and light samples were mixed at a 1:1 ratio and stored at -80°C for the following experiments. A total of 50µg of protein was run on 4-12% NuPAGE gradient gel (Invitrogen). After SDS-PAGE, the silver staining was adopted to visualize bands.

2.2.7 In-gel digestion

The whole lane from the SDS-PAGE gel was cut into 10 pieces for in-gel digestion. The gel pieces were washed with destaining solution several times until the color totally disappeared, followed by dehydration with 100% acetonitrile and vacuum dried. Subsequently, the proteins were reduced with 10mM dithiothreitol at 56°C for 1hr, followed by alkylation with 55mM iodoacetamide at room temperature for 45min. Gel pieces were dehydrated again with 100% acetonitrile and vacuum dried. In-gel digestion was carried out by trypsin (Sigma-Aldrich) at 1:100 (m/m), and at 37°C overnight. The supernatant was then transferred to a new 1.5ml tube and the peptides were extracted with extraction buffer (50% acetonitrile + 5% acetic acid). Gel pieces were vortexed for 10 seconds and sonicated for 30min. The supernatant was collected into the same tube. The extraction steps were repeated several times for complete peptides extraction. Peptides were vacuum dried and stored at -80°C until use.

2.2.8 LC-MS/MS analysis

Digested peptides were analyzed in an LTQ-FT Ultra mass spectrometer (Thermo Fisher, Waltham, Massachusetts, USA) coupled to a ProminenceTM HPLC unit (Shimadzu, Kyoto, Japan) as described previously¹⁶³. Briefly, peptide samples were injected from an auto-sampler (Shimadzu) and concentrated in a Zorbax peptide trap (Agilent Technologies, Santa Clara, California, USA), and subsequently resolved in a capillary column (200 μ m ID x 10 cm) packed with C18 AQ (5- μ m particle size, 100-Å pore size, Michrom BioResources, Auburn, California, USA). The samples were ionized in an ADVANCETM CaptiveSprayTM Source (Michrom BioResources) with an electrospray potential of 1.5 kV. The LTQ-FT Ultra was set to perform data acquisition in the positive ion mode. The 10 most intense ions above a 500 count threshold were selected for fragmentation. A full MS scan (350–1600 m/z range) was acquired in the FT-ICR cell at a resolution of 100 000 and a maximum ion accumulation time of 1000 ms. The automatic gain control target for FT was set at 1×10^6 , and precursor ion charge state screening was activated. The linear ion trap was used to collect peptides and to measure peptide fragments generated by CID. The default automatic gain control setting was used (full MS target at 3.0×10^4 , MSn at 1×10^4) in the linear ion trap. The 10 most intense ions above a 500-count threshold were selected for fragmentation in CID (MS2), which was performed concurrently with a maximum ion accumulation time of 200 ms and a dynamic range of 30 s. For CID, the activation Q was set at 0.25, isolation width (m/z) was 2.0, activation time was 30 ms, and normalized collision energy was 35%.

2.2.9 Proteomic data analysis

The MS raw files were converted to mzXML format and mgf format using Trans-Proteome Pipeline. Protein database search was performed by uploading mgf files to an in-house Mascot cluster server (version 2.2.07) (Matrix Science, Boston, MA) against a concatenated target and decoy version of manually annotated non-redundant UniProt Knowledgebase protein sequence database (40516 sequences, downloaded on 8 October 2010). The decoy sequences were generated by reversing the UniProt protein sequences. The search was limited to a maximum of 2 missed trypsin cleavages; #13C of 2; mass tolerance of 20 ppm for peptide precursors; and 0.8 Da mass tolerance for fragment ions. Fixed modification was carbamidomethyl at Cys residues, while variable modifications include oxidation at methionine residues, phosphorylation at serine, threonine and tyrosine, heavy lysine and

arginine. False discovery rates, calculated as 2 times the percentage of decoy matches in total matches, at both peptide and protein levels were below 1%. Extracted ion chromatograph (XIC) of the identified peptides was generated using in-house scripts. A tolerance of 0.05 Th was used for XIC generation. The ratio for SILAC pairs was calculated based on the area of XIC of each peptide. Ratios calculated from both replicate experiments were average in log scale, and error factors, where applicable, were calculated in log scale as well.

2.2.10 RNA extraction and DNA microarray data analysis

The microarray experiment was performed by Genotypic (Bangalore, India). Briefly, total RNA was extracted from two sets of MDA-MB-231 (control) and MDA-MB-231-POPX2 knockdown cells using Qiagen RNeasy kit. The RNA was labeled with Cy3 or Cy5 using Agilent Low Input RNA Amplification Kit. Agilent human whole genome 4x44k array slide was used for the hybridization. Array normalization was done using GeneSpring GX (Agilent) with the recommended Per Spot and Per Chip intensity dependent (lowness) normalization. Fold change analysis and gene ontology analysis were done using GeneSpring GX10 and Biointerpreter (Genotypic Technology) respectively.

2.2.11 Bioinformatics

Pathway analysis

The differentially expressed genes and proteins were submitted to DAVID (Database for Annotation, Visualization and Integrated Discovery)¹⁶⁴ separately using their Agilent ID (transcriptome) and official_gene_symbol (proteome). The functional annotations of each gene or protein were downloaded and their KEGG pathway annotations were manually distributed. The fisher exact P-value was adopted to examine the pathway enrichment. Genes or proteins involved in the same KEGG pathway were counted. For each pathway, the fisher exact P-value was calculated based on the following numbers:

- (1) Hit number: the number of genes (or proteins) in our query list involved in this pathway.
- (2) Hit total number: the number of genes (or proteins) in our query list mapped to KEGG pathway database.

(3) Background hit number: in human genes background, the number of genes annotated to this pathway.

(4) Background hit total number: in human genes background, the number of genes annotated to KEGG pathway database.

Motif analysis

Motif-X¹⁶⁵ was used for the kinase motif analysis. Parameters were set up as follows: the significance was set as 0.000001 with an occurrence of 5. The IPI human proteome database was used as background. The sequences of all phospho-peptides were submitted and centered on phospho-serine with at least ± 6 amino acids extended from the phosphorylation sites. No phospho-threonine or phospho-tyrosine motifs were extracted, which might be due to the low numbers of phospho-threonine or phospho-tyrosine peptides in our dataset.

STRING analysis

Protein network was analyzed using the STRING database¹⁶⁶. The differentially expressed phospho-proteins were submitted to the STRING database and a medium confidence setting (0.400) was used. Post-translational modification (pink) and binding (blue) functional link between two proteins were used to visualize protein relationships.

Secreted proteins prediction

Proteins secreted by the classical secretory pathway were predicted with SignalP¹¹⁷ with signal peptide probability of ≥ 0.9 set as the threshold. Proteins secreted by the non-classical secretory pathway were predicted using SecretomeP¹²¹ with a NN score ≥ 0.5 set as the cut-off. It is also possible shed membrane receptors were released to extracellular space. Thus this class of proteins was predicted with TMHMM¹²². Proteins associated with exosome were obtained from ExoCarta database¹²³. Proteins localized in extracellular space were also retrieved from Gene Ontology.

2.2.12 Cell transfection

HeLa, COS-7 cultured in 6-well dishes grown to approximately 80-90% confluence were transiently transfected with 1 µg of plasmids using Lipofectamine 2000 mixed in optiMEM. After 4-6hrs, cell was changed to fresh medium. Cell lysates were collected after 24 hrs.

2.2.13 siRNA transfection

The siRNA specific to human RhoGDI1 and RhoGDI2, including control siRNA were 25nt (Stealth), ordered from Invitrogen. RhoGDI1 siRNA sequences were 5'-3' CAG AAG AGC AUC CAG GAG AUC CAGG (designated GDI1siRNA1)¹⁶⁷, 5'-3' CAG GAA AGG CGU CAA GAU UGA CAAG (designated GDI1siRNA2)¹⁴¹. RhoGDI2 siRNA sequences were 5'-3' CAA AGA UGA UGA GAG UCU AAU UAAG (designated GDI2siRNA1), 5'-3' GAA AGU GGA UAA AGC AAC AUU UAUG (designated GDI2siRNA2)¹⁶⁸. Firstly, HeLa cells were cultured in 6-well dishes grown to approximately 60-70% confluence. Then transient transfections of RNA interference duplexes were carried out using Lipofectamine 2000 (Invitrogen). 48hrs post-transfection, cells were lysed.

2.2.14 Cloning

The full length human RhoGDI1 and RhoGDI2 (designated GDI^{FL}) were amplified using cDNA extracted from HeLa and MDA-MB-231, respectively. Then RhoGDI1 was cloned into XhoI and BglII restriction sites of pXJ-GFP-FLAG vector, and RhoGDI2 was cloned into HindIII and XhoI restriction sites of pXJ-GFP-FLAG vector. The N-terminal and C-terminal truncated RhoGDIs (designated GDI^{NT} and GDI^{CT}) were amplified using the RhoGDIs plasmids as templates and cloned into the same sites as full length RhoGDIs into the same vector. All the RhoA^{WT}, Cdc42^{WT}, Rac1^{WT}, the dominant active mutants (RhoA^{V14}, Cdc42^{V12}, Rac1^{V12} and RhoA^{L63}, Cdc42^{L61}, Rac1^{L61}) the dominant negative mutants (RhoA^{N19}, Cdc42^{N17}, Rac1^{N17}) were sub-cloned into BamHI and BglII sites of pXJ-HA vector.

2.2.15 Site-directed mutagenesis

The following mutants: RhoGDI1^{D45A}, RhoGDI1^{D185A}, RhoGDI1^{I177N}, RhoGDI1^{L55S,L56S}, RhoGDI2^{N174I}, RhoGDI2^{L52S,L53S} were generated using the QuikChange[®] Site-Directed Mutagenesis Kit from STRATAGENE. The primers were designed using the online tool provided by the STRATAGENE. The PCR reaction was carried out following the protocol of the mutagenesis kit.

2.2.16 Flag immunoprecipitation

Cell lysate were harvested from HeLa or COS-7. The same amount of cell lysate from different samples was incubated with Flag beads at 4°C, 2hrs. 2hrs later, beads were washed thrice with protein lysis buffer, 5min each time. Then the protein complexes bound to beads were analysed by SDS-PAGE.

2.2.17 Western blot

Fifty µg protein from each cells lines was subjected to reducing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 12% resolving gels for 10-70 KDa proteins of interest or 10% resolving gels for 20-100 KDa protein of interest. SDS-PAGE was performed at 120v at room temperature. Resolved proteins were subsequently transferred to nitrocellulose membranes (Bio-Rad, USA) at 80v for 2hrs at room temperature. Blot was blocked with 5% (w/v) skim milk in 0.05% (v/v) Tween-20/Tris-buffered saline for 1hr at room temperature, and blotted with respective antibodies, followed by secondary antibodies dissolved in 5% (w/v) skim milk. Blots were developed with either Novex[®] ECL Chemiluminescent Substrate Reagent Kit (Invitrogen) or Lumigen[®] (GE Healthcare).

2.2.18 Immunofluorescence

Similar number of cells were grown on coverslips in 6-well until 90% confluence before fixing with 5% paraformaldehyde for 20min at room temperature. Then the fixed cells were permeabilized with 0.1% TritonX-100/PBS for 10min at room temperature, followed by blocking in 4% BSA/PBS for at least 1hr at room temperature. Primary antibodies were 1:100

diluted in 1% TritonX-100/PBS and incubated with cells at 4°C overnight. Secondary antibodies were also diluted 1:100 in 1% TritonX-100/PBS and incubated with cells at room temperature for 1hr, followed by mounting with DAPI. Images were captured using an Axio Observer microscope (Carl Zeiss, Germany) and analyzed by Image J software.

2.2.19 Scratch wound assay

Similar numbers of cells (6.5×10^5) were seeded in six-well dishes. When cells were grown to a monolayer (24 hrs), five scratch wounds were generated per well using white pipette tips. The cells were washed with 1×PBS, incubated in fresh medium and allowed to migrate into the wound gap for 24 hrs. Phase contrast images were acquired at time-0 and time-24 and analyzed using Image J software. Three independent experiments were performed.

2.2.20 Cell adhesion assay

A 96-well culture dish was pre-coated with BSA (10mg/ml), fibronectin (10µg/ml) or collagen (100µg/ml). Similar number (2×10^4) of MDA-MB-231 cells stably expressing either Control siRNA or POPX2 siRNA1/2 were seeded in the coated 96-well dish and incubated for 30min for attachment. After cells attachment, the culture dish was rinsed with 1 × PBS to remove the non-adhering cells. Cells were then fixed with 5% Glutaraldehyde/PBS at room temperature for 20min before washing three times with distilled water, followed by staining with 0.1% (w/v) crystal violet at room temperature for 1hr. The crystal violet was then solubilised using 10% acetic acid and absorbance was read by spectrophotometry at 570nm.

2.2.21 *In vitro* tube formation assay

The *in vitro* tube formation assay was optimized based on reported methods^{124, 169}. The 24-well plate was coated with 100µl matrigel per well. Similar number of HUVECs (human umbilical vein endothelial cells) were plated on the matrigel-coated wells and incubated in EGM-2 (complete endothelial cell medium) for 40min for attachment. Then HUVECs were changed to respective conditioned medium for 4hrs to allow tube formation. The tubular

structures were captured after 4hrs using an Axio Observer microscope (Carl Zeiss, Germany) and analyzed by Image J software. Ten different fields per condition were captured and quantified. Measures were expressed as the total tube length and the number of branch points.

Chapter 3 Results

3.1 An Integrative Transcriptome and Proteome Study to Identify the Signaling Network Regulated by POPX2 Phosphatase

(All Figures in results part 3.1 were obtained from Zhang, S., 2013¹⁷⁰)

POPX2 is recently reported to be highly expressed in the more invasive MDA-MB-231 breast cancer cells. POPX2 knockdown resulted in lower cell migratory and invasive abilities. How POPX2 can regulate cell motility is still remained to be investigated. In this study, we embarked on a combined approach to investigate the genomic and proteomic changes in response to silencing POPX2 in MDA-MB-231 breast cancer cells to decipher signaling molecules or pathways which were related to POPX2 signaling. Our study suggests that POPX2 participates in the focal adhesions regulation and cytoskeletal dynamics. POPX2 is also implicated in the control of MAP kinase signaling. We found that silencing of POPX2 led to decreased MAPK1/3 and GSK3 α/β activities. The activation status of these kinases could impact on the arrangement of cytoskeleton and transcription, leading to changes in cell motility. The phosphoproteome associated with the silencing of POPX2 was also investigated. The level of phospho-stathmin was reduced in the breast cancer cells with POPX2 knockdown. Stathmin's activity in promoting microtubule depolymerization could be turned off through phosphorylation. Consistent with the lower levels of phospho-stathmin, the amount of acetylated-microtubules, which corresponds to stable microtubules, were reduced in the POPX2 knockdown cells. This could explain the decreased cell motility obtained in the POPX2 knockdown cells.

3.1.1 POPX2 silencing resulted in perturbed transcriptome

The cell model used for this study was a stable POPX2-knockdown-MDA-MB-231 breast cancer cell line, which was described previously¹. The endogenous POPX2 levels were successfully reduced in MDA-MB-231 cells as shown in Figure 14A.

A

B

Figure 14. Characterization of POPX2 silencing in breast cancer cells and experimental work-flow. (A) Total protein lysates were harvested from either control or POPX2-silencing cells. Similar amount of proteins were subjected for western blot analysis using anti-POPX2 antibody (Koh lab) and anti-actin antibody. (B) Experimental set-up for transcriptome and proteome analysis.

Firstly, changes of the transcriptome in response to POPX2 silencing were investigated using the Agilent DNA microarray system (Figure 14B). Total RNA extracted from the POPX2 knockdown and control cells were sent for Agilent DNA microarray analysis and two independent datasets were obtained. In total, the DNA microarray system reported 22604 transcripts with respective expression ratios (Supplementary Table 1_transcriptome, provided in the link: <http://pubs.acs.org/doi/suppl/10.1021/pr301113c>). As shown in Figure 15A, the transcriptome expression ratio was distributed and plotted. Typically, a two-fold cut-off was applied for the transcriptome analysis¹⁷¹. Therefore, among the 22604 total transcripts, 1163 genes were classified as up-regulated hits ($\text{Log}_2\text{Ratio} > 1$), and 639 genes were classified as down-regulated hits ($\text{Log}_2\text{Ratio} < -1$).

3.1.2 Proteomic changes induced by POPX2 silencing

Next, we went on to study the proteome alterations caused by reduced POPX2 expression in our current breast cancer cell model used in the transcriptome analysis described above. We have opted to use a SILAC-based quantitative proteomics system (Figure 14B). Equal amounts of proteins harvested from both control and POPX2 knockdown samples were combined. The total digested peptides were then fractionated using the ERLIC method and subsequently subjected for mass spectrometry-based identification and quantification. We have also conducted a technical repeat. Proteomic quantification reported 1775 proteins from technical replicate 1 and 1894 proteins from technical replicate 2 (Supplementary Tables 2-7_proteome, provided in the link: <http://pubs.acs.org/doi/suppl/10.1021/pr301113c>). In total, 2146 unique proteins were identified and quantified. We considered a difference of at least 1.8-fold change as significant. Accordingly, a total of 110 proteins were classified as up-regulated hits ($\text{Log}_2 \text{Ratio} > 0.85$), and 797 proteins were classified as down-regulated hits ($\text{Log}_2 \text{Ratio} < -0.84$) (Figure 15B).

Figure 15. Total identified genomic and proteomic expression ratios were distributed and plotted. Heavy vs Light ratio was calculated for every identified transcript or protein, and was subsequently converted to $\text{Log}_2 \text{ Ratio}$ and plotted for the transcriptome (A) and proteome (B).

3.1.3 Integrative pathway analysis uncovered the involvement of POPX2 in cancer-related signaling pathways

The goal of our study is to determine the role of POPX2 in cancer-related cellular processes. To this end, the genes and proteins changes were translated into pathways alterations. Integrative pathways analysis was carried out using the KEGG pathway database¹⁷² and the DAVID tool¹⁶⁴ (Table 3). “Focal adhesion” and “cell cycle” were found to be highly and commonly over-represented from our pathway analysis ($p < 0.05$). The coordination among actin cytoskeleton, microtubule dynamics as well as focal adhesion regulation is essential during cell migration¹⁷³, which is the primary POPX2-related phenotype that we are investigating in this thesis. Although cell cycle regulatory pathways are over-represented, the role of POPX2 in cell cycle progression requires further study and will not be covered in this thesis. Accordingly, only the focal adhesion signaling map was drawn from the KEGG database (Figure 16). Several focal adhesion-related signaling pathways (Figure 16) were also over-represented from our integrative pathway analysis (Table 3), including those regulating the cells cycle, actin cytoskeleton as well as MAPK signaling.

Table 3. KEGG pathway enrichment. Enriched pathways with *P*-value < 0.05 are shown in bold face.

KEGG pathway	<i>P</i> -value		Hit number	
	Transcriptome	Proteome	Transcriptome	Proteome
Pathway in cancer	0.0153	0.6218	31	24
Focal adhesion	0.0303	0.0329	20	23
Regulation of actin cytoskeleton	0.1328	0.0350	18	24
MAPK signaling pathway	0.4805	0.9656	18	14
Cell cycle	0.0264	0.0027	14	19
Axon guidance	0.0684	0.5414	13	10
Endocytosis	0.5370	0.2113	13	19
Tight junction	0.1381	0.0024	12	20
Viral myocarditis	0.0056	0.0003	11	15
Wnt signaling pathway	0.3749	0.8346	11	9
Spliceosome	0.2959	<0.0001	10	27
Leukocyte transendothelial	0.2083	0.4044	10	10
Pathogenic Escherichia coli	0.0076	<0.0001	9	14
p53 signaling pathway	0.0283	0.0754	9	9
Arginine and proline metabolism	0.0276	0.6507	8	4
Lysine degradation	0.0350	0.3256	7	5
Lysosome	<0.0001	0.6505	19	7
Systemic lupus erythematosus	0.0299	0.9613	15	6
Antigen processing and presentation	0.0003	0.0300	14	11
Type I diabetes mellitus	0.0003	0.6552	10	3
Graft-versus-host disease	0.0009	0.8362	9	2
Allograft rejection	0.0108	0.8147	7	2
Thyroid cancer	0.0090	0.3894	6	3
Ribosome	0.9380	<0.0001	3	25
Huntington's disease	0.9816	0.0433	6	21
Oocyte meiosis	0.5844	0.0003	7	20
Glycolysis / Gluconeogenesis	0.6193	<0.0001	4	19
Pyruvate metabolism	0.2816	<0.0001	4	13
RNA degradation	0.0826	0.0026	8	13
Progesterone-mediated oocyte	0.4770	0.0310	6	12
Gap junction	0.5109	0.0392	6	12
Citrate cycle (TCA cycle)	0.8726	<0.0001	1	10
Propanoate metabolism	0.3376	0.0023	3	8
Nucleotide excision repair	0.8043	0.0226	2	8
Fatty acid metabolism	0.3110	0.0491	4	7
Pentose phosphate pathway	0.5442	0.0177	2	6

Figure 16. Focal adhesion signaling pathway extracted from KEGG pathway database. Up-regulated hits in our datasets are colored in red while down-regulated hits are colored in green. ERK1/2 and GSK3 β are highlighted with orange rectangles. “+p” represents phosphorylation, “-p” represents dephosphorylation, straight arrow lines represent direct stimulatory modification, dashed arrow lines represent indirect stimulatory modification.

Affected signaling pathways could be highly associated with phenotypic variations. Therefore, we went on to explore if POPX2 was link to phenotypes relevant to actin cytoskeletal changes and focal adhesion dynamics in MDA-MB-231 cells. POPX2 knockdown in MDA-MB-231 cells resulted in a loss of stress fiber (Figure 17A). Lower POPX2 expression in breast cancer cells also reduced the amount of focal adhesions. In particular, there was absence of focal adhesion in the cell center (Figure 17B). In our earlier study, we also observed loss of stress fiber and focal adhesion when POPX2 was reduced in MCF, another breast cancer cells¹. Similar phenotypes were also observed in HeLa when POPX2 was silenced (Figure 18). Conversely, we found enhanced stress fibers and focal adhesions when POPX2 was overexpressed in HeLa (Figure 18). X2R is the siRNA resistant construct containing a silent mutation in the POPX2-siRNA-targeting sequence. Overexpression of X2R in the POPX2-knockdown cells is able to rescue the loss of stress fibres and loss of focal adhesions (Figure 18). We also included immunoblots to confirm POPX2 knockdown or enhanced expression levels in HeLa (Figure 18E). Re-arrangement and changes in the actin cytoskeleton and focal adhesions could contribute to the cell motility observed in POPX2 knockdown cells.

Figure 17. The amount of stress fibers and focal adhesions were reduced in the POPX2 knockdown MDA-MB-231 cells. Similar amount of Control and POPX2 knockdown MDA-MB-231 cells were seeded and fixed, followed by immunostaining with anti-phalloidin (A) and anti-vinculin (B). Stress fiber presence was indicated by arrows. Experiments were conducted in triplicates and representative images were shown here. Scale bar, 30 μ m. Red is phalloidin, Green is GFP, Blue is DAPI.

Figure 18. The amount of stress fibers and focal adhesions were reduced in the POPX2 knockdown HeLa cells. HeLa cells transfected with POPX2 siRNA , control siRNA, X2-siRNA+X2R (A) or GFP-Flag-POPX2 cDNA constructs (B) were immunostained with anti-phalloidin and anti-vinculin antibodies. Scale bar, 30 μ m. (C) The percentage of cells showing loss of stress fibers was quantified from three independent experiments, and at least 100 cells were counted per condition ($n \geq 100$). (D) The number of focal adhesions were counted from three independent experiments ($n=20$). The error bars represent standard deviation. The significance of difference was calculated by Students's t-test. * indicated P value<0.05; ** indicated P value<0.01; *** indicated P value<0.001; NS indicated P value>0.05, not significant. (E) Western analysis to determine the efficiency of knockdown and overexpression. Actin was used as a loading control.

3.1.4 Motif analysis implicated the involvement of POPX2 in MAPK1/3- and GSK3-related signaling

PAK and CaMKII are two well-characterized POPX2 substrates, which are effectively dephosphorylated and inactivated by POPX2 phosphatase^{126, 128}. It is possible that POPX2 could impact on phosphoproteome either directly through dephosphorylation of other candidate substrates or indirectly through regulating PAK and CaMKII kinases activities and their respective substrates. In our present study, we found 250 phospho-sites corresponding to 276 phospho-peptides from 133 phospho-proteins using mass spectrometry-based identification and quantification. Up to half of the 250 phospho-sites were novel sites compared to the PhosphoSite¹⁷⁴ and Phospho.ELM databases¹⁷⁵.

Since protein phosphorylation can regulate the functions of many proteins, it is of interest to investigate the phospho-proteins affected by POPX2 knockdown. Over-represented phosphorylation patterns extracted from our peptide sequences could shed light on POPX2 substrates or proteins whose phosphorylation status is affected indirectly by POPX2. Motif-X was employed to determine the over-represented phosphorylation motifs and the respective upstream kinases¹⁶⁵. The two enriched proline-directed kinase motifs found, along with their motif scores were shown in Figure 19A. The extracted kinase motifs were compared with PhosphoMotif Finder¹⁷⁶. PXpSP was identified to be GSK3, MAPK3 (ERK1), MAPK1 (ERK3) and CDK5 kinase substrate motif¹⁷⁷ and the pSP was identified to be MAPK3 (ERK1) and MAPK1 (ERK2) kinase substrate motif¹⁶⁵. Moreover, MAPK1 (ERK2) itself were also present in our proteomic data with the two identified phosphorylation sites: T185 and Y187. Phosphorylation of these two critical sites are reported to be significant for the MAPK1 activation¹⁷⁸. Reduced phosphorylation at T185/Y187 of MAPK1 (T202/Y204 of MAPK3) induced by POPX2 knockdown was validated by western blot analysis using the phospho-specific antibodies (Figure 19B and C). GSK3, acting as the switch for numerous signaling pathways, is composed of two isoforms: GSK3 α and GSK3 β ^{179, 180}. S21/S9 appeared to be the phospho-inhibitory sites for GSK3 α/β , respectively¹⁸¹. Enhanced phosphorylation levels at S21/S9 of GSK3 α/β in response to POPX2 knockdown were detected using the western blot analysis with the respective phospho-specific antibodies (Figure 19D and E). Taken together, our proteome data suggests that MAPK1/3 and GSK3 α/β kinase activities are reduced when POPX2 is silenced.

Figure 19. Motif analysis of the identified phospho-peptides. (A) Motif-X was adopted to analyse the over-represented motif patterns. Logo-like representation of motifs was shown with the respective motif score obtained. Motif score represents the statistical significance of every motif extracted. The higher the motif score, the more over-represented of the motif. Total cell lysates were harvested for western blot analysis using the phospho-site-specific and total MAPK1/3 (B) or GSK3 α/β (D) antibodies. The phospho-MAPK1/3 (C) and phospho-GSK3 α/β (E) levels were quantified and plotted. For (C) and (E), western blots were performed and quantified in triplicates. Error bars represent standard deviations. Students's t-test was used. * indicated P value<0.05; ** indicated P value<0.01; *** indicated P value<0.001; NS indicated P value>0.05, not significant.

3.1.5 A MAPK1 sub-network was over-represented from proteomic analysis

Since MAPK1 was included in our proteomic data, we proceeded to extract MAPK1 substrates from our proteomic data list based on the STRING database¹⁶⁶ and published literature. A MAPK1 sub-network was over-represented (Figure 20A). Within our data list, we found many downstream substrates of MAPK1, including stathmin (STMN1), cysteine sulfinic acid decarboxylase (CSDA), dynamin 1 (DNM1), myristoylated alanine-rich C-kinase substrate (MARCKS), dynamin 2 (DNM2), and paxillin (PXN), cortactin (CTTN). IQGAP1, a MAPK1 interaction partner, was also found within our data list. Reduced phosphorylation levels of these MAPK1 substrates were obtained from the proteomic quantification of POPX2 knockdown. The findings were consistent with lower MAPK1 activity. These MAPK1 substrates were all reported to be involved in the regulation of actin cytoskeleton, focal adhesion and microtubule-based biological processes, which were required during cell migration¹⁸².

Cortactin cooperates with Arp2/3 to participate in actin cytoskeleton regulation.

Phosphorylation and its reversible process play a critical role in this process¹⁸³.

Phosphorylation of cortactin at S417 and S418 appeared to be much less in POPX2 knockdown cells compared to that in control cells. Particularly, S418 is identified to be the MAPK1/3 targeting site¹⁸⁴. Phosphorylation of cortactin by MAPK1/3 enables cortactin's binding to N-WASP, which further enhances the N-WASP-Arp2/3-mediated cytoskeletal regulation¹⁸⁵.

Figure 20. The MAPK1/3 sub-network extraction and phospho-stathmin level examination. (A) The proteomic data was sent into STRING database for network analysis. The over-represented MAPK1 sub-network was shown. The relationships between MAPK1 and its substrates were indicated with different colors: post-translational modification was represented in pink and binding was represented in blue. (B) Phospho-stathmin and total stathmin expression levels were examined in western blot analysis. (C) The phospho-stathmin level was statistically quantified and plotted. The experiments were conducted in triplicates. The error bars represent standard deviations. Student's t test was used. P values were indicated as in Figure 19.

Two distinct models have been proposed for the stathmin-mediated microtubule depolymerization. In the tubulin-sequestering model, stathmin associates with free tubulin to

block its incorporation into the microtubules. The second model proposes that stathmin work by promoting microtubule catastrophe. Stathmin also facilitates microtubule depolymerization through binding the polymerized tubulin and promoting their dissociation from microtubules⁶¹. Three distinct phosphorylation sites on stathmin were identified through proteomic analysis. They were S16, S25 and S38. We found decreased phosphorylation levels of these three phosphorylation sites in response to reduced POPX2 expression. The ability of stathmin in promoting microtubule depolymerization was turned off through MAPK1/3-mediated phosphorylation⁶². Microtubule stability in turn is also linked to cell migration^{63, 66, 186}. To validate our proteomics data, we have confirmed that POPX2 knockdown resulted in reduced phospho-stathmin level using the phospho-stathmin antibody (Figure 20B and C). Interestingly, we also observed that total stathmin levels were reduced in response to lower POPX2 expression. Thus the ratio of phospho-stathmin vs total stathmin in POPX2 knockdown cells was similar to the ratio obtained in control cells (Figure 20C).

3.1.6 Reduced acetylated-microtubules was observed in the POPX2 knockdown cells

It is reported that changes in stathmin phosphorylation level can impact on microtubule stability which in turn results in perturbation of cell motility⁶⁶. In order to find out if POPX2 can affect microtubule stability and to validate our proteomic and western results in the cells, we next performed immunostaining with antibody against acetylated- α -tubulin to determine the relative amount of stable microtubules at the migrating front of the cells⁶⁰. The migration speed was also measured using the scratch wound assays. Less dense acetylated microtubules were observed in the migrating cells with lower POPX2 expression, particularly at the cell leading edge (Figure 21A and B). The amount of acetylated- α -tubulin level was similar in both control and POPX2 knockdown cells as shown in western blot results (Figure 21C and D). The total microtubules levels remained unchanged (Figure 21B-D). Indeed, POPX2 silencing resulted in decreased cell motility in breast cancer cells (Figure 21E). Similarly, the amount of acetylated microtubules was also reduced in non-migrating MDA-MB-231 (Figure 22A and B) or HeLa cells with POPX2 knockdown (Figure 23). Total microtubules remained similar in control and POPX2 knockdown cells. These findings suggest that re-arrangement of the acetylated (stable) microtubules resulted from lower POPX2 expression could be a contributing factor to the decrease in cell motility observed in POPX2 knockdown cells.

Figure 21. POPX2 silencing induced decreased acetylated-microtubules accumulation at the cell migrating front. Scratch wound assay and immunostaining analysis were performed to monitor the changes on acetylated-microtubules (A) and total microtubules (B) at the cell migrating front. The migrating edges were indicated by white curves. Three independent experiments were carried out. Scale bar, 30 μ m. (C) The amount of acetylated-microtubules and total microtubules were also examined in the western blot analysis. (D) Acetylated-microtubules and total microtubules were quantified and plotted. Western blots were conducted and quantified in triplicates. The error bars represent standard deviations. Student's t test was used. NS indicated P value>0.05, not significant. (E) Reduced cell motility in POPX2 knockdown cells were confirmed with the scratch wound assay. The wound edges are indicated by black lines. Scratch wound assays were performed three times. Scale bar, 200 μ m.

Figure 22. Less dense acetylated-microtubules were observed in the non-migrating breast cancer cells in response to POPX2 silencing. The immunostaining was carried out to monitor changes on acetylated-microtubules (A) and total microtubules (B) in the non-migrating breast cancer cells with or without POPX2 depletion. Experiments were conducted in triplicates. Scale bar, 30 μ m.

Figure 23. The amount of acetylated-microtubules was perturbed in HeLa cells with decreased and increased POPX2 expression. (A) POPX2 knockdown cells and (B) POPX2 over-expressing cells were fixed and immunostained for acetylated-microtubules and total microtubules. Scale bar, 30 μ m. (C) Ac- α -tubulin quantification. The percentage of cells showing strong Ac- α -tubulin was quantified from three independent experiments ($n \geq 100$). Error bars represent standard deviations. Student's t test was used. *** indicated P value < 0.001 .

3.1.7 POPX2 knockdown impacted on transcriptional regulation

Figure 24. POPX2 knockdown impacted on transcriptional regulation. The up- or down-regulated transcripts were sent for DAVID analysis to extract the over-represented transcription factors. PhosphoSite and NetworkKIN databases were employed to analyse if these enriched transcription factors were regulated by MAPK1/3 or GSK3β. Relationships extracted from combined genomic and proteomic analysis were shown here. POPX2 knockdown resulted in decreased phospho-MAPK1/3 and increased phospho-GSK3β. CEBPA, FOXO3, GR, HSF1 and SREBP1 are activated in the presence of decreased MAPK1/3 and GSK3β activities, contributing to up-regulated target expression. ATF2, CREB, ELK1, and ER are inhibited in the presence of decreased MAPK1/3 and GSK3β activities, contributing to the down-regulated target expression.

MAPK1/3 and GSK3 β play key roles in transcriptional regulation through modulating transcription factors directly as the upstream kinases or indirectly through cooperation with other signaling proteins^{179, 187-189}. Next, we went on to examine whether POPX2 could impact on transcriptome through modulating the transcription factors and their upstream kinases, MAPK1/3 and GSK3 β . Briefly, the “bottom-up” strategy was adopted. The differentially regulated hits obtained from DNA microarray data analysis were analysed by DAVID to predict and to over-represent the upstream transcription factors. The enriched transcription factors, which control the transcripts expression, were then analyzed using PhosphoSite¹⁷⁴ and NetworKIN databases¹⁹⁰ to determine if they have been reported to be the direct substrates of MAPK1/3 or GSK3 β . We found the following over-represented transcription factors which corresponded to up-regulated transcription activity in the presence of decreased MAPK1/3 and GSK3 β activities: CCAAT/Enhancer-binding protein α (C/EBP α), forkhead box O3 (FOXO3), glucocorticoid receptor (GR), heat shock factor 1 (HSF1), and sterol regulatory element-binding protein 1 (SREBP1). Among the transcription factors which led to down-regulation of target expression when MAPK1/3 and GSK3 β activities were inhibited are: activating transcription factor 2 (ATF2), cAMP responsive element-binding protein (CREB), ELK-1 belonging to the ternary complex factor family of transcription factors, and estrogen receptor (ER). Figure 24 showed the possible regulation of transcription factors by MAPK1/3 and GSK3 β , which were in turn regulated by POPX2 levels. The involvement of these transcription factors in apoptosis and cell proliferation is well-recorded.

3.1.8 Conclusion for Part I study

In this study, an integrative genomic and proteomic approaches were adopted to elucidate POPX2-related signaling pathway. POPX2 could participate in the coordination of actin cytoskeleton, microtubule and focal adhesion through modulating MAPK1/3 kinase activity and its substrates, leading to perturbation on breast cancer cell motility. Moreover, POPX2 could also involve in apoptosis and proliferation through regulating MAPK1/3-mediated transcriptional regulation (Figure 25).

Figure 25. Working model of how POPX2 could affect transcriptome and proteome through modulating MAPK1/3 and their substrates, leading to changes on multiple cellular processes.

3.2 An Integrative Phosphoproteome and Secretome Study to Elucidate the Effects of POPX2 phosphatase on Tumor Angiogenesis

Another significant phenotype observed by our group was that the conditioned medium from POPX2 knockdown cells contributed to angiogenesis. The effects of POPX2 knockdown on angiogenesis was firstly examined using the well-established *in vivo* CAM assay system¹⁹¹. Briefly, experiments were carried out in which the conditioned medium from Control and two separate POPX2 knockdown cell lines were mixed with collagen and randomly placed on CAM for 3 days to observe angiogenesis development. Conditioned medium from POPX2 knockdown cells induced 1.8- and 2.4-fold elevation in angiogenesis compared to that of Control cells, respectively (H Chan and CG Koh, personal communication). Accordingly, proteins secreted from POPX2 silencing breast cancer cells induced angiogenesis *in vivo*. Apart from the *in vivo* angiogenesis system, we have also used an *in vitro* angiogenesis system to confirm the role of POPX2 in angiogenesis (Figure 26) using human umbilical vein endothelial cells (HUVECs) which are capable of aggregating to form capillary-like structure rapidly when culturing on matrigel in the presence of FBS and growth factors *in vitro*. This process is thought to mimic the angiogenesis process *in vivo*¹⁶⁹. Endothelial cells maintained in basal media served as the negative control. They were unable to form regular tubes. Endothelial cells maintained in complete media with FBS and growth factors served as the positive control. They were able to form regular tubular networks. In order to examine how POPX2 affect angiogenesis through breast cancer secretome, conditioned media collected from both control and POPX2 knockdown MDA-MB-231 cells were used for the *in vitro* angiogenesis assay. Total tube length and branch points were calculated. We found that the tubular network was more regular when endothelial cells were cultured in the conditioned medium obtained from POPX2 knockdown cells compared to that from Control (Figure 26A). In addition, the total tube length and number of branch points were increased in response to the secretome collected from POPX2 knockdown cells (Figure 26B and C). Based on these observations, we concluded that POPX2 knockdown induces *in vitro* angiogenesis through impacting on breast cancer cell secretome. How POPX2 can affect angiogenesis is not clear. Therefore, we proceeded to use a combined phosphoproteome and secretome strategy to further investigate the signaling pathways related to POPX2 and angiogenesis.

A

B

C

Figure 26. POPX2 knockdown induces *in vitro* angiogenesis through impacting on breast cancer cells secretome. (A) Similar number of HUVECs (human umbilical vein endothelial cells) were incubated in EBM (endothelial cells basal medium), EGM (EBM + 10% FBS + essential endothelial cells growth factors), Control-CM, and X2-shRNA1/2-CM (conditioned medium obtained from control and two separate POPX2-knockdown MDA-MB-231 cells, respectively). Tube formation on matrigel was captured after 4hrs incubation. Representative images from three biological replicates were shown. Scale bar, 200µm. (B) Total tube length and (C) the number of branch points were quantified from at least 10 random fields for each sample. Results were average from three biological replicates. The error bars represent standard deviation. The significance of difference was calculated by Student's t-test. * indicated P value<0.05; ** indicated P value<0.01; *** indicated P value<0.001.

3.2.1 Phosphoproteome of POPX2-knockdown MDA-MB-231 cells

To find possible substrates of the POPX2 phosphatase, the SILAC-based phosphoproteome quantification were carried out. Control cells (MDA-MB-231) were maintained in heavy medium and the POPX2-knockdown cells were maintained in light medium. Cell lysates were harvested after seven passages to insure complete heavy amino acids incorporation. Phosphopeptides were extracted using a combination of three strategies to enhance the enrichment efficiency: ERLIC¹¹⁴, IMAC¹¹¹ and TiO₂¹¹³. Enriched phosphopeptides were sent for mass spectrometry-based identification and quantification. Phosphoproteome analysis was conducted in two technical replicates (Figure 27). Totally, 1265 phosphopeptides and 1005 unique phospho-sites, corresponding to 541 phospho-proteins were identified from the phosphoproteome analysis (Figure 28A). Up to 20% (189) of the 1005 phospho-sites were novel sites when compared to PhosphoSite¹⁷⁴ and Phospho.ELM¹⁷⁵ databases (Figure 28B).

3.2.2 Altered CDK1 kinase activity implicated the involvement of POPX2 in cell cycle process

To simplify the identification of phospho-protein profiles and extraction of the potential targets for our subsequent work, all the significantly up- or down-regulated phospho-proteins were identified and categorized into several distinct protein classes, including enzyme, kinase, transcription regulator, ion channel, transporter, translation regulator, phosphatase, and transmembrane receptor. The identified phospho-peptides were also listed and the identified phosphorylation-sites were colored in red with annotations (Table 4 and 5). Interestingly, phosphorylation of Cyclin dependent kinase 1(CDK1) at T14 and Y15 was significantly down-regulated in the POPX2 knockdown cells (Table 4). CDK1 acts as the critical switch in cell cycle signaling¹⁹². T14 and Y15 are two well-characterized phosphorylation sites, involved in cell cycle progression. Phosphorylation at T14 and Y15 by Myt1 inhibit CDK1 kinase activity¹⁹³. Decreased phosphorylation levels of T14 and Y15 on CDK1 suggested the higher CDK1 kinase activity when POPX2 was knockdown. Preliminary data unveiled that POPX2 knockdown cells proliferated much faster than Control cells. These observations led us to further investigate the CDK1 kinase activity in POPX2 knockdown cells and the effects of silencing POPX2 on cell cycle progression in breast cancer (ongoing: to be followed up by Dr. Kelvin Wong Kin Kwan).

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Figure 27. Work-flow of phosphoproteome analysis. The SILAC-based quantitative phosphoproteomic study was carried out. Control cells were grown in “heavy” media and POPX2 knockdown cells were grown in “light” media. The cells harvested were mixed in 1:1 ratio, lysed and digested by trypsin. Phospho-peptides were enriched by ERLIC, IMAC and TiO_2 . Enriched phospho-peptides were then subjected to LC-MS/MS for identification and quantification.

Figure 28. Overview of phosphopeptides analysis. (A) The number of phosphopeptides, phospho-proteins and phospho-sites quantified by SILAC-based mass spectrometry were shown. (B) Phospho-sites annotation. Totally, 1005 unique phospho-sites were annotated according to PhosphoSite and Phospho.ELM databases. The number of novel phospho-sites (upper, light green) and annotated phospho-sites (lower, dark green) were quantified. pS: phospho-serine; pT: phospho-threonine; pY: phospho-tyrosine.

Table 4. Phosphoproteins which were significantly down-regulated in POPX2 knockdown cells were categorized into several protein classes. Identified phosphosites were highlighted in red and annotated in PhosphoSite database.

Gene	Phosphopeptide	PhosphoSite
Enzyme		
ATL1	TYEW ^{ss} EEEEPVkk	novel/novel
CDC23	rV ^s PLNLSSV ^t P	S588/T596
CRY2	L ^t FEYDSEPFGKEr	novel
CYB561	rP ^s QAEEQALSmDFK	novel
DPYSL5	GVN ^s FQmFmTYk	novel
FTSJ3	QP ^s KEEEEEEEEEQLNQ ^t LAEMk	S356/T371
GBP6	kFmET ^t MNKk	novel
MCM2	E ^s MATGSIPITVr	S754
MX1	NPGSVAENNL ^s QyEEk	novel/novel
NDUFV3	^t FrLNEIDkESQKPFEVK	novel
NIT1	I ^y NcHVLLNSk	Y143
PCNA	YYLAPkIEDEEG ^s	S261
PGM1	^s mPTSGALDRVASATk	novel
POLR1A	EkQEEEV ^s DYEsEEEErEGEENDDEDMQEEr	S1429
PTGES3	AkLNWLSVDFNNWkDWEDD ^s DEDMSNFDr	S113
TMX1	kVEEEQEAD ^s EEDV ^s EEEAESkEGTNk	S247
TOP1MT	GP ^y FAPP ^y EPLPDGVr	novel/novel
TOP1MT	rPA ^s RGVPG ^s RR	novel/novel
Kinase		
CDK1	IEkIGEG ^{ty} GVVYk	T14/Y15
OXSRI	LHkTEDGGWEW ^s DDEFDEESEEGk	S339
OXSRI	LHkTEDGGWEW ^s DDEFDEESEEGkAAISQLr	S339
PRPF4B	EQPEMEDAN ^s Ek ^s INEENGEVSEDQSQNk	S20/S23
ROCK1	rNLE ^s TVSQIEK	novel
TJP2	^s FsPEEr	S413/S514
WNK2	^s VVESDQEEPPGLEAAEAPGPQPPQPLQr	S45
Transcription regulator		

BDP1	AtGNESsPr	novel/novel
GLIS3	mPsGGGMAPQNNVAEsr	novel/novel
HTATSF1	LFEEsDDkEDEDEDADGkEVEDADEk	S676
REXO1	EVDFDsDPMEEcLr	S610
SUPT6H	kMsDDEDDDEEEYGkEEHEk	S125
TARDBP	mDETDAssAVkVK	S91/S92
TRRAP	LPEMGsNVELsNLYLk	novel/novel
UHRF1	DSELsDTDSGccLGQSESDk	S91
ZNF236	VFKcDtEKAFAKPSQLer	novel
ZRANB2	LDEDEDEDDADLSkYNLDAsEEEDSNk	S188
Ion channel		
CACNG4	AsSSSPYArMPsYrYR	novel/S235
CUL5	LALPADsVNIKILNAGAWsr	novel/novel
KCNH8	GcLrsLSLHIK	novel
Transporter		
SLC7A5	MLAAKsADGsAPAGEGEGVtLQr	S31/S35/T45
Translation regulator		
EEF1D	QVEPPAKKPATPAEDDEDDDDIDLFGsDNEEEDk	S162
Phosphatase		
PPP1R7	VEsEEsGDEEGkk	S24/S27
Transmembrane receptor		
HLA-E	FISVGyVDDTQFVrFFLr	novel

Table 5. Phosphoproteins which were significantly up-regulated in POPX2 knockdown cells were categorized into several protein classes. Identified phosphosites were highlighted in red and annotated in PhosphoSite database.

Gene Name	Phosphopeptide	PhosphoSite
Enzyme		
COIL	LIIEsPSNTSSTEPA	S566
F13A1	LsIQsSPK	novel/novel
FKBP4	SNTAGsQSQVETEA	S451
LAMB2	ySEIEPSTEGEVIyR	novel/novel
MAN1B1	xWAVATTVVmyPPPPPPPHR	Y38
MANEA	INsETNTK	novel
MYO5A	VSVSFIRtIQmR	novel
NAPRT1	LQALVNSLcAGQsP	S537
PDCL	NSATcHsEDSDLEID	S293
PRMT6	LESGGGGEGGEGtEEEDGAER	T21
RAD9A	SPQGSPVLAEDsEGEG	S387
RPAP3	FAVAEsDcNLAVALNR	novel
TRMT112	GIPNMLLsEEETES	S119
ZMYND17	FsGTVRGAEMsITVVQSAR	novel/novel
Transcription regulator		
ATF7	AAAGPLDMsLPsTPDIK	S108/S111
CCNH	HEEEEWtDDDLVESL	T315
CSDA	AGEAPTENPAPPTQQSsAE	S370
FOSL2	SSSSGDQSSDSLNsPTLLAL	S320
HCFC1	ySNDLYELQASR	novel
NAA15	ITVNGDSsAEAEELANEI	S856
PURB	GGSGGGGEEsEGEEVDED	S304
RELB	EAAFGGGLLsPGPEAT	S573
TRIM28	FAVLVEPPPMSLPGAGLsSQELSGGPGDGP	S823
YBX1	AADPPAENSsAPEAEQGGAE	S314
ZEB2	sDHEEDNMEDGM	S1203
ZNF593	LAVPTEVSTEVPEMDTsT	S133

ZRANB2	ENVEYIEREEsDGEYDEFGR	S120
Transporter		
ARPP19	YFDsGDYNMAK	S62
ATP2B1	SATSSSPGsPLHSLEtsL	S1249/novel/S1257
SLC5A9	m sKELAAMGPGASGDGVR	novel
SORCS3	EQEMIGsVSQSENAPK	novel
SORT1	SGYHDDsDEDLLE	S825
TOMM20	IVSAQsLAEDDVE	S138
VPS26A	FEsPESQASAEQPEM	S823
Kinase		
MAP2K4	ILDQMPATPSsPMYVD	S394
MAP3K4	LEsEDDSLGLWGAPDWSTEAGFSR	S499
NUAK1	ENDFAQSGQDAVPEsPSK	S388
NUCKS1	sGKNSQEDsEDsEDKDVK	S50/S58/S61
Phosphatase		
PTPN4	tLELYGVEFHAR	novel
PTPRG	NGAVLIADEsDPAESMESLV	S1435
PTPRZ1	FPLEMQIycFDADRFssFEEAVK	novel/novel/novel
SSH2	TTNPFYNTM	T1422
Transmembrane receptor		
IGF2R	LVsFHDDsDEDLLHI	S2479/S2484
HLA-B	GGSYSQAACsD sAQGSVDVSLTA	novel
MPL	AtVSDtcEEVEPSLLEILPK	novel/T559
Translation regulator		
EIF3K	IDFDSVSSIMASsQ	S217
USP15	GASAATGIPLEsDEDSNDNDNDIENENcMHTN	S961
Ion channel		
TMEM38A	mELLSALSGLGELALsFsR	novel/novel

3.2.3 Quantitative mass-spectrometry analysis revealed secretomic alterations in POPX2 knockdown cells

Based on the *in vivo* CAM assays and *in vitro* tube formation assays conducted, POPX2 affected tumor angiogenesis possibly through exerting impact on the secretomic profiles of breast cancer cells. Next, changes on secretome in POPX2-depleted cells were studied using SILAC-based quantitative proteomics¹⁰¹⁻¹⁰³ (Figure 29A). Control or POPX2 knockdown cells, which were previously described in our earlier work, served as our model system^{1, 170}. The Control cells were maintained in heavy medium supplemented with heavy arginine and lysine whereas the POPX2 knockdown cells were grown in light medium supplemented with light arginine and lysine. Media from multiple independently grown cells were conditioned for 24hrs before harvesting for secretome analysis. Secretome analysis was conducted in two technical replicates. Totally, 7221 peptides corresponding to 919 proteins from the first round of technical replicate and 7264 peptides corresponding to 924 proteins from the second round of technical replicate were identified and quantified. Moreover, a total of 1016 unique proteins were identified and quantified from the two secretome technical replicates. Identified protein expression ratios were calculated as Control vs POPX2 knockdown (heavy vs light) and plotted in histogram (Figure 29B). A two-fold change cut-off was adopted for the secretome analysis. Accordingly, a total of 465 proteins were classified as up-regulated hits whereas a total of 391 proteins were classified as down-regulated hits in response to POPX2 silencing.

Figure 29. Work-flow and overview of secretome analysis. (A) The conditioned media harvested from both Control and POPX2 knockdown cells were used for secretome analysis. SILAC-based quantitative proteomics approach was adopted. (B) The protein expression ratios were calculated as Control vs POPX2 knockdown (heavy vs light). The Log₂ Ratio was calculated for every identified hit. The frequencies (vertical axis) of the Log₂ Ratio (horizontal axis) were plotted in histograms for secretome.

3.2.4 Secretome analysis unveiled the enrichment of exosome in the secretome of POPX2 knockdown cells

Actually, not all the identified proteins are really localized in the extracellular space. Contamination by non-secreted proteins released following cell lysis or cell death is unavoidable. Accordingly, secreted protein prediction by distinctive bioinformatics tools is necessary¹¹⁶. In total, up to 85% of the total identified proteins are predicted to be secreted (861 out of 1016 total identified proteins) (Figure 30A). The majority of the proteins are secreted in the highly evolved classical secretory pathway. These proteins contain the N-terminal signal peptide which directs them to the extracellular space¹¹⁶. This class of proteins is predicted by SignalP¹¹⁷. A total of 258 identified proteins are predicted to be secreted by the classical secretory pathway. Protein secretion independent of the N-terminal signal peptide by the non-classical secretory pathway has also been documented¹¹⁸⁻¹²⁰. This class of proteins is predicted by SecretomeP¹²¹. And a total of 478 identified proteins are predicted to be secreted through the non-classical secretory pathway. Some of the secreted proteins could be shed membrane receptors, which are predicted by TMHMM¹²². The number predicted from TMHMM is 124. In addition, 172 identified proteins reported to localize at the extracellular space are retrieved from Gene Ontology.

Apart from the secretory pathways described above, proteins are also released through exosome. Interestingly, up to 631 exosome associated proteins are obtained through ExoCarta database¹²³. Moreover, the secretion level of CD81, a well-characterized exosome marker, was found to be much higher in the secretome obtained from POPX2 knockdown cells. It has been reported that exosomes secreted by tumor cells participate in promoting angiogenesis, tumor growth and facilitating dissemination of tumor cells^{194, 195}. In order to investigate how POPX2 regulates the exosome secretion level, we first performed western blot analysis to

examine the secretion levels of two critical exosome markers, CD81 and CD9^{194, 196}. Antibodies specifically against CD81 and CD9 implicated a significantly enrichment of exosomes in the secretome obtained from POPX2 knockdown cells (Figure 30B).

Figure 30. Secreted proteins prediction and exosome validation. (A) The sub-cellular localizations of the proteins identified from the secretome were predicted using multiple bioinformatic tools. Secreted proteins undergoing classical secretory pathway were predicted with SignalP. Secreted proteins undergoing non-classical secretory pathway were predicted with SecretomeP. Shed membrane receptors that were released to extracellular space were predicted with TMHMM. Proteins associated with exosome were obtained from ExoCarta database. Proteins localized in extracellular space were also retrieved from Gene Ontology. (B) Both the conditioned medium and cell lysate were harvested for western blot examination. Exosome secretion level was investigated using specific antibodies against two critical exosome markers, CD81 and CD9. Actin was used as a loading control. Representative blot from three independent biological replicates were shown.

3.2.5 Pathway analysis implicated the involvement of POPX2 in breast cancer cell-ECM and cell-cell interaction process

To determine POPX2 related pathways, functional cluster examination of differentially-regulated secretome was performed using DAVID tool¹⁶⁴. The KEGG pathway¹⁷² annotation information was further extracted and plotted with their respective p-value (Figure 31). Several cancer metastasis and angiogenesis related signaling pathways are over-represented, including “ECM-receptor interaction”, “Focal adhesion”, “Adheren junction” as well as “Regulation of actin cytoskeleton”. POPX2 was already reported to be linked to focal adhesion and actin cytoskeleton^{1, 131, 170}. Therefore, in our current study, we were more interested in exploring the effects of POPX2 silencing on breast cancer cell-ECM and cell-cell interaction processes.

To determine whether there was a link between POPX2 and cell-ECM, cell-cell interaction, we went on to look at the specific deferentially regulated hits identified from the secretome which were distributed to these biological functional clusters (Table 6). In addition, these differentially regulated secreted hits were also sent for KEGG pathway analysis, and the “ECM-receptor interaction” pathway and “Adheren junction” pathway were extracted. All POPX2 knockdown induced secreted hits were colored in red whereas the reduced secreted hits were colored in green (Figure 32). We observed a dramatic decline of several of the following ECM components in the POPX2 knockdown secretome (Figure 32): Agrin (AGRN), collagen type IV alpha 1 (COL4A1), collagen type IV alpha 2 (COL4A2), collagen type V alpha 1 (COL5A1), Laminin alpha 5 (LAMA5), Laminin beta 1 (LAMB1), Laminin beta 2 (LAMB2), Laminin beta 3 (LAMB3), Laminin gamma 2 (LAMC2), heparan sulfate proteoglycan 2 or Perlecan (HSPG2)^{197, 198}. We also found decreased levels of Thrombospondin 1 (THBS1) and Tenascin (TNC), which are potent inhibitors of angiogenesis^{199, 200}. Another two ECM components were shown to be up-regulated in the POPX2 knockdown secretome (Figure 32), Fibronectin (FN1), promoting microvessel elongation during angiogenesis²⁰¹; and Vitronectin (VTN), facilitating angiogenesis through protecting endothelial cells from apoptosis²⁰².

Figure 31. KEGG pathway analysis of differentially regulated secretome. The differentially regulated hits obtained from secretome were submitted to DAVID using their official_gene_symbol. The KEGG pathway annotations were downloaded and plotted. P-value for each KEGG pathway was -Log converted.

Table 6. Differentially regulated proteins distributed to the over-represented KEGG pathways.

Number	Accession Number	Gene Symbol	Log₂ Ratio (H/L)	KEGG pathway
1	P24666	ACP1	-20.5368	Adheren junction
2	P60709	ACTB	2.723476	Focal adhesion; Adheren junction; Regulation of actin cytoskeleton
3	P35609	ACTN2	1.094153	Focal adhesion; Adheren junction; Regulation of actin cytoskeleton
4	O00468	AGRN	5.839179	ECM-receptor interaction
5	Q13017	ARHGAP5	-19.174	Focal adhesion
6	O15143	ARPC1B	-21.6997	Regulation of actin cytoskeleton
7	O15144	ARPC2	-21.725	Regulation of actin cytoskeleton
8	O15145	ARPC3	3.324484	Regulation of actin cytoskeleton
9	P59998	ARPC4	3.008018	Regulation of actin cytoskeleton
10	O15511	ARPC5	3.833704	Regulation of actin cytoskeleton
11	Q9BPX5	ARPC5L	3.726186	Regulation of actin cytoskeleton
12	P16070	CD44	2.673675	ECM-receptor interaction
13	P60953	CDC42	-21.0565	Focal adhesion; Adheren junction; Regulation of actin cytoskeleton
14	P23528	CFL1	2.11616	Regulation of actin cytoskeleton
15	Q9Y281	CFL2	1.724731	Regulation of actin cytoskeleton
16	P02462	COL4A1	16.90653	ECM-receptor interaction; Focal adhesion
17	P08572	COL4A2	2.330857	ECM-receptor interaction; Focal adhesion
18	P20908	COL5A1	3.388425	ECM-receptor interaction; Focal adhesion
19	P46108	CRK	-22.9499	Focal adhesion; Regulation of actin cytoskeleton
20	P04626	ERBB2	-24.9006	Focal adhesion; Adheren junction
21	P21333	FLNA	1.813865	Focal adhesion
22	O75369	FLNB	2.738806	Focal adhesion
23	Q14315	FLNC	3.876103	Focal adhesion

24	P02751	FN1	-1.23532	ECM-receptor interaction; Focal adhesion; Regulation of actin cytoskeleton
25	Q9UBI6	GNG12	2.033891	Regulation of actin cytoskeleton
26	P06396	GSN	-21.6587	Regulation of actin cytoskeleton
27	P98160	HSPG2	1.964292	ECM-receptor interaction
28	P46940	IQGAP1	2.863221	Adheren junction; Regulation of actin cytoskeleton
29	P05556	ITGB1	-1.42644	ECM-receptor interaction; Focal adhesion; Regulation of actin cytoskeleton
30	Q16363	LAMA4	-20.0044	ECM-receptor interaction; Focal adhesion
31	O15230	LAMA5	1.329623	ECM-receptor interaction; Focal adhesion
32	P07942	LAMB1	1.232189	ECM-receptor interaction; Focal adhesion
33	P55268	LAMB2	2.607251	ECM-receptor interaction; Focal adhesion
34	Q13751	LAMB3	18.26436	ECM-receptor interaction; Focal adhesion
35	Q13753	LAMC2	17.99777	ECM-receptor interaction; Focal adhesion
36	P28482	MAPK1	-21.5131	Focal adhesion; Adheren junction; Regulation of actin cytoskeleton
37	P27361	MAPK3	-20.7619	Focal adhesion; Adheren junction; Regulation of actin cytoskeleton
38	P08581	MET	4.157448	Focal adhesion; Adheren junction
39	Q7Z406	MYH14	-22.2645	Regulation of actin cytoskeleton
40	P35579	MYH9	2.024985	Regulation of actin cytoskeleton
41	P07737	PFN1	1.667893	Regulation of actin cytoskeleton
42	P60673	PFN3	-24.4905	Regulation of actin cytoskeleton
43	Q92692	PVRL2	19.68767	Adheren Junction

44	P35241	RDX	1.279314	Regulation of actin cytoskeleton
45	P61586	RHOA	4.163998	Focal adhesion; Adheren junction; Regulation of actin cytoskeleton
46	P18827	SDC1	1.457186	ECM-receptor interaction
47	P31431	SDC4	1.54454	ECM-receptor interaction
48	P07996	THBS1	2.792647	ECM-receptor interaction; Focal adhesion
49	Q9Y490	TLN1	2.030635	Focal adhesion
50	P24821	TNC	20.29635	ECM-receptor interaction; Focal adhesion
51	P50552	VASP	-24.1442	Focal adhesion
52	P18206	VCL	1.220079	Focal adhesion; Adheren junction; Regulation of actin cytoskeleton
53	P15692	VEGFA	23.26539	Focal adhesion
54	P04004	VTN	-25.9747	ECM-receptor interaction; Focal adhesion

ECM-RECEPTOR INTERACTION

Figure 32. ECM-receptor interaction map extracted from KEGG pathway database. Up-regulated hits in our datasets are colored in red while down-regulated hits are colored in green.

Figure 33. Adherens junction map extracted from KEGG pathway database. Up-regulated hits in our datasets are colored in red while down-regulated hits are colored in green.

In the “Adherens junction” pathway, a dramatic decrease of Poliovirus receptor-related 2 (PVRL2), also known as Nectin 2, was reported from the secretome quantification analysis (Figure 33). Nectins and Cadherins are all major cellular adhesion molecules, maintaining regular cell-cell interaction^{203, 204}. A decrease in these ECM components and adhesion molecules indicated the impact of POPX2 silencing on cell-ECM and cell-cell interaction processes.

3.2.6 POPX2 knockdown perturbed cell adhesiveness to extracellular matrix and promoted epithelial-mesenchymal transition

To determine whether POPX2 knockdown affect cell-ECM and cell-cell interaction process, cell adhesiveness to extracellular matrix and EMT were examined in both control and POPX2 knockdown cells. The adhesive ability of POPX2 knockdown cells to both fibronectin and collagen were dramatically reduced compared with that of control (Figure 34). Moreover, POPX2 silencing in another non-metastatic breast cancer cells, MCF7, also showed significant defects in cell attachment to extracellular matrix¹.

Figure 34. Cell adhesion assay. The Control and two separate POPX2 knockdown cells were plated on the bovine serum albumin (BSA), fibronectin and collagen coated 96-well and allowed to attach for 30min before fixing. Attached cells were stained with crystal violet and quantified through measuring the absorbance at 570nm. The results were averaged from adhesion assays conducted in triplicates. Error bars represent standard deviations. The significance of difference was calculated by Students's t-test. P value was indicated as in Figure 19.

EMT describes the transition from epithelial cells to mesenchymal cells, with the loss of tight junction, adherens junction and desmosome, which is essential during cancer metastasis and angiogenesis⁹³. Next, we went on to explore the effects of POPX2 knockdown on EMT process. A number of EMT markers are documented⁹⁵. Among which, E-cadherin and β -catenin are classified as epithelial markers, whereas Vimentin and N-cadherin are grouped into mesenchymal markers. In our study, the expression of various EMT markers were examined in both MCF7 (epithelial cell type) and MDA-MB-231 (mesenchymal cell type) cells with or without POPX2 depletion. Significantly loss of E-cadherin and β -catenin (epithelial markers) localization at the cell-cell junction in POPX2-knockdown MCF7 cells were observed from immunostaining (Figure 35A). MDA-MB-231 did not express E-cadherin (Figure 35B and C). The localization of β -catenin at the cell periphery was also reduced in MDA-POPX2 knockdown cells (Figure 35B). On the contrary, increased Vimentin and N-cadherin (mesenchymal markers) expression were observed upon POPX2 knockdown in MDA-MB-231 (Figure 35B). Western blot analysis was also performed to confirm the POPX2 knockdown effects on Vimentin and N-cadherin expression in MDA-MB-231 (Figure 35C). MCF-7 did not express Vimentin (Figure 35A and C). Increased N-cadherin was observed in the MCF-7 with POPX2 knocked down (Figure 35A and C). These observations suggest that POPX2 knockdown promoted EMT progression in breast cancer cells which could further lead to induced angiogenesis.

Figure 35. Examination of the effects of POPX2 knockdown on EMT process. Immunostaining with antibodies against various EMT markers, including the epithelial markers (E-cadherin, β -catenin) and the mesenchymal markers (Vimentin, N-cadherin), were carried out in both MCF7 (A) (epithelial cell type) and MDA-MB-231 (B) (mesenchymal cell type) with or without POPX2 knockdown. The experiments were conducted in triplicates. Scale bar, 30 μ m. (C) Cell lysate obtained from both MCF7 and MDA-MB-231 with or without POPX2 knockdown were subjected for western blot analysis to observe the expression of various EMT markers using their respective specific antibodies. Actin was used as a loading control. Results are represented from three independent biological replicates.

3.2.7 Conclusion for Part II study

To explore how POPX2 affect angiogenesis, both SILAC-based quantitative phosphoproteome and secretome studies were carried out, in combination with extensive biological validation and investigation. POPX2 knockdown influenced the phosphoproteome in breast cancer cells. A dramatic decreased phosphorylation levels at T14 and Y15 of CDK1 corresponding to higher CDK1 kinase activity were reported from the SILAC-based phosphoproteome quantification results, which implicated the involvement of POPX2 in cell cycle progression in breast cancer leading to faster tumor growth. Moreover, POPX2 knockdown also altered the secretomic profile, leading to changes on several pro-angiogenic processes, including enriched exosome secretion, defects on cell-ECM interaction, as well as faster EMT progression in breast cancer.

3.3 Functional study of RhoGDIs, Candidate Proteins Regulated by POPX2 phosphatase Related Pathways

In our current study using the integrative transcriptome and proteome approaches, we were able to elucidate the role of POPX2 in breast cancer cell motility and actin cytoskeleton regulation. Moreover, through phosphoproteome and secretome study, we also uncovered the involvement of POPX2 in angiogenesis. Last but not the least, we have undertaken the candidate approach to study the biological functions of candidate genes. The level of RhoGDI has been reported to be affected by POPX2 silencing in both MCF-7 and MDA-MB-231 breast cancer cells¹. RhoGDIs are well-known inhibitors of RhoGTPases and are involved in several actin cytoskeleton related biological processes^{135, 160}. They are also reported to be linked to cancer cell migration and invasion^{205, 206}. The RhoGDIs might be interesting candidate proteins regulated by POPX2 phosphatase related pathways.

3.3.1 Overexpression of RhoGDIs in HeLa cells resulted in rounded cells

We firstly investigated the effects of RhoGDIs on actin cytoskeleton through overexpression of these two constructs (pXJ-GFP-Flag-RhoGDI1 and 2) in HeLa cells. The endogenous RhoGDI1 appeared as a 25KD band, whereas the endogenous expression of RhoGDI2 in HeLa was quite low. The overexpression level of RhoGDI1 appeared almost the same as the endogenous level (Figure 36A and B). Interestingly, when HeLa cells were transfected with either RhoGDI1 or RhoGDI2, we observed cells rounding to different extend. More rounded cells were observed in RhoGDI1-transfected cells (Figure 36C).

Figure 36. Overexpression of RhoGDIs in HeLa cells. (A) and (B) HeLa cells were transiently transfected with Control, RhoGDI1 and RhoGDI2 plasmids. 24 hrs after transfection, cells were harvested for western blot analysis using RhoGDI1 and RhoGDI2 specific antibodies, separately. β -tubulin was used as the loading control. (C) 24 hrs after transfection, GFP images were captured using the fluorescent microscope. Scale bar, 200 μ m.

3.3.2 Overexpression of RhoGDIs led to reduced stress fibers and focal adhesions

To determine whether RhoGDIs affect actin cytoskeleton, RhoGDIs-transfected HeLa cells were immuno-stained with phalloidin antibody to examine the cytoskeletal changes. Control cells were well-spread with regular stress fiber. Both GFP-RhoGDI1/2 were distributed in the cytosol and resulted in cells which were either spindle-shaped or rounded. Only few stress fibers were observed in the spindle-shaped cells, whereas no stress fiber was found in the rounded cells (Figure 37A).

Focal adhesions link the stress fibers to the extracellular matrix. To determine the effects of RhoGDI1/2 overexpression on focal adhesion, immunostaining with two focal adhesion proteins, vinculin and paxillin, were carried out. Vinculin and paxillin distributed throughout the Control cells. On the contrary, the number of focal adhesion was reduced in the spindle-shaped cells induced by RhoGDI1/2 overexpression. Consistently, no focal adhesion could be observed in the rounded cells resulted from RhoGDI1/2 overexpression (Figure 37B and C).

RhoGDI1 and RhoGDI2 were able to induce morphological changes to spindle-shaped or rounded cells with loss of stress fibers and focal adhesions to different extends. The percentage of deformed cells upon RhoGDI1/2 overexpression over that of Control was plotted. Overexpression of RhoGDI1 resulted in more spindle-liked (24%) and rounded cells (50%) compared to RhoGDI2 overexpression (16% for spindle-liked cells and 21% for rounded cells) (Figure 37D). The effects of RhoGDI2 on cell cytoskeleton were less prominent than that of RhoGDI1.

B

C

D

Figure 37. Overexpression of RhoGDIs in HeLa resulted in cells rounding with reduced stress fibers and focal adhesions. Control and RhoGDI1/2 transfected HeLa cells were immunostained with phalloidin (A), Vinculin (B) and Paxillin (C) to visualize stress fibers and focal adhesions.

Representative images were shown here. Scale bar, 30 μ m. (D) The spindle-like cells and rounded cells resulted from RhoGDI1/2 overexpression were counted using 40 $\times$ objective in 100 cells from at least four different fields of every coverslips. Experiments were conducted in triplicates. Student's t-test was used. P value was indicated as in Figure 19.

3.3.3 RhoGDI1 knockdown did not affect actin cytoskeleton

The effects of silencing RhoGDI1/2 on the actin cytoskeleton have not been documented in mammalian cells. As shown in Figure 36A and B, the endogenous level of RhoGDI1 in HeLa was high, whereas the endogenous level of RhoGDI2 in HeLa could not be detected in western blot using the specific antibodies. We next proceeded to determine the effects of RhoGDI1 depletion using siRNA targeting two distinct regions of RhoGDI1 (designated as siRhoGDI1_1 and siRhoGDI1_2). Immunostaining with phalloidin, vinculin, FAK and paxillin were carried out to visualize actin cytoskeletal changes. Both siRNAs reduced

endogenous RhoGDI1 without affecting endogenous level of RhoGDI2 (Figure 38A). The siRNA were co-transfected with Invitrogen BLOCK-iT™ Alexa Fluor® Red Fluorescent Oligo to indicate transfection efficiency. siRNA transfection efficiency in HeLa cells was up to 90%. Compared to the luciferase-siRNA transfected control cells, the cell morphology was unaffected by RhoGDI1 silencing (Figure 38B).

Figure 38. RhoGDI1 knockdown in HeLa cells. (A) Total protein lysates were harvested from HeLa cells transiently transfected with siRNA-Luciferase (control), siRNA-RhoGDI1_1 and siRNA-RhoGDI1_2. 48 hrs after transfection, cells were harvested and equal amount of proteins were subjected to western blot analysis using RhoGDI1-specific antibody. β-tubulin was used as the loading control. (B) HeLa cells were transiently co-transfected with siLuc control, siRhoGDI1_1/2 and Invitrogen BLOCK-iT™ Alexa Fluor® Red Fluorescent Oligo to indicate transfection efficiency. 48 hrs later, bright field (BF) and rhodamin (Rhod) images were captured using fluorescent microscope. Scale bar, 200μm.

No significant differences of the actin cytoskeleton were observed between the control and RhoGDI1 knockdown cells (Figure 39A-D). The number of focal adhesions in RhoGDI1 knockdown cells also remained the similar as in the control cells (Figure 39E). The expression levels of vinculin, FAK and paxillin were unaffected by RhoGDI1 depletion (Figure 39F). In conclusion, RhoGDI1 knockdown showed no prominent effects on the actin cytoskeleton and focal adhesions.

Figure 39. RhoGDI1 knockdown did not affect the actin cytoskeleton. HeLa cells were transiently transfected with luciferase siRNA, siRhoGDI1_2 and stained with phalloidin (A), vinculin (B), FAK (C), paxillin (D) to visualize stress fibers and focal adhesions. Representative images were shown here. Scale bar, 30 μ m. (E) The number of focal adhesions were counted in every 15 cells from 15 distinctive fields on each coverslips. Results were averaged from three independent experiments. Error bars represent standard deviations. (F) Focal adhesion proteins, vinculin, FAK and paxillin were also examined by western blot using their respective specific antibodies. β -tubulin was used as the loading control. Representative images from three biological replicates were shown here.

3.3.4 RhoGDIs associated with RhoGTPases *in vitro*

RhoGTPases are key regulators of the actin cytoskeleton. Active RhoA induces the formation of stress fibers and cell contraction, whereas active Cdc42 and Rac1 result in filopodia and lamellipodia formation, respectively²⁰⁷. To elucidate the mechanism through which RhoGDIs affect the actin cytoskeleton, we firstly examine RhoGDIs' association with Rho proteins using the *in vitro* Flag pull-down assay (Figure 40). In contrast to RhoGDI1, which formed complexes with RhoA, Cdc42 and Rac1, RhoGDI2 only associated with Rac1. Nevertheless, the complex formation between RhoGDI2 and Cdc42, although quite weak, could still be detected. RhoGDI2 could not bind RhoA. Accordingly, effects of RhoGDI1 on actin cytoskeleton were more significant than RhoGDI2, which was possibly due to their differential binding affinity to the Rho proteins.

Figure 40. RhoGDIs associated with Rho proteins. HeLa was co-transfected with Flag tagged RhoGDI1/2 and HA tagged RhoA, Cdc42 or Rac1. 24hrs after transfection, cell lysates were harvested and subjected to Flag pull-down, SDS-PAGE and western blot analysis.

3.3.5 A single isoleucine residue at the C-terminal region of RhoGDI1 was critical to Cdc42 binding

Although RhoGDI1 and RhoGDI2 share 73.6% identity (Figure 41A), they show preferential binding to different Rho proteins. What contributes to the binding differences between RhoGDI1 and RhoGDI2? Do their associations with Rho proteins contribute to actin cytoskeletal changes? Site-directed mutagenesis shows that a single change at residue N174 of RhoGDI2 to the corresponding residue of RhoGDI1 (I177) can impart nearly full RhoGDI activity for inhibition of GDP dissociation from Cdc42^{208, 209}. To verify whether a single residue change could affect the complex formation between RhoGDIs and Cdc42, the GFP-Flag-RhoGDI1 (I177N) and GFP-Flag-RhoGDI2 (N174I) mutants were generated by site-directed mutagenesis. Flag pull-down assay was carried out. Indeed, RhoGDI1 (I177N) showed much weaker affinity to Cdc42 compared to RhoGDI1 WT, whereas the RhoGDI2 (N174I) formed stronger complex with Cdc42 than RhoGDI2 WT (Figure 41B). The data suggest that the presence of isoleucine at amino acid residue 177 of RhoGDI1 and the corresponding 174 of RhoGDI2 is important for Cdc42 interaction.

3.3.6 The two conserved leucine residues at the N-terminal region of RhoGDIs were important for binding RhoGTPases

NMR spectroscopy study of the structure of RhoGDI1-Rac1 complex suggests that a particular region in the N-terminal (residue 48 to 58) of RhoGDI1 contributes to complex formation between RhoGDI1 and Rac1¹⁴⁸. Further structural analysis of this region found two leucine residues, L55 and L56, which are responsible for stabilizing the complex²¹⁰. Through sequence alignment, these two leucine residues were also present in the corresponding sites in RhoGDI2 (L52 and L53) (Figure 42A). Next, to examine whether these two conserved leucine residues could be responsible for both RhoGDI1 and 2's association with Rac1, RhoA and Cdc42, two mutants were generated using site-directed mutagenesis method (RhoGDI1^{L55S,L56S} and RhoGDI2^{L52S,L53S}). These mutants were co-transfected with HA-tagged RhoA, Cdc42 and Rac1, and cell lysates were harvested for Flag pull-down assay. We observed mutation of the two leucine residues to serine resulted in defects in complexes formation between the RhoGDIs and all three Rho proteins (Figure 42B). Therefore, the two conserved leucine residues were important for Rho protein binding.

A

B

Figure 41. A single isoleucine residue at the C-terminal region of RhoGDI1 is critical to RhoGTPases binding. (A) RhoGDIs structures. (B) COS-7 cells were co-transfected with Flag tagged RhoGDI1/2 (WT or mutants) and HA tagged Cdc42. 24 hrs after transfection, cell lysate were harvested for Flag pull-down analysis, and 10% of total lysate was loaded.

A

B

Figure 42. The two conserved leucine residues at the N-terminal region of RhoGDIs are essential for Rho protein binding. (A) RhoGDIs structures. (B) COS-7 cells were co-transfected with Flag tagged RhoGDI1/2 (WT or various mutants) and HA tagged RhoA, Cdc42 and Rac1. 24 hrs after transfection, cell lysate were harvested for Flag pull-down analysis, and 10% of total lysate was loaded.

3.3.7 RhoGDIs affected actin cytoskeleton through RhoGTPases

Next, these RhoGDIs mutants (RhoGDI1^{I177N}, RhoGDI2^{N174I}, RhoGDI1^{L55S,L56S}, RhoGDI2^{L52S,L53S}), were utilized to examine whether RhoGDIs affect actin cytoskeleton through their binding to RhoGTPases. HeLa cells transfected with control plasmids, RhoGDI1/2^{WT} and RhoGDIs mutants were subjected to immunostaining with phalloidin, vinculin and paxillin to visualize actin cytoskeletal changes. Enhanced RhoGDI1/2^{WT} expression perturbed actin cytoskeleton with cells exhibiting loss of stress fibers and focal adhesions. On the contrary, RhoGDIs mutants (RhoGDI1^{I177N}, RhoGDI1^{L55S,L56S}, RhoGDI2^{L52S,L53S}), which showed defects in forming complexes with Rho proteins, exhibited normal cell morphology. Cells transfected with these mutants spreaded well like the control cells, and the stress fibers and focal adhesions were well-distributed and organized. In addition, the RhoGDIs mutant (RhoGDI2^{N174I}), which showed enhanced binding to Rho proteins, still led to both spindle-shaped or rounded cells with concomittant loss of stress fibers and focal adhesions (Figure 43A-C).

To better understand the link between effects of RhoGDIs on actin cytoskeleton and their binding efficiency to Rho proteins, the number of deformed cells induced either by RhoGDI1/2^{WT} or mutants were counted and plotted along with their binding efficiency to Rho proteins. We observed less cytoskeletal disruption in cells when the binding between RhoGDIs and Rho proteins are weaker (Figure 43D). Our observations suggest that RhoGDIs affect actin cytoskeleton through their interaction with RhoGTPases.

A

C

Figure 43. RhoGDI1/2 affected actin cytoskeleton through RhoGTPases binding. HeLa cells were transiently transfected with control, RhoGDI_1/2, and RhoGDIs mutants and immuno-stained with phalloidin (A), vinculin (B), paxillin (C) to visualize stress fibers and focal adhesions. Representative images were shown here. Scale bar, 30 μ m. (D) The spindle-like cells and rounded cells resulted from RhoGDI1/2 and RhoGDIs mutants overexpression were counted using 40 $\times$ objective in 100 cells from at least four different fields of every coverslips. Experiments were performed three times.

3.3.8 Both domains of RhoGDIs contributed to RhoGTPases binding

As shown in Figure 44A, RhoGDI1/2 contain two domains, the N-terminal flexible domain and the C-terminal folded domain¹³⁷⁻¹³⁹. Next, we went on to explore which domain contributed to the overexpression phenotype. Both the N- and C-terminal domains were sub-cloned into the pXJ-GFP-Flag vector and co-transfected with the HA-tagged Rho proteins. Flag pull-down was carried out subsequently. Consistently, RhoGDI1^{FL} obtained stronger

binding affinity to Rho proteins compared to that of RhoGDI2^{FL}. We found that none of the domains alone could interact with the Rho proteins (Figure 44B). Accordingly, both the N- and C-terminal domains of RhoGDIs contributed to RhoGTPases binding.

Figure 44. Both the N- and C-terminal domains of RhoGDIs contributed to Rho proteins binding. (A) RhoGDIs domain structures. (B) COS-7 cells were co-transfected with Flag tagged RhoGDI1/2 (full length or N- and C-terminal domains) and HA tagged RhoA, Cdc42 and Rac1. 24 hrs after transfection, cell lysates were harvested for Flag pull-down analysis, and 10% of total lysate was loaded.

3.3.9 Both domains of RhoGDIs contributed to actin cytoskeletal changes and cell rounding

We next investigated if the N- or C-terminal domains of RhoGDI can affect the actin cytoskeleton on their own. HeLa cells were transfected with either RhoGDIs^{FL} as positive control or domain constructs alone. Immunostaining on actin cytoskeleton using phalloidin, vinculin and paxillin were carried out. Enhanced RhoGDI1/2^{FL} expression perturbed the actin cytoskeleton with cells showing loss of stress fibers and focal adhesions. The N- and C-terminal domains, which failed to bind the Rho proteins on their own, were also unable to induce the rounded and spindle-like morphology (Figure 45A-C). The number of spindle-like and rounded cells also dramatically decreased when only RhoGDIs domains were overexpressed (Figure 45D). In conclusion, both the N- and C-terminal domains contributed to the regulation of the actin cytoskeleton. Moreover, these findings also supported that RhoGDIs were not able to affect the actin cytoskeleton without forming complexes with Rho proteins.

A

B

C

Figure 45. Both the N- and C-terminal domains contributed to actin cytoskeletal changes and cell rounding. HeLa cells were transiently transfected with Control plasmid, RhoGDI_1/2 full length, and RhoGDI domain mutants and immuno-stained with phalloidin (A), vinculin (B), paxillin (C) to visualize stress fibers and focal adhesions. Representative images were shown here. Scale bar, 30 μ m. (D) The spindle-like and rounded cells resulted from RhoGDI1/2 full length and RhoGDI domain mutants overexpression were counted using 40 $\times$ objective in 100 cells from at least four different fields of every coverslips. Experiments were performed three times.

3.3.10 Conclusion for Part III

In order to investigate the role of POPX2 in breast cancer cell motility, an integrative transcriptome and proteome study was carried out. In addition, we also investigated the effects of POPX2 on angiogenesis, and elucidated the underlying mechanism through combined phosphoproteome and secretome analysis. Meanwhile, we also took a candidate approach to investigate RhoGDIs because our earlier work suggested that the levels of RhoGDIs could be affected by POPX2 silencing. We have determined RhoGDI1 and RhoGDI2 affected actin cytoskeleton to different extent, the effects of RhoGDI2 was less prominent. Further characterization of RhoGDIs' interaction with the Rho GTPases revealed that RhoGDI1 and RhoGDI2 associated with different RhoGTPases. We have also verified that a single residue at the C-terminal region could affect RhoGDIs binding affinity to Cdc42. We also found two conserved leucine residues, which were essential to RhoGTPase binding. Both the N- and C-terminal domains of RhoGDIs contributed to Rho protein binding and regulation of the actin cytoskeleton.

Chapter 4 Discussion

4.1 An Integrative Transcriptome and Proteome Study to Identify the Signaling Network Regulated by POPX2 Phosphatase¹⁷⁰

4.1.1 Integrative of transcriptome and proteome study

In this part of the project, we focus on the role of POPX2 in breast cancer cell migration and further map POPX2 related signaling pathways in breast cancer. A global gene expression profiling and proteomics analysis helps us to achieve objectives efficiently. A number of commonly differentially-regulated pathways were over-represented through our integrative approach of protein and gene profiling. The implicated pathways included those involved in the regulation of the following: “focal adhesion”, “cell cycle”, “actin cytoskeleton”, “MAPK1/3 kinase signaling pathway” (Table 3). Meanwhile, several other integrative transcriptome and proteome studies at the pathway level have also been published recently. In the study of androgen receptor signaling²¹¹, changes of candidate proteins are also examined at the transcriptional levels using RT-PCR. Although the protein and mRNA changes do not correlate well in study by Martyniuk et al., 2009, both transcriptomic and proteomic approaches shed lights on the underlying regulatory mechanisms at the pathway level and provide complementary answers. In another investigation to identify protein and gene changes during activation of dendritic cells (DCs)²¹², the differentially regulated genes and proteins are mapped to functional signaling pathways and significantly enhance the predictive power of the combined proteomic and transcriptomic analysis.

In our combined study, we noticed that the numbers of down regulated genes corresponded to the numbers of down-regulated proteins. However, the numbers of up-regulated genes and proteins were not similar. In addition, the overall transcriptome changes in response to POPX2 knockdown did not strongly correlated with the overall proteome changes. We also compared the significantly up- or down-regulated proteome and transcriptome. Unfortunately, the comparison results showed a low overlap. One possibility is that POPX2 knockdown might enhance the transcription of some genes but has little effect on protein translation. In addition, the turnover of proteins might be differently regulated. Alternatively, the up-regulated proteins might be of low abundance and could not be easily detected. Such poor

correlation was also observed by several other recent reports. A combined analysis of proteome and transcriptome obtained from *Thermoplasma acidophilum* cultured under aerobic and anaerobic conditions has been reported²¹³. A very weak correlation between the transcript and protein changes is observed, suggesting the existence of extensive post-translational modification. Hence, it would be informative to have combined and integrative study of proteome and transcriptome to achieve a more comprehensive understanding of the system. In another paper which described the identification of tyrosine-phosphorylated proteins upon stimulation of ErbB receptor ligands, EGF or heregulin in the non-invasive breast cancer cell line (MCF7)²¹⁴, the SILAC-based quantitative mass spectrometry and Affymetrix gene expression array are adopted concurrently. The overlapping hits between the proteomic and transcriptomic data-sets also appear to be very low, which may due to transcript or protein instability, and multiple post-translational modification and regulation.

4.1.2 MAPK1/3 and GSK3 α / β kinase activity were reduced in POPX2 knockdown cells

Two proline-directed kinases motifs (PXpSP motif and pSP motif) were overrepresented through motif analysis when we analysed the phosphoproteome data (Figure 19A). MAPK1/3 and GSK3 α / β are the two kinases, which phosphorylate these two motifs. The overrepresented kinases motifs suggest that POPX2 might regulate phosphorylation through these two kinases. Our follow-up western blot validation confirmed that in response to POPX2 knockdown there were decreased phosphorylation levels at T185/Y187 of MAPK1 (T202/Y204 of MAPK3) and increased phosphorylation levels at S21/S9 of GSK3 α / β (Figure 19B-E). Phosphorylation at T185/Y187 of MAPK1 (T202/Y204 of MAPK3) renders the kinase active whereas S21/S9 of GSK3 α / β are the phospho-inhibitory sites. The results we obtained from both proteomic analysis and western blot analysis suggest that the kinase activities of MAPK1/3 and GSK3 α / β are inhibited when POPX2 is silenced.

MAPK1/3 kinase signaling pathway is one of the most well studied mammalian signaling pathway. The MAP kinase pathway has been found to regulate many biological activities such as cell proliferation, apoptosis, and transcription regulation²¹⁵. In addition to its role in these biological activities, MAPK1/3 signaling is also highlighted in a number of cancer cell

migration and metastasis-related processes²¹⁶. Activation of MAPK signaling, in cooperation with other signaling pathways and functional proteins, promote cancer cell migration, invasiveness and metastasis²¹⁷⁻²¹⁹. Consistently, we found MAPK1/3 to be less phosphorylated and hence less active in POPX2 knockdown cells, which corresponded to slower cell motility observed.

GSK α/β are mainly involved in Wnt/ β -catenin signaling and the PI3K/Akt signaling pathway. They also play an indispensable role in adherens junction dynamics, cell cycle progression, apoptosis and microtubule dynamics¹⁷⁹. The role of GSK3 β in cancer progression is also heavily studied. It is reported that GSK3 β inhibition enhances angiogenesis and tube formation in *in vivo* matrigel plug assays²²⁰. Increased phosphorylation levels at S9 corresponds to decreased GSK3 β kinase activity, which leads to inhibition of gastric cancer cell migration²²¹. In our current study, we also found elevated phosphorylation of S9 along with reduced breast cancer cell migration. Our findings are consistent with the reported cancer-related observations. It is highly possible that POPX2 regulates breast cancer motility through MAPK1/3 and GSK3 β , in cooperation with other signaling pathways. In contrast to what we observed in POPX2 knockdown MDA-MB-231 breast cancer cells, a higher phosphorylation level at S9 and thus more inactive GSK3 β were reported in POPX2-overexpressing NIH3T3 cells¹³². The contradictory observations could be due to different cell types used. In the NIH 3T3 cells, POPX2 may affect GSK3 β activity through the AKT signaling pathway²²². Apart from AKT, several other kinases have also been found to phosphorylate GSK3 α and GSK3 β at S21 and S9, respectively²²³.

4.1.3 Cell migration and microtubule dynamics

From our proteome analysis and western blot validation using specific antibodies, we found lower stathmin expression level in POPX2 knockdown cells (Figure 20B-C). Stathmin is mainly responsible for microtubule destabilization. Microtubule stability is critical for cell migration, thus perturbed microtubule dynamics is reported to affect cell migration significantly⁶⁶. The microtubule-destabilizing activity of stathmin is turned off by phosphorylation in various combinations of four different phosphorylation-sites: S16, S25, S38 and S63⁶². Decreased phosphorylation level at S38 and less acetylated-microtubule

(stable microtubule) were observed in the POPX2 knockdown cells concurrently (Figure 20B-C and Figure 21). In addition, the S25 and S38 of stathmin are reported target sites of MAPK1/3²²⁴. Since POPX2 knockdown resulted in declined MAPK1 phosphorylation level and thus lower kinase activity, it is possible that silencing POPX2 led to lower stathmin phosphorylation in addition to lower levels of stathmin itself. All in all, the overrepresented MAPK1 sub-network and microtubule stability perturbation further supported the involvement of POPX2 in various actin, focal adhesion and microtubule-based biological processes through MAPK1/3 and their downstream substrates, possibly leading to the decreased cell migration eventually.

4.1.4 POPX2 knockdown impacted on transcriptional regulation

Several transcription factors which are regulated by MAPK1/3 and GSK3 β were overrepresented from the transcriptome analysis (Figure 24). CEBPA, FOXO3, GR, HSF1 and SREBP1 are reported to be inactivated by increased MAPK1/3 and GSK3 β phosphorylation. Thus higher transcriptional activities of this class of transcription factors are expected in the POPX2 knockdown cells, resulting from less active MAPK1/3 and GSK3 β . All these five transcription factors are crucially involved in proliferation, apoptosis, and cell cycle processes through MAPK1/3 and GSK3 β -dependent phosphorylation modulation²²⁵⁻²³¹. In addition, ATF2, CREB, ELK1 and ER are reported to be positively regulated by MAPK1/3 and GSK3 β phosphorylation. Thus lower transcriptional activities of this class of transcription factors are expected in the POPX2 knockdown cells, possibly due to the inactivation of MAPK1/3 and GSK3 β by silencing POPX2. These four transcription factors are also reported to be key components in cell cycle, cell growth and apoptosis, especially CREB, which is actively involved in MAPK kinase-led cell growth and differentiation^{222, 232-236}. Our earlier work has already shown that POPX2 is involved in the regulation of Serum Response Factor (SRF) mediated transcription²³⁷. Hence it is likely that POPX2 is involved in other gene regulation processes through modulating the transcription factors downstream of MAPK1/3 and GSK3 β .

4.2 An Integrative Phosphoproteome and Secretome Study to Elucidate the Effects of POPX2 phosphatase on Tumor Angiogenesis

4.2.1 CDK1 kinase activity is affected by POPX2 knockdown

In order to decipher the role of POPX2 in tumor angiogenesis, SILAC-based quantitative phosphoproteomic approach was adopted. POPX2 knockdown significantly impacted phosphoproteome in breast cancer cells, especially those phosphoproteins classified into the kinases category. From our phosphoproteome analysis, we found less phosphorylation on T14 and Y15 on CDK1 (cyclin dependent kinase 1) in POPX2 knockdown cells (Table 4). Phosphorylation levels of these two residues affect the kinase activity of CDK1¹⁹³. CDK1 plays a crucial role in cell cycle regulation¹⁹². Decreased phosphorylation levels at T14 and Y15 implicated more active CDK1 in POPX2 knockdown condition, suggesting that POPX2 might play a role in regulating cell cycle and cell proliferation.

The role of CDK1 in tumor growth has also been recorded²³⁸. Highly active CDK1 is reported to promote tumor cell proliferation and cell survival through phosphorylation and inactivation of the transcription factor FOXO1²³⁹. In a recent report, CDK1 is found to be highly expressed in lung tumor tissues and correlated to the lung cancer survival²⁴⁰. It is conceivable that POPX2 might be involved on the regulation of cell cycle progression and cell proliferation through the modulation of CDK1 activity.

4.2.2 Exosome enrichment in the secretome of POPX2 knockdown cells

Angiogenesis is not only affected by pro- or anti-angiogenic factors but also by other micro-effectors, such as exosomes²⁴¹. Exosomes are 30-100nm membrane vesicles derived from endosome, and reported to be secreted by several cell types, including B cells, dendritic cells, mast cells, T cells and tumor cells²⁴². Numerous proteins have been reported to be associated with the exosomes. The ExoCarta database remains the most popular database for information related to the exosomes^{123, 243}. The presence of exosomes in the secretome is usually verified by western blot analysis using exosome marker like CD81, CD9, CD63, Alix^{194, 196}. Based on our secretome analysis (ExoCarta database), we observed that a high percentage of identified hits were associated with the exosomes (Figure 30A). We have also

validated the conditioned media collected by western blot analysis using antibodies specific to the known exosome markers, CD81 and CD9. We confirmed that a high amount of exosomes was found in the conditioned media of POPX2 knockdown cells (Figure 30B).

It has been reported that exosomes are involved in promoting angiogenesis, tumor growth and facilitating dissemination of tumor cells^{194, 195}. Proteomic analysis of the tumor-derived exosomes implicates the presence of DEL-1 (developmental endothelial locus-1) in exosomes. DEL-1 is a strong pro-angiogenic factor and is able to increase vascular development²⁴⁴. In lung cancer cells, tumor derived exosomes also induce VEGF expression and promote angiogenesis, which finally contributes to cancer metastasis²⁴⁵. It has also been reported recently that exosomes are able to affect angiogenesis by associating with nano-tube structures suggesting that exosome might provide a communication link between tumor cells and endothelial cells. In addition, exosome is also able to control the cytokine expression profiles²⁴⁶. In summary, exosome secretion plays important roles in angiogenesis and tumor growth²⁴⁷⁻²⁴⁹. Since we found enrichment of exosomes in POPX2 knockdown cells, it is of interest to pursue the roles of POPX2 in the regulation of angiogenesis for future research.

4.2.3 EMT and angiogenesis

Several cancer-related signaling pathways are overrepresented from KEGG pathway analysis, among which the “ECM-receptor interaction” and “Adherens junction” proteins appear to be particularly affected in our secretome dataset (Figure 31-33). To examine the role of POPX2 in cell-ECM and cell-cell interactions, we further investigated cell attachment to fibronectin (and collagen) and epithelial-mesenchymal transition. Our validation work using western blot analysis and cell staining confirmed that cell attachment to the ECM was perturbed in POPX2 knockdown cells and silencing POPX2 promoted EMT in breast cancer cells (Figure 34-35).

EMT describes the transition from epithelial cells to mesenchymal cells, with the loss of tight junction, adherens junction and desmosome. EMT and its reversible process, MET, is an essential step during cancer metastasis and angiogenesis. E-cadherin, the critical epithelial marker, is significantly repressed when cells undergo EMT and also leads to increased cell

motility and invasiveness. In addition, E-cadherin repression results in β -catenin-VEGF activation, which finally contributes to tumor angiogenesis²⁵⁰. Based on these preliminary findings, it is recently reported that the epithelial-mesenchymal transition process facilitate angiogenesis via β -catenin activation and VEGF induction²⁵¹. Consistently, we observed an increased angiogenesis and accelerated EMT progression in the POPX2 knockdown cells, suggesting possible POPX2 involvement in angiogenesis through regulating EMT progression.

E-cadherin is required to maintain stable adherens junctions in the epithelial cells and its expression is repressed during EMT process^{93, 95}. E-cadherin transcriptional repression is resulted from the activation of some transcription repressors, e.g. Snail, Slug, Sip1 and Ets, which are regulated by MAPK and GSK3 β kinases^{96, 97}. Inhibited GSK3 β kinase activity stimulates Snail transcription to repress E-cadherin expression and to promote EMT progression in breast tumor²⁵². Moreover, another study carried out in breast cancer implicates the suppression of E-cadherin expression through decreased GSK3 β activity and nuclear accumulation of Slug²⁵³. Consistent with these findings, an elevated phosphorylation of S9 corresponding to a lower GSK3 β kinase activity is observed in POXP2 knockdown cells (Figure 19) and these cells also appear to have suppressed E-cadherin expression and increased EMT progression (Figure 35). It is probable that POPX2 could regulate E-cadherin expression at transcript level via affecting GSK3 β activity and its downstream transcription factors.

4.3 Functional study of RhoGDIs, Candidate Proteins Regulated by POPX2 phosphatase Related Pathways

4.3.1 Effects of RhoGDIs on actin cytoskeleton

We have decided to investigate the roles of RhoGDIs because our earlier work has shown that in POPX2 knockdown cells the levels of RhoGDIs are also affected¹. Overexpression of either RhoGDI1 or RhoGDI2 affected actin cytoskeleton to different extent in HeLa cells. The effects of RhoGDI2 was less prominent than RhoGDI1 (Figure 36-37). Our observation is consistent with what has been reported¹³⁵. However, the mechanism through which RhoGDIs exerts destructive effects on actin cytoskeleton is not clear and we have explored further in this study.

It was interesting that overexpression of RhoGDIs exerted strong effects on actin cytoskeleton, however, silencing of RhoGDI1 in HeLa cells had no prominent effects on either stress fiber or focal adhesions (Figure 38-39). In mammalian cells, only effects of silencing RhoGDIs on cell migration and invasion have been reported. The effect of knocking down RhoGDI on cell morphology is not documented. In yeast, silencing of RhoGDI1 does not affect cell morphology but overexpression blocks cell budding dramatically²⁵⁴.

4.3.2 RhoGDIs associated with Rho proteins

Since RhoGDIs are one of the main regulators of the Rho GTPases²⁵⁵. We investigated how the binding of Rho GTPases to RhoGDIs can modulate the actin cytoskeleton. In HeLa cells, RhoGDI1 associated with RhoA, Cdc42 and Rac1 while RhoGDI2 preferentially associated with Rac1 (Figure 40). The stronger affinity to RhoGTPases might explain why RhoGDI1 exerted more prominent effect on actin cytoskeleton.

In order to elucidate what contributed to the binding differences between RhoGDI1 and RhoGDI2, several mutants were generated. It was reported that a single swap of residues from I to N of residue 177 in RhoGDI1 and N174 to I in Rho GDI2 could change the ability of RhoGDI1 and RhoGDI2 on inhibiting GDP dissociation from Cdc42^{136, 147}. Our results

further verified that this single residue change could affect RhoGDIs' binding affinity to Cdc42 (Figure 41). We also checked whether a single residue substitution can change RhoGDIs' binding affinity to RhoA and Rac1, but no significant changes were observed.

Structural analysis suggests that the two leucine residues in the N-terminal of RhoGDI1 are responsible for stabilizing RhoGDI1-Rac1 complex¹⁴⁹. We observed that these two leucine residues were conserved in RhoGDI2. We went on to show that these two conserved leucine residues were critical to RhoGTPases binding, not only to Rac1 but also to RhoA and Cdc42 (Figure 42). Although these two mutants (GDI1^{L55S, L55S}, GDI2^{L52S, L53S}) are defective in RhoGTPases binding, whether they can serve as dominant negative RhoGDIs still remains to be investigated. However, these mutants helped us to elucidate that RhoGDIs affected actin cytoskeleton via their interaction with RhoGTPases. RhoGDI1^{L55S, L56S} and RhoGDI2^{L52S, L53S}, which showed defects in forming complexes with Rho proteins, are not able to induce rounded and spindle-like morphology (Figure 43). Future work includes experiments to determine whether the mutants can block the roles of RhoGDIs in cancer cell migration and invasion.

RhoGDI2 is up-regulated in response to POPX2 knockdown at both transcript and protein levels (from our DNA microarray and proteome quantification analysis). It is possible that they are involved in the same or related signaling pathways to regulate the actin cytoskeleton. From our candidate approach, we are able to show that both RhoGDI2 overexpression and POPX2 knockdown lead to loss of actin stress fibers. However, we have not been able to decipher the links between RhoGDI2 and POPX2 through our analysis of the proteomic and genomic data.

Chapter 5 Conclusions and future directions

Conclusions

In this study, we explore the biological functions of POPX2 and its roles in cancer cell motility and angiogenesis by three different approaches: genomic, proteomic and candidate. DNA microarray was adopted for the genomic approach, which we later combined with the proteomic work into an integrated study. We were able to identify key signaling pathways involved. Our data suggest that silencing of POPX2 could lead to down-regulation of MAPK1/3 and GSK3 activities, which in turn affect cell motility. This is consistent with the slower migration speed observed for POPX2 knockdown MDA-MB-231 cells.

Since we have earlier observed that POPX2 knockdown MDA-MB-231 cells enhanced angiogenesis in the CAM assays (Chan and Koh, unpublished data), we proceeded to investigate the secretome and phosphoproteome of these cells. Our phosphoproteome study implicates the involvement of POPX2 in the regulation of CDK1 phosphorylation status and thus activity. Further analysis of the role of POPX2 in cell cycle progression is on-going. The secretome analysis and the follow-up biological validation suggest the enrichment of exosome and accelerated EMT progression in response to POPX2 silencing. These results could further explain the increased angiogenesis observed.

Meanwhile, we also undertook a candidate approach to investigate RhoGDIs because our earlier work showed that the levels of RhoGDIs could be affected by POPX2. We have determined that RhoGDI1 and RhoGDI2 affect actin cytoskeleton to different extent. The effects of RhoGDI2 are less prominent. Further characterization of RhoGDIs' interaction with the Rho GTPases reveals that RhoGDI1 and RhoGDI2 bind to different RhoGTPases. We also verified that a single residue in the C-terminal region could swap RhoGDIs binding affinity to Cdc42. More interestingly, we found two conserved leucine residues which are critical to RhoGTPase binding. These GDIs-mutants are then used to elucidate that RhoGDIs affect actin cytoskeleton via their efficient binding to RhoGTPases.

In this study, we are able to explain how the lower motility of POPX2 knockdown cells could be related to the lower activity of MAPK1/3 and GSK3, and we are able to reconcile our data to the observations that higher levels of POPX2 are found in the more invasive cancer cell lines. However, one paradox remains; higher POPX2 levels are found in more invasive cancer but lower POPX2 levels appear to favour angiogenesis and cell proliferation. Could it be that for cancer cells to be invasive, high levels of POPX2 are required? On the other hand, POPX2 levels need to be downregulated for angiogenesis to occur and the tumour to proliferate at a secondary site. We hypothesize that there exists a “switch”, which leads to changes in POPX2 levels at different stages of cancer development. Future work will focus on the elucidation of the “switch”.

Future Directions

Future work which arises from our current project includes

1. To study how POPX2 can modulate CDK1 activities and thus cell cycle progression and cell proliferation. Since the critical T14 and Y15 are not direct substrates of POPX2, the phosphatase must have exerted its effect on other kinases or phosphatases to regulate the phosphorylation status of T14 and Y15 of CDK1.
2. To investigate how POPX2 can regulate exosome secretion and angiogenesis. Since exosome secretion increases in the conditioned medium obtained from POPX2 knockdown cells, we propose to examine the roles of POPX2 silencing-induced exosomes in regulating angiogenesis and tumor growth.
3. To examine how POPX2 can affect the pro-angiogenic factors secretion which in turn regulate angiogenesis. Although we have found that silencing POPX2 leads to increased exosome secretion, this may not be the only mechanism that controls angiogenesis in POPX2 related context. We propose to study some common cytokines and angiogenic factors using cytokine arrays to further narrow down the cytokines or angiogenic factors involved.
4. To determine if there is a “switch” which might regulate the levels of POPX2 at different stages of cancer development.

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