Understanding the biological functions of POPX2 phosphatase through its interactome

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**Understanding the biological functions of POPX2
phosphatase through its interactome**

Kim Purum

SCHOOL OF BIOLOGICAL SCIENCES

Aug 2019

**Understanding the biological functions of POPX2
phosphatase through its interactome**

Kim Purum

SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to the Nanyang Technological
University in partial fulfilment of the requirement for
the degree of Doctor of Philosophy

Aug 2019

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Abbreviations

ABP	Actin binding protein
BSA	Bovine Serum Albumin
CA	Calyculin A
CaMKII	Calcium/calmodulin-dependent protein kinase II
Cdc42	Cell division cycle 42
Chk1	Check point kinase 1
CK2	Casein kinase 2
CPT	Camptothecin
DDR	DNA damage response
DMEM	Dulbecco's modified Eagle's medium
DN	Dominant Negative
DSBs	Double-strand DNA breaks
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial to Mesenchymal Transition
FBS	Fetal Bovine Serum
GAP	GTPase-activating protein
GBM	Glioblastoma
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GSK3	Glycogen synthase kinase 3
GST	Glutathion S-transferase
HNSCC	Head and neck squamous cell carcinoma
HRP	Horseradish peroxidase
HU	Hydroxyurea
IR	γ -irradiation
MAL	Megakaryoblastic leukemia
MFS	Metastasis free survival
MMP9	Matrix metalloproteinase 9
MS	Mass spectrometry
PAK	P21-activated kinase
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
POPX	Partner of PIX
RhoGDI	RHO protein GDP dissociation inhibitor of Rho proteins
SCLC	Small cell lung carcinoma
SRF	Serum response factor
SSBs	Single-strand DNA breaks
TNBC	Triple Negative Breast Cancer
YB-1	Y-box binding protein-1
β PIX	PAK-interacting exchange protein

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Abstract

POPX2 (Partner of PIX 2) is a serine/threonine phosphatase known to dephosphorylate PAK1, CaMKII and TAK1. POPX2 has been reported to be positively associated with cell motility and invasiveness of breast cancer cells. In order to further investigate the roles and functions of POPX2 in the cells, we have adopted two approaches to identify POPX2-interacting proteins. In the first approach, we pulled down Flag-tagged POPX2 and determined the co-precipitated proteins using mass spectrometry (MS). We discovered Coronin 1C (Coro 1C) as a potential POPX2-interacting protein. In the second approach, we used a combination of SILAC-MS proteomics and bioinformatic analysis and identified Check point kinase 1 (Chk1) as a binding partner and possible substrate of POPX2.

Coro 1C binds to F-actin and regulates the cellular actin network through cross-linking and bundling. Here, we confirmed the biological interaction between POPX2 and Coro 1C. We found that POPX2 binds to the C-terminus of Coro 1C. Furthermore, Coro 1C is dephosphorylated by POPX2 *in vivo* and *in vitro*, suggesting that Coro 1C is a substrate of POPX2. As POPX2 interacts with the C-terminus of Coro 1C, we further identified two potential phospho-sites of Coro 1C (Thr415 and Ser463) that could be dephosphorylated by POPX2. In order to study the functional role of phosphorylation on Coro 1C, we generated phospho-mutants (T415E, T415A, S463D, and S463A). Cell migration assay reveals that overexpression of phospho-dead mutant (T415A and S463A) leads to higher cell motility than phospho-mimic mutant (T415E and S463D), implying that dephosphorylation on Coro 1C promotes cell migration. Subsequently, we

observed that POPX2 promotes the localization of Coro 1C to the leading edge of the cells, which results in enhanced cellular protrusion and spreading. Taken together, we propose that POPX2 promotes cell spreading and motility through dephosphorylation of Coro 1C and its resultant translocation to the leading edge of the cells.

A combination of SILAC-MS proteomics and bioinformatic analysis allows us to identify Chk1 as a potential target of POPX2. Chk1 is an essential regulator of DNA damage pathway and cell cycle progression. Activation of Chk1 induces cell cycle arrest at G2 phase for DNA repair or apoptosis. Here, we discovered that the PP2C domain of POPX2 interacts with the Pkinase domain through domain-domain interaction. Subsequently, we narrowed down possible substrates with the Pkinase domain from SILAC-MS data and discovered 46 possible substrates. Out of the 46 proteins identified, Chk1 exhibits similar phylogeny as known targets of POPX2. Thus, we investigated the possible interaction between POPX2 and Chk1. Our results show that POPX2 interacts with Chk1 and dephosphorylates Chk1 at Ser317 and Ser345 in response to DNA damage. Our approach led us to identify POPX2 as a regulator of Chk1 in response to DNA damaging drug.

1. Introduction

1.1 The POPX phosphatases (Partner of PIX)

1.1.1 POPX2 is a PP2C phosphatase

Type 2C Protein phosphatases (PP2C) are the main enzyme subtype of protein phosphatase Mg^{2+} or Mn^{2+} dependent (PPM) family and dephosphorylate a broad range of substrates. The activity of PP2C requires Mn^{2+} or Mg^{2+} , and its activity is not sensitive to general phosphatase inhibitors such as Calyculin A and Okadaic acid, which inhibit PP1 and PP2A (Cohen, 1989).

The POPX (Partner of PIX) phosphatases belong to the PP2C phosphatase family and consist of POPX1 (PPM1E) and POPX2 (PPM1F), which are of 757 and 454 amino acids, respectively. The POPX phosphatases were first identified in a two-hybrid screen using full-length PIX. Expression of POPX1 is found to be enriched in brain and testis tissues, while POPX2 is ubiquitously expressed in most human tissues (Koh et al., 2002). Rat POPX2 is also known as rat Ca^{2+} /calmodulin-dependent protein kinase phosphatase (rCaMKPase). Rat POPX2 was first identified from rat brain protein extract in a phosphatase screen using the phosphopeptide corresponding to a fragment of Ca^{2+} /calmodulin kinase II (CaMKII) (Ishida et al., 1998). CaMKII α is a serine/threonine kinase enriched in brain and has been implicated in learning, memory and neural plasticity (Irvine et al., 2006; J. Lisman et al., 2002; John Lisman et al., 2012; Lucchesi et al., 2011) and also in Ca^{2+} homeostasis in cardiac myocytes (Grueter et al., 2007). CaMKII is found to be dephosphorylated by POPX2 at its auto-phosphorylation site, Thr286 (Tan et al., 2001). Overexpression of POPX2 in fibroblasts reduces CaMKII α activity and phosphorylation levels of its downstream substrate

vimentin (Harvey et al., 2004). POPX2 has also been reported as a human homologue of FEM-2 from *C. elegans* (hFEM-2), sharing 79% of amino acid identity with rCaMKPase and found to promote apoptosis in mammalian cells (Tan et al., 2001). Apart from CaMKII, other substrates of POPX2 have also been identified. They will be discussed in sections below.

1.1.2 The roles of POPX2 in signaling pathways regulated by CDC42 and RhoA

POPX proteins were identified as binding partners of PIX (CDC42/Rac-specific guanine nucleotide exchange factor) in a two-hybrid screen (Koh et al., 2002). It has been revealed that POPX2 forms a trimeric complex with β PIX and PAK1 (p21-activated Kinase 1). In this trimeric complex, we find an activator of CDC42 and Rac (β PIX), together with the effector of CDC42 and Rac (PAK) and a negative regulator of PAK (POPX2). The activity of PAK1 is activated by CDC42/Rac and inactivated by POPX2. Active CDC42/Rac interacts with PAK1 and leads to activation and auto-phosphorylation of PAK1 at Ser57 and Thr423 in the kinase activation loop. PAK1 is negatively regulated by POPX2 through dephosphorylation on Ser57 and Thr423. Active PAK1 induces stress fiber loss and the disassembly of focal adhesions in cells (Chong et al., 2001), whereas introduction of POPX2 into the cells resulted in robust stress fibers by inhibiting PAK1-induced stress fiber breakdown (Koh et al., 2002). Therefore, POPX2 plays a role in stress fibers maintenance via the CDC42- β PIX/PAK1 pathway.

POPX2 also plays a role in RhoA-mDia1 regulated signaling pathways. RhoA modulates stress fibers through mDia1 and ROCK/ROK (Watanabe et al., 1997). mDia1 belongs to the diaphanous family of formins, which catalyzes actin

polymerization at the plus end. mDia1 normally adopts a closed conformation through head-to-tail interaction. When active RhoA binds to mDia1, the auto-inhibition is relieved and mDia1 becomes activated (Alberts, 2001; Otomo et al., 2005). Expression of the dominant negative form of mDia1, mDia1-DN, induces stress fiber breakdown. POPX2 binds to mDia1-DN containing FH3 domain. Overexpression of POPX2 can block the effect of mDia1-DN on stress fibers, suggesting that POPX2 might be involved in the maintenance of stress fibers through in a cooperation of CDC42- β PIX/PAK1 and RhoA-mDia1 pathways.

RhoA also modulates transcription through Serum Response Factor (SRF). SRF-mediated transcription is sensitive to actin dynamics and the ratio of G- and F-actin in the cells (Posern et al., 2002; Sotiropoulos et al., 1999). RhoA-actin signaling regulates the subcellular localization of a myocardin-related SRF co-activator (MAL1). MAL monitors the levels of actin in the cytoplasm and coordinates SRF-mediated transcription (Miralles et al., 2003). The interaction between POPX2 and mDia1 leads to inhibition of SRF-mediated transcription by blocking the nuclear translocation of MAL1 (Xie et al., 2008), suggesting the negative role of POPX2 in SRF-mediated transcription.

1.1.3 The role of CaMKII /POPX2-KIF3A-N-cadherin pathway in cell-cell contacts

Cadherins, a family of Ca^{2+} -dependent cell adhesion molecules (CAMs), localize to the cell surface and mediate specific cell-cell contacts and communication through its homophilic binding between cells (Lodish et al., 2000). The kinesin-2 motor complex consists of two motor units KIF3A, KIF3B and one non-motor

unit KAP3. The KIF3 motor complex delivers N-cadherin and β -catenin to the cell-cell contacts (Hirokawa, 2000).

It has been reported that POPX2 perturbs KIF3A-mediated cargo transport of N-cadherin through dephosphorylation of KIF3A on Ser690 (Phang et al., 2014). Phosphorylation of KIF3A on Ser690 by CaMKII within the tail domain induces a conformational change and releases the tail domain from auto-inhibition, whereas dephosphorylation of KIF3A on Ser690 by POPX2 leads to auto-inhibition of KIF3A and affects its role in cargo delivery (Fig 1) (K. Chen et al., 2018). Thus, POPX2 overexpressing cells have impaired cell-cell contacts due to the lack of N-cadherin transport to the cell periphery (Phang et al., 2014). Perturbed cell-cell contacts can contribute to cancer progression, for instance, loss of cadherins on cell surface affects cell adhesion and migration in cancer (Lodish et al., 2000). Furthermore, it could cause impaired contact inhibition and anchorage independent growth in cancer cells (Ozawa, 2015).

Figure 1. Schematic diagram of Kinesin-2 activation.

Kinesin-2 consists of KAP3, KIF3A and KIF3B. KIF3A is phosphorylated at S690 (S690 for human, S689 for mouse) by CaMKII and dephosphorylated by

POPX2. Phosphorylation of KIF3A results in release of auto-inhibition and cargo transport on microtubule networks, whereas dephosphorylation of KIF3A induces close conformation. (The image was retrieved from Chen et al., 2018)

1.1.4 The role of CaMKII /POPX2-KIF3A-N-cadherin pathway in cell polarity

N-cadherin transport to the cell periphery is important for mediating cell-cell adhesion as well as establishment of cell polarity. Cell polarity is essential for directional migration, differentiation of stem cells, wound healing and immune response. Par-3 and N-cadherin are cell polarity regulators and they are transported to the cell periphery by the KIF3 motor (Dupin et al., 2009; Schmoranzer et al., 2009; Suzuki & Ohno, 2006). In migrating fibroblast, cells move in a polarized manner with the centrosomes positioned between the leading edge of the cells and the nuclei. Changing the localization of N-cadherin may affect cell polarity through the alterations of the centrosome-nucleus axis (Dupin et al., 2009). Overexpression of POPX2 leads to impaired Par-3 and N-cadherin transport to the cell periphery due to defective KIF3 trafficking, resulting in cell migrating in random directions rather than straight into the wound in scratch wound assays (Hoon et al., 2014).

Deficiency in cell-cell contacts and random migration are hallmark features of metastasis. Loss of cell contacts leads to dissemination of tumor cells from the epithelial layer and neighboring cells. Moreover, loss of intrinsic cell polarity might cause tumor cells to become more sensitive to external chemotactic factors secreted by blood vessels in the primary tumor, resulting in intravasation (Condeelis & Segall, 2003; Ozawa, 2015; Shestakova et al., 2001). Thus, high

levels of POPX2 in the cells may promote early stages of cancer progression; dissemination and invasion through loss of cell-cell contacts and random migration (Fig 2).

Figure 2. Schematic illustration of cancer metastasis regulated by POPX2.

Cancer metastasis is driven by the ability of tumour cells to disseminate from the primary site to the secondary site. After dissemination, tumour cells invade into basement membrane and migrate towards blood vessels, follow by intravasation, circulation and extravasation. We postulate that 1) high levels of POPX2 in cells can promote dissemination through impaired cell-cell contacts mediated N-cadherin. 2) High levels of POPX2 in cells display random cell migration through alternation of the centrosome-nucleus axis.

1.1.5 The role of POPX2 in the Hippo pathway and anoikis resistance

The Hippo pathway plays a role in organ size control and has been implicated in cancer metastasis (Pan, 2010). Cancer cells with mutated members of the Hippo pathway acquire the capability of anoikis resistance and anchorage independency.

The Hippo pathway kinase cassette consists of kinases including MST1/2, LATS1/2 and NDR1/2. MST phosphorylates LATS and subsequently, active LATS phosphorylates the transcription co-activators, YAP/TAZ (Hergovich et al., 2006; B. Zhao et al., 2010). Phosphorylated YAP/TAZ are retained in the cytoplasm and subjected to proteasome degradation, whereas non-phosphorylated YAP/TAZ can translocate to the nucleus and interacts with the transcription factor, TEAD to induce gene expression. Many of the YAP/TAZ-TEAD target genes are involved in the regulation of cell proliferation and survival (Lin et al., 2017).

POPX2 participates in the regulation of the Hippo pathway through binding to the core kinases including MST1, LATS1 and NDR1. POPX2 negatively regulates the activity of LATS1 through dephosphorylation on Thr1079. This will result in more non-phosphorylated YAP/TAZ which might translocate to the nuclei. Nuclear YAP/TAZ binds to transcription factor TEAD and induce gene expression involving in cell proliferation and anchorage independent growth. Up-regulation of TAZ target gene expression has been implicated in promoting epithelial-mesenchymal transition (EMT) (Lei et al., 2008). On the other hand, depletion of POPX2 in the cells will result in decreased TAZ-target gene expression and decreases anchorage independent growth (Rahmat et al., 2019). Overall, POPX2 may play a role in anoikis resistance and anchorage independency, possibly through suppressing the Hippo pathway through inhibition of LATS (Fig 3).

Figure 3. Schematic illustration of the role of POPX2 in the Hippo pathway.

POPX2 dephosphorylates LATS1 at Thr1079, leading to translocation of YAP/TAZ into the nucleus. Nuclear YAP/TAZ interacts with transcription factor, TEAD and increases target gene expression involving in cell proliferation and anchorage independent growth. On the other hand, LATS1 remains active in POPX2-knockout cells and YAP/TAZ go through degradation, resulting in down-regulated TEAD target gene expression. (The figure was retrieved from Rahmat et al., 2019)

1.1.6 Dual regulatory roles of POPX2 in breast cancer metastasis

Screening of POPX2 expression in different types of breast cancer cell lines reveals that the levels of POPX2 are high in invasive cell lines, such as MDA-MB-231, while the levels of POPX2 are low in non-invasive cell lines, such as MCF-7. Depletion of POPX2 in MDA-MB-231 cells significantly reduces cell motility and invasiveness, possibly by modulating the GSK3 and ERK (MAPK) pathways (Susila et al., 2010; Zhang et al., 2013).

In this context, POPX2-knockdown can inhibit tumor progression via reduced cell motility and invasiveness. However, this is contrasted in late metastasis. Mice injected with POPX2-knockdown MDA-MB-231 cells exhibit larger and

more numerous tumour nodules at metastatic sites compared with mice injected with control cells. It was found that silencing POPX2 in MDA-MB-231 cells enhances tumor morbidity and metastasis through the secretion of proteins (Zhang et al., 2017). It has been reported that the secretome derived from POPX2-knockdown cells contained enriched exosome-associated proteins as well as increased cytokines and pro-angiogenesis factors. Consistently, *in vitro* angiogenesis assays show that the conditioned media collected from POPX2-knockdown cells increases tube length and vessel branch points, suggesting that silencing POPX2 leads to increased angiogenesis through induction of pro-angiogenetic cytokines (Zhang et al., 2017).

This is further supported by data from cancer patient samples. Information extracted from Oncomine, a web-based cancer microarray database, shows that POPX2 gene expression is high in triple negative breast cancer (TNBC) compared with non-TNBC (Fig 4A). Interestingly, POPX2 gene expression is low in metastatic sites compared to primary cancer sites in many different types of cancers (Fig 4B). These findings suggest that POPX2 might have dual regulatory roles in early and late stages of metastasis through regulating different signaling pathways including CaMKII-KIF3A pathway, MAPK pathway, Hippo pathway and secretion of cytokines.

A

B

Figure 4. PPM1F (POPX2) gene expression in patient samples from Oncomine database.

(A) POPX2 gene expression in TNBC and non-TNBC samples. (B) POPX2 gene expression in primary and metastatic sites in different types of cancers. (The figure was retrieved from Zhang et al., 2017)

1.1.7 POPX2 regulates apoptosis through the TAK1-IKK-NF- κ B pathway

The TAK1-IKK-NF- κ B pathway is activated in response to genotoxic stress and mediates the balance between anti-apoptotic and pro-apoptotic gene expression.

The binding of TAB1 to TGF- β activated kinase1 (TAK1) promotes auto-

phosphorylation and phosphorylation of TAK1 on Thr187 in the activation loop (Kishimoto et al., 2000; Shibuya et al., 1996). Activated TAK1 phosphorylates IKK and leads to dissociation of NF- κ B from I κ B. TAK1 acts as an anti-apoptosis protein by promoting anti-apoptotic gene transcription through translocation of NF- κ B to the nucleus (Z. J. Chen et al., 2006; Simeonidis et al., 1999).

The TAB1-TAK1 complex is discovered as a binding partner of POPX2. It has been reported that POPX2 can dephosphorylate TAK1 at phospho-Thr187. Therefore, POPX2-knockdown cells have increased TAK1 activity and up-regulated anti-apoptotic gene expression mediated by NF- κ B in the nucleus. On the other hand, high levels of POPX2 in cells have reduced levels of phosphorylated TAK1 and down-regulated anti-apoptotic gene expression. Hence, low levels of POPX2 in cells could lead to higher cell viability via the TAK1-IKK-NF- κ B pathway in response to DNA damaging agents (Fig 5) (Weng and Koh, 2017).

Figure 5. Proposed model of the TAK1-IKK-NF-kB pathway regulation by POPX2.

The TAK1-IKK-NF-kB pathway is activated in response to DNA damage to regulate the balance of anti-apoptosis and pro-apoptosis. When POPX2 is present, POPX2 dephosphorylates TAK1 at Thr187 and inhibits its downstream targets, leading to down-regulation of anti-apoptotic gene expression and decreased cell viability. On the other hand, POPX2-knockdown cells have increased cell viability through enhanced TAK1 activity in response to replication stress (Weng and Koh, 2017). (The image was generated using Biorender software)

1.1.8 POPX2 participates in a myriad of signaling pathways

In summary, POPX2 regulates various signaling pathways through interacting with different target proteins. To date, 7 binding partners of POPX2 have been reported (Table 1). Of these, 5 of them are also substrates of POPX2. So far, POPX2 has been implicated in: (1) maintenance of stress fibers through in a

cooperation of CDC42- β PIX/PAK1 and RhoA-mDia1 pathways; (2) promotion of apoptosis via dephosphorylation of CaMKII and TAK1; (3) regulation of cell adhesion and polarity through N-cadherin cargo transport by CaMKII-KIF3A pathway; (4) enhancement of cell migration through GSK3 and ERK (MAPK) pathways; and (5) anoikis resistance and anchorage independency through dephosphorylation of LATS1 in the Hippo pathway.

Binding protein	Pathway	Function	Reference
β PIX,PAK1 (S57,T423)	CDC42- β PIX-PAK1	Stress fiber	(Koh et al., 2002)
CAMKII (T286)	?	Apoptosis	(Tan et al., 2001)
mDia1	RhoA-mDia1	Stress fiber	(Xie et al., 2008)
?	GSK3-ERK (MAPK)	Cell motility	(Zhang et al., 2012)
CAMKII,KIF3A (S690)	KIF3A-N-cadherin	N-cadherin transport	(Phang et al., 2014)
		Cell polarity	(Hoon et al., 2014)
TAK1 (T187)	TAK1-IKK-NF-kB	Apoptosis	(Weng and Koh, 2017)
LATS1 (T1079)	Hippo pathway	Anchorage-dependent growth	(Rahmat et al., 2019)

Table 1. Known substrates and partners of POPX2 and their related pathways and functions.

1.2 Coronin 1C

1.2.1 Coronin in actin cytoskeleton and coronin domain structure

Cytoskeleton includes actin filaments, microtubules and intermediate filaments. Actin filaments are double-stranded helical polymers made up of monomeric G-actin subunits. The spatial organization of actin networks is mediated by a number of actin binding proteins (ABPs) including (1) actin nucleation and capping proteins, (2) actin severing proteins, (3) actin branching proteins, and (4) cross-linking and bundling proteins.

Formins promote polymerization at barbed ends of actin filaments (Zigmond, 2004) and cofilin depolymerizes actin filaments at pointed ends (McGough et al., 1997). The actin-related protein 2/3 (Arp2/3) complex consists of seven subunits (Arp2, Arp3, P40, P34, P20, P21 and P16) and associates at the side of a pre-existing filament to nucleate a daughter filament (Higgs & Pollard, 2001). Actin bundling is mediated by fimbrin and coronins. Fimbrin contains a calcium binding domain and a pair of actin binding domains (ABDs), facilitating cross-linking of actin filaments into rigid bundles (Bretscher & Weber, 1980). Coronins cross-link the filaments through dimer- or trimer-oligomerization (B. L. Goode et al., 1999). ABPs mediate actin turnover and cellular processes including migration, cell division and endocytosis.

Coronins were first identified in *Dictyostelium discoideum*, where they localize to actin-rich regions (de Hostos et al., 1991). Coronins have been implicated in actin-based processes including cell migration, phagocytosis and micropinocytosis (de Hostos, 1999). The protein contains a conserved N-terminal

domain with WD40 repeats, which is known to regulate protein-protein interactions, followed by a unique region and a C-terminal coiled coil domain. WD40 repeats typically form four stranded anti-parallel β -sheet or blade (D. Li & Roberts, 2001; Smith et al., 1999). Crystal structure of murine Coronin 1 (Crnl) reveals that there are seven bladed β -propeller composed of five WD40 repeats and two non-canonical WD40 repeats (Fig 6A-B). The coiled coil domain at the C-terminus forms homotrimer that mediates actin bundling and cross-linking (Fig 6C) (Appleton et al., 2006; Kammerer et al., 2005).

Figure 6. Crystal structure of Murine Coronin 1 (Crn1) and homotrimer of coiled coil domains.

(A) The individual blades are named from one to seven including the N-terminus (indicated as blue, yellow and green). The C-terminus has a coiled coil domain (coloured red). The strands in each blade are numbered from A to D in blade 4. (B) Side view of Crn1 crystal structure. (C) Crystal structure of Crn1 homotrimer and the N-terminus is on top. The image was retrieved from PDB website. PDB ID: 2AQ5 (A, B) and 2AKF(C).

1.2.2 The mammalian coronin family

Mammalian coronin homologues (Coronin 1~7) are subdivided into two subfamilies (short and long coronins) based on sequence similarity. The first subfamily consists of Coronin 1~3 (Coro 1A-1C) and the second subfamily consists of Coronin 4~7 (Rybakin & Clemen, 2005). The mammalian coronins exhibit distinct expression patterns in cell types and tissues (Table 2). Coro 1A is expressed in hematopoietic tissues and cells (Oku et al., 2003), Coro 1B and Coro 1C are ubiquitously expressed in most tissues (Cai et al., 2005). Coronin 4 (Coro 2A) is expressed in testis, ovary and uterus, whereas Coronin 5 (Coro 2B) is enriched in the brain (Nakamura et al., 1999; Okumura et al., 1998). Coronin 6 is expressed in the brain and Coronin 7 is ubiquitously expressed but at lower levels than the other coronins (Rybakin et al., 2004).

Coronin	Synonyms	Protein	Accession number
Coronin 1	Coronin 1A, p57	461aa	NP_009005
Coronin 2	Coronin 1B	489aa	NP_065174
Coronin 3	Coronin 1C, Crn2	474aa	NP_055140
Coronin 4	Coronin 2A, ClipinB	525aa	NP_438171
Coronin 5	Coronin 2B, ClipinC	475aa	NP_006082
Coronin 6	ClipinE	431aa	NP_624355
Coronin 7	Crn7, POD	925aa	NP_078811

Table 2. The human Coronin family.

1.2.3 The subcellular localization and function of coronins in cells

Coronin localizes to the leading edge of migrating cells and deficiency of coronin in *Dictyostelium* exhibits defects in cytokinesis and cell motility, suggesting its

significance in actin related processes (de Hostos et al., 1993). *Saccharomyces cerevisiae* coronin binds to F-actin and Arp2/3 complex and localizes in actin patches (Heil-Chapdelaine et al., 1998; Humphries et al., 2002).

The function of mammalian coronins is mainly regulated by F-actin binding, oligomerization, phosphorylation and Arp2/3 binding. Coro 1C has been implicated in promoting cellular protrusion and cell motility through its association with actin filaments. Coro 1C has two actin binding sites, Arg28 and four lysine residues within the unique linker region at the C-terminus (Fig 7) (Chan et al., 2012). Deletion of the N-terminus and the C-terminus including two actin binding sites remarkably reduced the formation of lamellipodia and filopodia compared with full length Coro 1C, implying that F-actin binding modulates cellular function of Coro 1C (Rosentreter et al., 2007).

Coro 1C localizes to sub-membranous cytoskeleton, perinuclear region and the cytosol (Rosentreter et al., 2007). The N-terminus and C-terminus are required for the localization of Coro 1C to the cell periphery, suggesting two actin binding sites of Coro 1C may modulate its subcellular localization (Spoerl et al., 2002).

Figure 7. Schematic image of Coro 1C domains.

Coro 1C contains a β -propeller (N-terminus and WD40 repeats), conserved and unique C-terminus and coiled coil domain. The N-terminus has an actin binding site and GDP-Rac1 binding site at Arg28 and Arg31, respectively. Four lysine residues within unique linker bind to actin. The coiled coil domain interacts with Arp2/3 and also mediates oligomerization.

1.2.4 Phosphorylation of mammalian coronins

Phosphorylation is a regulatory mechanism in the interaction between coronins and Arp2/3 as well as coronin-mediated cell motility. Coro 1A is phosphorylated by PKC (Protein Kinase C) at Thr412. The phospho-mimic mutant (T412D) of Coro 1A displays lower affinity with actin compared with control (Oku et al., 2012). Coro 1B is phosphorylated by PKC at Ser2 and phosphorylated Coro 1B has weaker interaction with Arp2/3. Moreover, fibroblasts expressing phospho-mimic mutant (S2D) of Coro 1B have reduced ruffling and migration speed in response to PKC activator, phorbol 12-myristate 13-acetate (PMA) stimulation (Cai et al., 2005), suggesting that phosphorylation of Coro 1B by PKC negatively regulates interaction with Arp2/3 and cell migration.

Coro 1C has 6 potential serine/threonine phospho-sites as shown in Fig 8 and Coro 1C is phosphorylated by casein kinase 2 (CK2) at Ser463 within the coiled coil domain. Similar to Coro 1B, phosphorylated Coro 1C on Ser463 by CK2 shows weaker interaction with Arp2/3. Expression of phospho-mimic mutant (S463D) of Coro 1C in cells exhibits reduced F-actin bundling at the front of lamellipodial extensions, decreased cellular protrusions and motility compared with WT or phospho-dead mutant (S463A) transfected cells (Xavier et al., 2012). Taken together, phosphorylation of coronins by kinases disrupts the interaction with Arp2/3 and diminishes actin-based processes including membrane ruffles and cell motility.

Interestingly, it has been reported that the subcellular localization of Coro 1C is regulated by phosphorylation. Phosphorylated Coro 1C is found in the cytosol, while dephosphorylated Coro 1C localizes to the sub-membranous cytoskeleton in HEK293cells (Spoerl et al., 2002) and in Neuro-2a cells (Hasse et al., 2005). Similar pattern is observed for myristoylated alanine-rich protein kinase C substrate (MARCKS) protein. Dephosphorylated MARCKS localizes to the plasma membrane and promote actin polymerization and cross-linking, while phosphorylated MARCKS is sequestered at the cytosol (McNamara & Lenox, 1998). These findings suggest that phosphorylation on Coro 1C regulates its localization between cytosol and sub-membranous cytoskeleton and resultant roles in membrane ruffles and cell motility.

Figure 8. Phosphorylation sites on Coro 1C.

The structure of Coro 1C and its phospho-sites with more than 2 mass spectrometry (MS) references on PhosphositesPlus® website. There are total 6 phospho-sites; Ser187, Ser193, Ser299 in the WD40 domain, Ser354, Thr415 and Ser463 at the C-terminus.

1.2.5 Coronins are GDP-Rac1 binding proteins

Rac1 is a member of the Rho GTPases, which regulates cell cycle, cell-cell adhesion and migration. The activity of Rac1 is regulated by: (1) guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) proteins, (2) RHO protein GDP dissociation inhibitor of Rho proteins (RhoGDIs) in the cytosol, (3) RCC2 and Caveolin at the membrane (Williamson et al., 2015, 2014) and (4) Rac1 trafficking.

Mammalian Coro 1A and Coro 1C have been reported to interact with inactive form of Rac1 and regulate its activation. The binding sites of Coro 1A to Rac1 have not been reported. Arg31 within the N-terminus of Coro 1C interacts with Thr35 and Arg38 of Rac1 within its switch I loop (Tilley et al., 2015). Coro 1A and Coro 1C have different roles in Rac1 regulation. Coro 1A promotes dissociation of Rac1 from RhoGDI and facilitates Rac1-membrane association (Castro-Castro et al., 2011). Whereas Coro 1C releases inactive Rac1 from the lateral membrane (non-protrusive membrane) and redistributes Rac1 into the

leading edge for lamellipodia formation. Although Coro 1A promotes Rac1-membrane association and Coro 1C release Rac1 from the membrane, depletion of Coro 1A or Coro 1C causes reduced cell motility and loss of cell polarity due to the mislocalization of Rac1 (Williamson et al., 2014).

1.2.6 Involvement of Coro 1C in brain cancer

Glioblastoma multiforme (GBM) is the most aggressive cancer that develops from the glial cells that support the nervous system in the brain. GBM is often referred to as a grade IV astrocytoma and is able to invade into nearby regions of the brain (Bleeker et al., 2012). Generally, newly diagnosed GBM patients have a median survival of about 12 months (Stupp et al., 2005). Coro 1C is expressed in various types of neuronal cells and localizes to the outgrowing neurites and promotes neurite formation. Supporting evidence shows positive correlation of Coro 1C expression with malignancy phenotype of brain tumor. Expression of Coro 1C appears to be increased in higher WHO grade of GBM. Depletion of Coro 1C in GBM cells reduces invadopodia formation and invasion into extracellular matrix (ECM) (Thal et al., 2007), implying that Coro 1C is associated with malignancy through regulating the invasiveness of cancer.

Further studies demonstrate that the effects of Coro 1C on invasiveness of GBM is dependent on its phosphorylation status on Ser463 by CK2. Overexpression of wild-type (WT) or phospho-dead mutant (S463A) of Coro 1C increases matrix degradation and invasion, while knocking-down Coro 1C or overexpression of phospho-mimic mutant (S463D) of Coro 1C decreases the invadopodia like extensions. Moreover, WT and S463A overexpressing GBM cells have deeper tumor invasion infiltration compared with S463D in *ex vivo* experiments,

suggesting phosphorylation of Coro 1C at S463 negatively regulates invasiveness of brain cancer cells (Ziemann et al., 2013).

1.2.7 Involvement of Coronin 1C in gastric cancer

The stomach wall consists of 5 layers (mucosa, submucosa, muscularis propria, subserosa and serosa) and gastric cancer usually starts in the inner most layer, mucosa. As cancer grows into deeper layers, the stage of cancer becomes more advanced. Gastric cancer can invade normal tissues and spread to other parts of the body, especially through the lymphatic system. Therefore, lymph nodes are the most common metastatic sites (John et al., 2013). Gastric cancer is the fifth leading type of cancer and the third leading cause of death (Bernard W and Christopher P, 2014). Expression of Coro 1C has been found to be correlated with metastasis of gastric cancer. Coro 1C is expressed at higher levels in metastatic lymph node than primary gastric cancer tissue. In addition, higher expression of Coro 1C is associated with higher clinical stage and poor survival periods of gastric cancer patients. Stable knock-down of Coro 1C in gastric cancer cells reduces invasiveness and consequently metastasis *in vivo* by inhibiting matrix metalloproteinase 9 (MMP-9), type IV collagenase and cathepsin K protease (Ren et al., 2012).

The interaction between Coro 1C and Arp2/3 is phosphorylation-dependent and is significant for gastric cancer invasion and metastasis. Phosphorylation of Coro 1C on Ser463 weakens the interaction of Coro 1C with Arp2/3 (Xavier et al., 2012). Down-regulation of Arp2/3 antagonizes Coro 1C-mediated enhanced cell motility and invasion, implying that Arp2/3 and Coro 1C regulate cell migration and invasion cooperatively. In addition, gastric cancer patient samples with high

Coro 1C/high Arp2 are correlated with high mortality. Patient samples with high Coro 1C/low Arp2 and low Coro 1C/high Arp2 show similar extent of survival rate, suggesting that high levels of Arp2/3 and Coro 1C are implicated for mortality (Y. Sun et al., 2014).

1.2.8 Involvement of Coronin 1C in breast cancer

The breast is made up of ducts and lobes. Cancer developing from the ducts and lobes are known as ductal carcinomas and lobular carcinomas, respectively. The stage of breast cancer is based on the size and location of the primary tumor and metastasis to nearby lymph nodes or other parts of the body. Estrogen Receptor (ER), Progesterone Receptor (PR) and Human Epidermal Growth Factor Receptor (HER) are used as biomarkers for breast cancer cells. Breast cancer cells without these three biomarkers are called Triple Negative Breast Cancer cells (TNBC). The presence of biomarkers is important in determining the types of drugs used to block the binding of hormone to receptors. However, TNBC does not respond to hormonal therapy medicines due to the lack of the three hormone receptors (*Breast cancer treatment by the National Cancer Institute*, 2013).

Cell motility and invasiveness of TNBC cells are positively associated with Coro 1C. Depletion of Coro 1C in MDA-MB-231 cells reduces cell migration and invasion, while overexpression of Coro 1C enhances cell motility and invasion (Lim et al., 2017). Expression of Coro 1C is regulated by non-coding RNA, miR-206 and transcription factor, YB-1 (Y-box binding protein-1) in TNBC. miR-206 inhibits Coro 1C-mediated cell motility through inhibition of Coro 1C expression by targeting 3'-UTR region of Coro 1C in TNBC cells (Jun Wang et al., 2014).

In addition, YB-1 is a conserved transcription factor that targets the gene expression of Coro 1C (Lim et al., 2017). Expression of YB-1 and Coro 1C are elevated in breast cancer cells, suggesting that Coro 1C expression is associated with cancer progression.

Analysis of 30 different breast cancer patient samples shows that expression pattern of membrane type I matrix metalloproteinase (MT1-MMP) and Coro 1C is similar in TNBC. Coro 1C and MT1-MMP are found to accumulate at proteolytically active invadopodia and are involved in ECM proteolysis activity, implying that Coro 1C may participate in invasion during metastasis. In addition, cortactin and Coro 1C localize to the lamellipodia at the edge of invasion protrusion extending within 3D collagen gel environment (Castagnino et al., 2018). Therefore, it is likely that Coro 1C promotes metastasis through MT1-MMP-mediated invasion of breast cancer cells.

1.3 The relationship between POPX2 and Coro 1C

Previous studies have reported that both POPX2 and Coro 1C are positively correlated with invasiveness and motility in TNBC. POPX2 expression is high in invasive breast cancer cells and low in non-invasive breast cancer cells (Susila et al., 2010). Similarly, the expression of Coro 1C increases cell motility and invasiveness of cancer cells (Lim et al., 2017; Ren et al., 2012; Ziemann et al., 2013). Since the function of Coro 1C is regulated by phosphorylation, we hypothesize that POPX2 phosphatase may regulate Coro 1C through dephosphorylation. In this study, we aim to unveil whether Coro 1C and POPX2 participate together in cancer metastasis through enhancing cell motility and invasiveness.

1.4 Checkpoint Kinase 1 (Chk1)

1.4.1 DNA damage response (DDR) pathways

DNA damage can be caused exogenously by genotoxic stress or radiation, and endogenously by reactive oxygen species (ROS), by-products of metabolism (Lindahl & Barnes, 2000). The evolutionally conserved DNA damage response (DDR) can preserve genome integrity through activation of cell cycle checkpoints and consequently cell cycle arrest. The activation of checkpoints slows down cell cycle progression to allow cells to repair abnormally structured DNA and pass accurate copies of their genomes to the daughter cells. DNA damage pathway is characterized by cascades of protein phosphorylation events. Ataxia telangiectasia and Rad3-related (ATR) and Ataxia telangiectasia mutated (ATM) are members of the phosphoinositide 3-kinase-related kinases (PIKKs) family and central components of DNA damage pathways (Lovejoy & Cortez, 2009). In addition to these kinases, Checkpoint kinase 1 (Chk1) and Checkpoint kinase 2 (Chk2) are downstream targets of ATM/ATR and both are implicated in the DNA damage repair pathways (Blasina et al., 1999; Q. Liu et al., 2000; Sanchez et al., 1997).

ATM deficient mice are viable, but exhibit infertility and cancer predisposition, while ATR deficiency in mice shows embryonic lethality (Barlow et al., 1996; Brown & Baltimore, 2000). Deletion of ATM or ATR abrogates cell cycle arrest after DNA damage. Cells lacking ATM are sensitive to γ -irradiation (IR) and overexpression of inactive ATR in cells show hypersensitive to UV, Hydroxyurea (HU) and IR (Barlow et al., 1996; Cliby et al., 1998; Wright et al.,

1998; Y. Xu et al., 1996). Chk1 deficiency in embryonic stem cells shows defective G2 checkpoint in response to IR (Liu et al., 2000).

ATM related pathway is mainly triggered by double-strand DNA breaks (DSBs) and ATR is mostly activated by single-strand DNA breaks (SSBs). ATM/ATR phosphorylates Chk1 and Chk2 at serine/threonine residues followed by Gln (SQ or TQ motif) (Traven & Heierhorst, 2005). ATR phosphorylates Chk1 at two SQ sites, Ser317 and Ser345 and ATM is also able to activate Chk1 at Ser345 in response to DNA damage (Liu et al., 2000; Zhao and Piwnica-Worms, 2001), indicating crosstalk between ATR and ATM pathways (Fig 9).

Although Chk1 and Chk2 do not share structural similarity, they can be activated by ATM/ATR and cooperate to prevent unscheduled DNA replication by targeting CDC25 phosphatases in response to DNA damage. ATM/ATR-Chk1/Chk2 pathways regulate cell cycle arrest through two mechanisms: (1) degradation of CDC25 phosphatases (Donzelli et al., 2002; Uchida et al., 2011) and (2) phosphorylation of CDC25 phosphatases by Chk1/Chk2 kinases (Sanchez et al., 1997).

CDC25 phosphatases (CDC25A/B/C) activate Cyclin-Dependent Kinases (CDKs) by removing the inhibitory phosphate group in the active site, resulting in cell cycle progression. The activation can be reversed by Wee1/Myt1/Mik1 kinases which phosphorylate CDKs at Thr14 and Tyr15 (Pines, 1999). A well-studied mechanism of cell cycle arrest is that of the degradation of CDC25 phosphatases. CDC25 phosphatases are degraded through Skp1/Cullin/F-box

(SCF)-mediated ubiquitination in response to DNA damage. Ubiquitination of CDC25 phosphatases and their subsequent degradation lead to inactivation of CDKs, resulting in S or G2 checkpoints activation (Donzelli et al., 2002; Uchida et al., 2011)

Another reported mechanism of cell cycle arrest is through the phosphorylation of CDC25 phosphatases. Chk1 phosphorylates CDC25C at Ser216 and induces the binding of CDC25C to 14-3-3 protein for its cytoplasmic sequestration. Cytoplasmic CDC25C fails to activate CDC2/Cyclin B complex and leads to cell cycle arrest (Sanchez et al., 1997). CDC25A is phosphorylated at Ser178, Ser278 and Ser292 by Chk1 in response to DNA damage to arrest at S phase of the cell cycle (Sørensen et al., 2003). Chk1 also mediates doxorubicin induced G2 arrest through degradation of CDC25A (Z. Xiao et al., 2003), suggesting that Chk1 is able to activate both S and G2 checkpoints through CDC25 phosphatases depending on DNA damaging agents.

Figure 9. Overview of ATM/ATR-Chk1 DNA damage pathway.

ATM is activated by DSBs and ATR is activated by SSBs. Activate ATM/ATR phosphorylate Chk1 at Ser317 and Ser345 within SQ/TQ motif. Active Chk1 subsequently phosphorylates CDC25 and phosphorylated CDC25 binds to 14-3-3, leading to its cytoplasmic sequestration and subsequent ubiquitination. Inactive CDC25 prevents the activation of Cdc2/CyclinB and arrest cells at G2 phase in response to DNA damage. (The image was generated using Biorender software)

1.4.2 Phosphorylation and activation of Chk1

Chk1 is activated by several regulatory mechanisms including conformational change, cellular distribution, phosphorylation and proteasome-dependent

degradation. Chk1 contains a kinase domain, SQ/TQ domain, CM1 and CM2 motifs as indicated in Figure 10.

Cellular distribution of Chk1 between the nucleus and the cytoplasm is regulated by phosphorylation and intramolecular interaction. Although Chk1 primarily localizes in the nucleus, a significant amount of Chk1 is observed in the cytoplasm. It has been reported that Conserved Motifs (CM1 and CM2) at the C-terminus of Chk1 act as non-canonical nuclear export signal and nuclear localization signal, respectively (Wang et al., 2012). Chk1 exists in a closed conformation through an intramolecular interaction between the kinase domain and the CM2 motif under normal condition. Upon DNA damage, Chk1 is phosphorylated at Ser317 and Ser345 by ATM/ATR, which induces conformational change by disrupting intramolecular interaction between the kinase domain and the CM2 motif. Loss of inhibitory effect by intramolecular interaction leads to dissociation of Chk1 from the chromatin (Wang et al., 2012). Chk1 in open conformation is phosphorylated at Ser280 by AKT and can translocate to the cytoplasm (Puc et al., 2005). Apart from phosphorylation of Chk1 on Ser280, Chk1 translocates to the cytoplasm after phosphorylation on Ser286 and Ser301 by CDKs, which in turn promotes mitosis entry under normal condition (N. Xu et al., 2012).

It has been reported that auto-phosphorylation of Chk1 leads to the activation of Chk1. Chk1 is auto-phosphorylated at Ser296, Thr378 and Thr382. Auto-phosphorylation of Chk1 on Ser296 does not affect phosphorylation of Chk1 on Ser317 and Ser345 (Okita et al., 2012), whereas auto-phosphorylation of Chk1 on Thr378 and Thr382 is linked to Chk1 activation and proteasomal degradation (Gong et al., 2018). Overexpression of constitutively active mutant of Chk1 (Thr378D/382D) arrests cells at G2 phase in the absence of DNA damage and induces rapid proteasomal degradation of Chk1 (Gong et al., 2018). It is likely that Thr378/382 localized within the CM1 motif binds to Fbx6-containing SCF complex that mediates Chk1 ubiquitination (Y.-W. Zhang et al., 2009).

Figure 10. Schematic diagram of Chk1 activation and phosphorylation.

Chk1 contains a kinase domain, SQ domain, CM1 and CM2 motifs. Chk1 is phosphorylated at Ser280 (AKT), Ser286 (CDK), Ser296 and Ser301 (Chk1), Ser317 and Ser345 (ATM/ATR), Thr378 and Thr382 (Chk1). Inactive Chk1 has an intramolecular interaction between the kinase domain and the CM2 motif resulted in a closed conformation. Once ATM/ATR phosphorylate Chk1 at Ser317 and Ser345, Chk1 adopts an open conformation, resulting in Chk1 release

from chromatin association. CM1 acts as a non-canonical nuclear export signal and CM2 serves as a non-canonical nuclear localization signal.

1.4.3 Chk1-binding proteins

Chk1 has numerous binding proteins that regulate its activity directly or indirectly as shown in Table 3. Claspin has been reported as a regulator of Chk1 pathway (Kumagai & Dunphy, 2000). Claspin binds to Chk1 in response to DNA damage and their binding is required for Chk1 activation (Chini & Chen, 2003). Claspin binds to four residues within the kinase domain (Lys54, Arg129, Thr153 and Arg162) and the C-terminus of Chk1 (Jeong et al., 2003). Phosphorylation of Claspin is required for interaction with Chk1 (Chini & Chen, 2003). Although Claspin contains a number of SQ/TQ motifs (Kastan & Lim, 2000) and phosphorylation of Claspin is inhibited by caffeine, ATM/ATR inhibitor (Chini & Chen, 2003), direct phosphorylation of Claspin by ATM/ATR has not been reported.

Protein phosphatase 1 (PP1) and PPM1D (Wip1) are known phosphatases of Chk1 (Küntziger et al., 2011; Lu et al., 2005). PPM1D phosphatase interacts with Chk1 and dephosphorylates Chk1 at Ser317 and -Ser345. PPM1D decreases the kinase activity of Chk1 through dephosphorylation and it leads to the reverse of Chk1-induced S and G2 checkpoints following completion of DNA repair (Lu et al., 2005).

FEM1B (human homolog of the *Caenorhabditis elegans* sex-determining FEM-1) has been identified as a Chk1-binding protein from a yeast two-hybrid screen. FEM1B participates in the activation of Chk1 as well as promoting the

association of Rad9 on chromatin in response to replication stress (Sun and Shieh, 2009). Moreover, nucleophosmin (NPM) and DNA mismatch repair protein Msh2/Msh6 (MutS α) also interact with Chk1 and regulate the activation of Chk1 through the chromatin loading (Chen et al., 2009; Liu et al., 2010).

Chk1 binding protein	Function	Reference
Claspin	Checkpoints	(Kumagai and Dunphy, 2000)
Timeless	G1/S arrest by UV	(Unsal-Kaçmaz et al., 2005)
PCNA	Checkpoints	(Scorah et al., 2008)
Pol α	Checkpoints	(Taricani et al., 2009)
Wip1 (PPM1D)	Checkpoints feedback	(Lu et al., 2005)
DNMT1	Chk1 cell localization	(Palii et al., 2008)
Fbx6	Chk1 degradation	(Zhang et al., 2009)
Cul4A/DDB1	Chk1 degradation	(Zhang et al., 2009)
NPM	Chromatin loading	(Chen et al., 2009)
MutS α	Chromatin loading	(Liu et al., 2010)
SpRfp1/Rnf4	Damage repair	(Kosoy et al., 2007)
FEM1B	Checkpoints	(Sun and Shieh, 2009)

Table 3. List of Chk1-binding proteins and function.

1.4.4 DNA damage pathways in cancer therapy

DNA damage response is closely associated with cancer development. Mutations in genes caused by unsuccessful DNA repair can contribute to cancer development and in fact, mutations in genes involved in DNA damage repair (DDR) result in hereditary cancer predispositions (E. L. Goode et al., 2002).

The DNA damage pathway is an attractive drug target because (1) increased DNA damage signaling pathway is associated with resistance to genotoxic therapies, and (2) many cancers have increased reliance on DNA damage pathway due to the defects in certain component of DDR (Weber & Ryan, 2015). As ATM and ATR are upstream kinases of the DNA damage pathway, ATM and ATR inhibitors have been well explored. There are a number of ATM and ATR inhibitors in preclinical and clinical development for cancer therapy (Table 4).

The compound CP-466822 is able to target ATM selectively and do not affect other PI3K or related PIKK family proteins (Rainey et al., 2008). Wortmannin is an inhibitor of PI3K family members and a potent inhibitor of ATM. However, this drug is not able to develop for clinical usage due to its toxicity and a lack of sensitivity (Karve et al., 2012).

VE-822, ATR inhibitor increases the sensitivity of pancreatic cancer cells to radiation and gemcitabine leads to reduced cancer cell survival after the treatment (Fokas et al., 2012). Schisandrin B (SchB), an active ingredient of *Fructus schisandrae* has been reported as an ATR inhibitor. SchB selectively inhibits ATR and its downstream targets, P53 and Chk1. Treatment of SchB significantly reduces the viability of lung cancer cells after UV exposure (Nishida et al., 2009).

Previously, Chk1 was considered to be a tumor suppressor as it halts replication when DNA is damaged or during genome stability. However, recent evidence shows that Chk1 can promote tumor progression (Zhang and Hunter, 2014). Chk1 has been postulated to cause chemotherapy resistance due to the ability of tumor cells to withstand higher levels of DNA damage with increased Chk1 levels (Liang et al., 2009). With this new role of Chk1 as oncogene in cancer cells, Chk1 inhibitors have recently found to be useful as sensitizers for chemotherapy and radiotherapy. Cisplatin is an anti-cancer drug used for treatment of many cancers by inhibiting DNA replication, but cisplatin-resistant cancer cases are on the rise. Interestingly, Chk1 inhibitors with anti-cancer drugs such as Cisplatin or Doxorubicin can decrease chemo-resistance in numerous

types of cancers such as small cell lung carcinoma (SCLC) (Thompson et al., 2012), head and neck squamous cell carcinoma (HNSCC) (Gadhikar et al., 2013), urothelial bladder cancer (Li et al., 2016), bone cancer cell (Koppenhafer et al., 2018) and melanoma (Hwang et al., 2018).

TNBC cells are often resistant to IR. Chk1 inhibitors use together with radiotherapy can sensitize cells to radiation and reduce the viability of TNBC cells (Dinkelborg et al., 2019). Therefore, Chk1 inhibitors can potentiate the efficacy of DNA damaging chemotherapies.

Compound (ATM inhibitor)	Reference	Clinical trial NCT number
CP-466822	(Rainey et al., 2008)	
KU-55933	(Hickson et al., 2004)	
KU-60019	(Golding et al., 2012)	
KU-559403	(Batey et al., 2013)	
Wortmannin	(Karve et al., 2012)	
Compound (ATR inhibitor)	Reference	Clinical trial NCT number
NU6027	(Peasland et al., 2011)	
VE-821	(Charrier et al., 2011)	
VE-822	(Fokas et al., 2012)	NCT02157792
AZ20	(Jacquemin et al., 2012)	
AZD6738	(Jones et al., 2013)	NCT02223923
Schisandrin B	(Nishida et al., 2009)	
Compound (Chk1 inhibitor)	Reference	Clinical trial NCT number
AZD7762	(Sausville et al., 2014)	NCT00413686
PF00477736	(Brega et al., 2010)	NCT00437203
LY2603618	(Doi et al., 2015)	NCT00839332
Sch 900776	(Daud et al., 2010)	NCT00779584
GDC0425	(Infante et al., 2017)	NCT01359696
GDC0575	(Italiano et al., 2018)	NCT01564251

Table 4. ATM/ATR/Chk1 inhibitors that in preclinical or clinical development with cytotoxic chemotherapy

1.5 The relationship between POPX2 and Chk1 in DNA damage response

DNA damage response consists of DNA repair, cell cycle checkpoint induction and DNA-damage induced apoptosis. POPX2 has been implicated in the regulation of cell survival and apoptosis. Overexpression of POPX2 enhances apoptosis in mammalian cells (Tan et al., 2001). Moreover, PAK1 and CaMKII promote cell survival and they are negatively regulated by POPX2 (Ong et al., 2011; C. Xiao et al., 2005). A mechanism for POPX2 in the regulation of apoptosis in response to DNA damage has been reported earlier. The TAK1-IKK-NF- κ B pathway is inhibited by POPX2 through dephosphorylation of TAK1 at Thr187. In POPX2-knockdown cells, there is increased activation of TAK1 and reduced apoptosis with genotoxic stress (Weng & Koh, 2017). In this study, we focus on the role of POPX2 in DNA damage pathway in terms of cell cycle arrest at G2 phase.

Aims of the study

Since POPX2 is implicated in cancer cell invasiveness and motility, we are interested to understand more about the functions mediated by POPX2 in the cells. One good strategy of understanding the role of a protein of interest is to identify its interacting partners. In this study, Coro 1C and Chk1 are identified as binding partners of POPX2 using two different approaches. Our aim is to determine the functional links between POPX2 and these two proteins. The project is divided into two main parts: (1) Coro 1C related and (2) Chk1 related.

Part 1. The functional relationship between Coro 1C and POPX2

The aims are (i) to identify the binding affinity between POPX2 and Coro 1C, (ii) to investigate whether POPX2 regulates Coro 1C through dephosphorylation and (iii) to understand the implication of the interaction between POPX2 and Coro 1C in actin-based processes.

Part 2. The role of POPX2 phosphatase in DNA damage pathway

The aims are (i) to investigate the binding of POPX2 and Chk1, (ii) to study the role of POPX2 in Chk1 activation in response to DNA damage and (iii) to identify the functional link of POPX2-Chk1 complex in DNA damage pathway.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals

1kb/100bp DNA ladder	NEB
Acetic acid	Merck
Acrylamide	Bio-Rad
Agarose	Bio-Rad
Ampicillin	USB
APS (Ammonium persulfate)	Bio-Rad
Bradford Protein Assay	Bio-Rad
Bromophenol blue	Sigma
BSA (bovine serum albumin)	Sigma
Calcium Chloride Dihydrate	Merck
Calyculin A	CST
Complete™ EDTA free Protease Inhibitor Cocktail Tablet	Roche
Coomassie R250	USB
Crystal Violate	Sigma
Deoxynucleotides (dG/A/T/CTP)	Roche
DMSO (Dimethyl Sulphoxide)	Sigma
DTT (Dithiothreitol)	Sigma
EDTA	Sigma
Ethanol	Merck
Ethidium bromide	Bio-Rad
Fibronectin	Sigma
Glycerol	Sigma
Glycine	1st BASE
HEPES	Sigma
Imidazole	Sigma
IPTG (Isopropyl β -D-1-thiogalactopyranoside)	Gibco
Isopropanol	Merck
L-Glutathione reduced	Amersham
Lysozyme	Sigma
Methanol	Merck
N-Lauroylsarcosine (Sarkosyl)	Sigma

Paraformaldehyde	Merck
PMSF (Phenylmethanesulfonyl Fluoride)	Sigma
Poly-L-lysine	Sigma
Ponceau S	Sigma
Potassium Chloride	Sigma
Precision plus protein dual color standards	Bio-Rad
Protease Inhibitor Cocktail	Roche
SDS (Sodium dodecyl sulfate)	USB
Sodium Chloride	BDH
Sodium Hydrogen Carbonate	Sigma
TEMED (N,N,N',N'-tetramethylethane-1,2-diamine)	Bio-Rad
Tris Base	Promega
Triton X-100	Bio-Rad
Tween 20	Bio-Rad
β -Mercaptoethanol	Merck

2.1.2 Commercial kits

QIAquick® Gel Extraction Kit Qiagen (Valencia, CA, USA)

QIAquick® PCR Purification Kit Qiagen (Valencia, CA, USA)

QIAquick® Spin Midiprep Kit Qiagen (Valencia, CA, USA)

AxyPrep Plasmid Miniprep Kit Axygen (Union City, CA, USA)

QuikChange Site-Directed Mutagenesis Kit Stratagene (La Jolla, CA, USA)

Amersham ECL Plus GE healthcare (Buckinghamshire, UK)

Subcellular protein fractionation kit (Thermo Scientific, MA, USA)

2.1.3 Bacteria and cell media

Ampicillin broth	LB medium containing 100 μ g/mL ampicillin
Ampicillin plate	LB agar plate containing 100 μ g/mL ampicillin
Cell culture media	Dulbecco's Modified Eagle's Medium with 4500 mg/L glucose
Cell freezing medium	10 % (v/v) DMSO in FBS
HeLa cell culture media	Minimum Essential Medium (MEM) supplemented with 2 mM L-glutamine
Kanamycin broth	LB medium containing 50 μ g/mL kanamycin

Kanamycin plate	LB agar plate containing 50 µg/mL kanamycin
LB agar	LB medium plus 1.5 % (w/v) bacto-agar
LB medium	1 % (w/v) Bacto-tryptone, 0.5 % (w/v) yeast extract, 1 % (w/v) NaCl
Opti-MEM™	Reduced serum media

2.1.4 Buffers

1× Nitrocellulose transfer buffer	100 ml 10× nitrocellulose transfer buffer 200 ml methanol 700 ml water
10× Nitrocellulose transfer buffer	30.3 g Tris base 144 g glycine 1 L water
10× PBS (phosphate buffered saline)	80 g NaCl 2 g KCl 14.4 g Na ₂ HPO ₄ 2.4 g KH ₂ PO ₄ 1 L water, pH 7.4
10× SDS-PAGE running buffer	30.2 g Tris base 144 g glycine 10 g SDS 1 L water
10× TBS	24.2 g Tris base 80 g NaCl 1 L water, pH 7.6
10x DNA loading buffer	10 mL TE buffer 10 mL glycerol Add bromophenol blue
50× TAE buffer	24.2 g Tris base 57.1 ml acetic acid 100 ml 0.5 M EDTA, pH 8.0 Top up to 1 L with water
6x SDS sample buffer	7 mL 4X Tris-Cl/SDS pH 6.8 3 mL glycerol 1 g SDS 0.93 g DTT

	Add bromophenol blue
Bacteria lysis buffer	10 mM Tris-Cl pH 7.5 150 mM NaCl 1 mM EDTA 0.5 mM PMSF 1 mM DTT 0.1 mg/mL Lysozyme 1.5 % Sarkosyl
Co-Immunoprecipitation (Co-IP) buffer	20 mM Tris-Cl pH 8.0 1% Triton-X 200 mM NaCl 1xTBS
Mammalian cell lysis buffer	50 mM Hepes pH 7.5 300 mM NaCl 1 mM MgCl ₂ 1 mM EGTA 10 mM β -glycerophosphate 1 mM Sodium orthovanadate 5% glycerol 5 mM DTT 0.5% TritonX-100 Add protease inhibitor and phosSTOP(Roche)
Protein storage buffer	20 mM Hepes pH 7.5 300mM NaCl 10% (v/v) glycerol
Protein elution buffer	<u>GST elution buffer</u> 10 mM Reduced L-glutamine in 50 mM Tris pH 8.0 <u>His wash and elution buffer</u> Wash Buffer: 20 mM Hepes, 500 mM NaCl, 10 mM Imidazole, 10 % (v/v) glycerol, pH 7.5 Elution Buffer: 20 mM Hepes, 500 mM NaCl, 500 mM Imidazole, 10 % (v/v) glycerol, pH 7.5

2.1.5 Bacterial strains and mammalian cell lines

Bacterial strains

DH5 α Chemically competent bacterial strain Invitrogen

BL21 Chemically competent bacterial strain NEB

Mammalian cell lines

MDA-MB-231 Human breast cancer epithelial ATCC HTB-26™

HEK293 Human embryonic kidney epithelial ATCC CRL-3216™

HeLa Human Cervix adenocarcinoma epithelial ATCC CCL-2™

NIH3T3 Mouse embryonic fibroblast ATCC CRL-1658™

NIH3T3-GFP Stable line overexpressing GFP Puromycin resistant.

NIH3T3-POPX2 Stable line overexpressing GFP-POPX2 Puromycin resistant.

NIH3T3-POPX2M Stable line overexpressing GFP-POPX2M Puromycin resistant.

U2-OS Human Bone Osteosarcoma epithelial ATCC HTB-96™

2.1.6 Enzymes for cloning and mammalian cell transfection reagents

Alkaline Phosphatase (CIP)	NEB
Q5® High-Fidelity DNA Polymerase	NEB
Restriction endonucleases	NEB
T4 DNA ligase	NEB
Lipofetamin ® 2000	Invitrogen

2.1.7 Affinity binding matrix

Anti-FLAG M2 agarose beads	Sigma
Glutathione Sepharose™ 4B beads	GE Healthcare
Ni Sepharose™ 6 Fast Flow	Roche

2.1.8 Primary antibodies

Antibody	Species	Source	Clonal	Cat #
Actin	mouse	Millipore	monoclonal	MAB1501R
Active Rac1	mouse	NewEast Biosciences	monoclonal	26903
CDC25A	mouse	SCBT	monoclonal	sc-7389
Chk1	rabbit	CST	monoclonal	SC-8408
Coronin 1C	rabbit	Abcam	polyclonal	Ab153954
Coronin 1C	mouse	SCBT	monoclonal	SC-130448

Flag	rabbit	Sigma	polyclonal	F7425
GAPDH	mouse			
GFP	rabbit	Invitrogen	polyclonal	11122
GST	rabbit	Bethyl Laboratories	polyclonal	A190-122A
His	mouse	CST	monoclonal	12698
Phospho Serine/Threonine	rabbit	ECM Biosciences	polyclonal	PP2551
Phospho- Chk1(S317)	rabbit	CST	polyclonal	2344
Phospho- Chk1(S345)	rabbit	CST	polyclonal	2341
POPX2	rabbit	Self-raised		
POPX2	mouse	Abcam	monoclonal	Ab56648
Rac1	mouse	Upstate	monoclonal	05-389

2.1.9 Secondary antibodies

Antibody	Company
Goat anti-mouse IgG, HRP conjugated	Dako Cytomation
Goat anti-rabbit IgG, HRP conjugated	Dako Cytomation
Goat anti-mouse IgG, Alexa Fluor 488	Molecular Probes
Goat anti-mouse IgG, Alexa Fluor 546	Molecular Probes
Goat anti-rabbit IgG, Alexa Fluor 488	Molecular Probes
Goat anti-rabbit IgG, Alexa Fluor 546	Molecular Probes
Alexa-Fluor 488 phalloidin	Invitrogen
Alexa-Fluor 546 phalloidin	Invitrogen

2.1.11 Plasmids

Plasmid	Insert	
pcDNA4-Chk1-Flag	Checkpoint kinase1	Full length 1-476
PET-His6-Coronin 1C ³⁴⁹⁻⁴⁷⁴	Human Coronin 1C	Amino acids 349-474
PET-POPX2	Protein phosphatase 1F	Full length 1-454

PET-POPX2M	Protein phosphatase 1F	Phosphatase mutant R362A, I338A
PGEX-6P1-POPX2	Protein phosphatase 1F	Full length 1-454
PXJ-Flag-Coronin 1C ¹⁻⁴⁴⁴	Human Coronin 1C	Amino acids 1-444
PXJ-Flag-Coronin 1C ⁷²⁻⁴⁷⁴	Human Coronin 1C	Amino acids 72-474
PXJ-Flag-Coronin 1C FL	Human Coronin 1C	Full length 1-474
PXJ-Flag-Coronin 1C ³⁰⁰⁻⁴⁴⁴	Human Coronin 1C	Amino acids 300-444
PXJ-Flag-Coronin 1C ³⁰⁰⁻⁴⁷⁴	Human Coronin 1C	Amino acids 300-474
PXJ-Flag-GFP	Flag-GFP tag	
PXJ-GFP	GFP tag	
PXJ-GFP-Coronin 1C	Human Coronin 1C	Full length 1-474
PXJ-GFP-Coronin 1C S463A	Human Coronin 1C	Full length, mutant S463A
PXJ-GFP-Coronin 1C S463D	Human Coronin 1C	Full length, mutant S463D
PXJ-GFP-Coronin 1C T415A	Human Coronin 1C	Full length, mutant T415A
PXJ-GFP-Coronin 1C T415E	Human Coronin 1C	Full length, mutant T415D
PXJ-GFP-POPX2	Protein phosphatase 1F	Full length 1-454
PXJ-GFP-POPX2M	Protein phosphatase 1F	Phosphatase mutant R362A, I338A
PXJ-GST	GST tag	
PXJ-GST-Coronin 1C CC	Human Coronin 1C	Amino acids 444-474
PXJ-GST-POPX2	Protein phosphatase 1F	Full length 1-454
PXJ-GST-POPX2M	Protein phosphatase 1F	Phosphatase mutant R362A, I338A
PXJ-mCherry	mCherry tag	
PXJ-mCherry-POPX2	Protein phosphatase 1F	Full length 1-454
PXJ-mCherry-POPX2M	Protein phosphatase 1F	Phosphatase mutant R362A, I338A

2.1.12 Primers

Name	Sequence (5'->3')
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Flag- Coronin 1C FL	Forward Primer ATATATAAGCTTATGAGGCGAGT Reverse Primer ATATATCTCGAGTCA GGCTGCTATCTTTGCCATC
Flag- Coronin 1C ⁷²⁻⁴⁷⁴	Forward Primer ATATAAGCTTATGTCTTACCCTACAGTATGTGG Reverse Primer ATATATCTCGAGTCA GGCTGCTATCTTTGCCATC
Flag- Coronin 1C ¹⁻⁴⁴⁴	Forward Primer ATATATAAGCTTATGAGGCGAGT Reverse Primer ATAT TCGAGTCAAATCTCATCCAACCTGGCTT
Flag- Coronin 1C ³⁰⁰⁻⁴⁷⁴	Forward Primer ATATATAAGCTTATG CCGTACGTCCACTACCTCAA Reverse Primer ATATATCTCGAGTCA GGCTGCTATCTTTGCCATC
Flag- Coronin 1C ³⁰⁰⁻⁴⁴⁴	Forward Primer ATATATAAGCTTATG CCGTACGTCCACTACCTCAA Reverse Primer ATAT TCGAGTCAAATCTCATCCAACCTGGCTT
GST- Coronin 1C ⁴⁴⁴⁻⁴⁷⁴	Forward Primer ATAT AAGCTTATGTAAAAGAGATCAAATCTAT Reverse Primer ATATATCTCGAGTCA GGCTGCTATCTTTGCCATC
Flag- Coronin 1C S463D	Forward Primer GCAATCAAGATGAGCGTATTGACAAGTTAGAACAGCAGATG GC Reverse Primer GCCATCTGCTGTTCTAACTTGTC AATACGCTCATCTTGATTGC
Flag- Coronin 1C S463A	Forward Primer GCAATCAAGATGAGCGTATTGCCAAGTTAGAACAGCAGATGG C Reverse Primer GCCATCTGCTGTTCTAACTTGGAATACGCTCATCTTGATTGC
Flag- Coronin	Forward Primer

1C T415E	ACATTCTGGATAGCAAGCCCGATGCAAACAAGAAGTGCGACC T Reverse Primer AGGTCGCACTTCTTGTTTGCATCGGGCTTGCTATCCAGAATGT
Flag- Coronin 1C T415A	Forward Primer ACATTCTGGATAGCAAGCCCGCTGCAAACAAGAAGTGCGACC T Reverse Primer AGGTCGCACTTCTTGTTTGCAGCGGGCTTGCTATCCAGAATGT

2.1.10 siRNAs

siRNA	Sequence (5'->3')
Luciferase (Control)	ACAUCACGUACGCGGAAUACUUCGA
POPX2 #1	ACCGCGCCUACUUUGCUGUGUUUGA
POPX2 #5	CCAAGAUGACCUGUUGUGUCAUAUA
Coronin 1C	GCACAAGACUGGUCGAAUU

2.2 Methods

2.2.1 Cell culture

MDA-MB-231, U2-OS, HEK293 and NIH3T3 fibroblasts cells were cultured in DMEM containing 4.5 g/L glucose supplemented with 3.7 g/L sodium bicarbonate and 10 % FBS. HeLa cells were grown in MEM supplemented with 2 mM L-glutamine and 10 % FBS. All mammalian cell lines were incubated at 37 °C with 5 % CO₂.

2.2.2 Cell lysis and Western blot

Cells were washed with 1x PBS and lysed with protein lysis buffer (50 mM Hepes pH 7.5, 300 mM NaCl, 1 mM MgCl₂, 1 mM EGTA, 10 mM β-glycerophosphate, 1 mM Sodium orthovanadate, 5 % glycerol, 5 mM DTT, 0.5 %

TritonX-100) supplemented with Protease inhibitor (Roche) and PhosSTOP (Roche). Cell lysates were centrifuged at 14,000 rpm for 10 min at 4 °C to clarify them. Subsequently, protein concentration determined with Bio-Rad protein assay kit. Equal amount of protein lysates in final 1x SDS sample buffer were heated at 100 °C for 10 min and loaded into each well on SDS-PAGE gel. The proteins were transferred to nitrocellulose membranes and blocking step was carried out for 1 h in 5 % skimmed milk or 3 % BSA for phospho-protein. Membranes were incubated with primary antibody at recommended dilutions at 4 °C overnight and secondary antibody at 1:4000 dilution for 1 h at room temperature (RT). The chemiluminescent signals were detected on X-ray film (Kodak) using Amersham ECL (GE healthcare).

2.2.3 Plasmids and siRNA transfection

Cells were seeded at 80-90 % confluency and transfected with 1-3 µg of plasmid DNA using Lipofectamin 2000™ and Opti-MEM™, which is a reduced serum medium according to manufacturer's instructions. For siRNA transfection, cells were seeded at 80 % confluency and transfected with 50 nM siRNA using Lipofectamin 2000™ and Opti-MEM™ according to manufacturer's protocol. The transfection efficiency was tested by either fluorescence microscopy or Western blot analysis. All the experiments were carried out 24~48 h post transfection.

2.2.4 Glutathione S-transferase (GST) - and Flag-pulldown assays

HEK293 cells were transfected with plasmids and lysed with Co-IP buffer (20 mM Tris-Cl, 150 mM NaCl, 1 % Triton-X in 1x TBS) supplemented with

Protease inhibitor and PhosSTOP 24 h post transfection. Cells were centrifuged at 14,000 rpm for 10 min at 4 °C and the supernatant was incubated with 30 µL of Glutathione Sepharose 4B™ (GE healthcare) or 20 µL anti-FLAG® M2 Affinity gel (Sigma) at 4 °C overnight with constant rotation. The beads were washed thrice with Co-IP buffer and precipitated proteins with beads was eluted with 1x SDS sample buffer by heating at 100 °C for 10 m. Precipitated proteins were loaded into each well on SDS-PAGE gel and Western blot was carried out as described earlier.

2.2.5 Co-Immunoprecipitation assays

HeLa cells were lysed with Co-IP buffer and centrifuged at 14,000 rpm for 10 m. For pre-clearing, the supernatant was added to 20 µL of protein G magnetic beads (Millipore) at 4 °C for 2 h under constant rotation. The pre-cleared supernatant was then incubated with a specific antibody at 4 °C overnight. Lysate-antibody mixture was incubated with protein G magnetic beads at 4 °C for 4 h. The beads were washed thrice with Co-IP buffer and the bound protein was eluted with 1x SDS sample buffer by boiling at 100 °C for 10 min. The eluted sample was loaded and Western blot was carried out as described earlier.

2.2.6 *In vivo* and *in vitro* de-phosphorylation assays

HeLa cells were co-transfected with plasmids encoding GST, GST-POPX2 or GST-POPX2M and Flag-Coro 1C. Cells were lysed with Co-IP buffer supplemented with Protease inhibitor and PhosSTOP 24 h post transfection. The equal amount of protein lysates was incubated with anti-FLAG® M2 Affinity gel at 4 °C overnight. Flag beads were washed thrice with Co-IP buffer and Flag-

Coro 1C protein was eluted by boiling and carried out Western blot analysis. Phosphorylated Coro 1C was detected using total serine/threonine antibody.

HeLa cells were transfected with Flag-Coro 1C and lysed 24 h post transfection. Cell lysates were added into anti-FLAG® Affinity gel at 4 °C overnight. The beads were washed thrice with Co-IP buffer and purified GST-POPX2 protein was added with phosphatase buffer (50 mM Hepes pH 7.3, 10 mM MgCl₂, 5 mM MnCl₂, 1 mM DTT, 0.05 % Triton-X-100) for 45 min at 30 °C. The reaction was ended by adding 1x SDS sample buffer and boiled at 100 °C for 10 min. Subsequently, Western blot was carried out and total serine/threonine antibody was used to detect phosphorylated Coro 1C.

2.2.7 Protein purification

PGEX-6P1-POPX2 and PET- His6-Coro 1C³⁴⁹⁻⁴⁷⁴ plasmids were transformed into *E.coli* strain BL21. GST-tagged POPX2 protein was purified by Glutathione Sepharose™ 4B beads and Ni Sepharose™ 6 Fast Flow (Roche) was used for His6-Coro 1C³⁴⁹⁻⁴⁷⁴ 1C purification. Bacterial LB culture media with either Ampicillin or Kanamycin were incubated at 37 °C overnight. Upon reaching OD value at 0.4 - 0.6, 1 mM IPTG was added and incubated at 160 rpm, 16 °C overnight. Bacterial media were centrifuged at 4000 rpm for 40 min and bacterial pellet were suspended in protein lysis buffer containing 50 mM Tris-Cl pH8.0, 200 mM NaCl, 10 % glycerol, 1.5 % Sarkosyl, 1 mM lysosome, supplied with 1 mM DTT and 1 mM PMSF and sonication at 25~30 % power for 1 min 3 times with 1 min interval. Clear lysate was loaded to beads and incubate for 2 h at 4 °C under constant rotation. GST-tagged protein was eluted with 20 mM reduced L-

glutathione in 50 mM Tris-Cl pH8.0. For His-tagged protein, the resin was washed with elution buffer containing 10 mM imidazole and the protein was eluted on a gradient from 50 to 500 mM imidazole. PD-10 desalting column (GE Healthcare) was used for storage buffer exchange and Centricon® centrifugal filter units (Millipore) were used for protein concentration.

2.2.8 Protein binding assay

Bacterially expressed GST or GST-POPX2 was incubated with bacterially expressed His-Coro 1C³⁴⁹⁻⁴⁷⁴ (plasmid was kindly provided from Protein Production Platform (PPP) at NTU). Ni Sepharose beads were used to precipitate His tagged proteins at 4 °C overnight and beads were washed thrice with Co-IP buffer to remove unspecific bindings. The samples were boiled and subjected to Western blot analysis.

2.2.9 Site-directed Mutagenesis

QuickChange™ Site-Directed Mutagenesis kit (Stratagene) was used to generate phospho-dead and phospho-mimic mutants. Mutagenesis reactions were carried out according to the manufacturer's protocol. DpnI enzyme was used to remove parental DNA template and mutagenesis reaction was used for transformation using heat shock method. Colonies were picked and incubated in LB broth with 100 µg/mL ampicillin. Plasmids were purified using AxyPrep™ Plasmid Miniprep Kit (Axygen) and verified by sequencing.

2.2.10 Immunofluorescence

HeLa and MDA-MB-231 cells were fixed with 4 % paraformaldehyde (PFA) and permeabilized in 0.2 % Triton-X-100 and blocked with 4 % BSA. Cells were

incubated with primary antibodies at 4 °C overnight and secondary antibodies at room temperature for 1 h. The primary antibody was diluted according to manufacturer's protocol and the secondary antibody was used at 1:100 dilution. Coverslips with cells were mounted using Vectashield® with DAPI (Vector Laboratories). Cell images were taken by Carl Zeiss Axiovert microscope with 40x/1.25 and 63x/1.4 objective and recorded on a Roper Scientific CoolSNAP CCD camera.

2.2.11 Fluorescence-activated cell sorting (FACS)

Cells were fixed with ice-cold 70 % ethanol at 4 °C overnight. Fixed cells were centrifuged for 5 min and washed five times with 1x PBS to remove fixing solution. Fixed cells were stained with Propidium Iodide for 1 h at RT. Stained samples were analysed with X-20 Fortessa (BD Biosciences) using FACSDiva software and FlowJo.

2.2.12 PDMS crossbow shaped-micro-patterning

PDMS gel was mixed with a curing agent in a 9:1 ratio and was degassed for overnight to remove bubbles in the mixture. The degassed gel was poured into the mould with crossbow shaped-micro-patterned and incubated at 80 °C to cure the mixture. PDMS stamps were plasma treated at 8.5kV for 5 min to decrease hydrophobicity of the stamps. Alexa-633 cy5 dye was diluted in fibronectin/collagen in 1x PBS at 1:100 to verify the shapes. The stamp was then inverted and stamped down onto the 27 mm Iwaki glass bottom. The glass bottom with specific shape was treated with 0.2 % pluronic acid for 1 h. The dish was

washed with 1x PBS thrice and 10,000 cells were seeded into micropatterned dish for 1 h and then fixed.

2.2.13 Flag-Coro 1C pulldown-mass spectrometry

Flag-Coro 1C plasmid was transfected into HeLa cells and cells were lysed 24 h post transfection. Cell lysates were immunoprecipiated using Flag beads at 4 °C overnight with constant rotation. Beads were washed with Co-IP buffer 5 times to remove unspecific binding and precipitated Coro 1C was subject to *in vitro* phosphatase assays as mention above (2.2.6). Final 1x SDS sample buffer was added to terminate the reaction and subjected to Western blot. SDS-PAGE gel was stained with Coomassie blue for 30 min with constant rotation at RT and bands were isolated. The samples were sent to LC/MS-MS service at NTU Mass Spectrometry core facility.

2.2.14 Wound healing assay

NIH3T3 fibroblasts were seed at 80% confluency and transfected with GFP, GFP-POPX2, GFP-Coro 1C-WT, GFP-Coro 1C-S463A, GFP-Coro 1C-S463D, GFP-Coro 1C-T415A, or GFP-Coro 1C-T415E. After 24 h, cells were seeded into the culture-insert (Ibidi). Once cells reach 100% confluency, the culture-insert was removed and the wound healing images were taken at indicated time points. Wound closure was quantified by Image J software.

2.2.15 Cloning and Polymerase chain reaction (PCR)

Primers with appropriate restriction enzyme recognition sites were designed to amplify the desired DNA sequence by PCR. Amplified products were subjected

to agarose gel electrophoresis and purified with Gel purification kit (Qiagen). Plasmid vectors and purified inserts were digested using two designed restriction enzymes for 1 h at 37 °C. PCR clean up kit (Qiagen) was used to remove enzymes and subjected to ligation at 4 °C overnight. The ligation reaction were transformed into DH5 α cells by heat shock method. The sequence of successful colonies were verified by sequencing.

2.2.16 Live cell imaging

Plasmid encoding mCherry, mCherry-POPX2 or mCherry-POPX2M was co-expressed with GFP-Coro 1C in NIH3T3 fibroblasts and cells were re-seeded onto a fibronectin coated dish 24 h post transfection. Live imaging was performed on Carl Zeiss Axiovert microscope at 37 °C with 63x/1.4 objective with 60 s interval.

2.2.17 Subcellular protein fractionation assay

Plasmid encoding GST or GST-POPX2 was transfected into HeLa cells and cells were lysed 24 h post transfection using subcellular protein fractionation kit (Thermo Scientific). Cells were lysed with ice-cold Cytoplasmic Extraction Buffer (CEB) supplemented with protease inhibitor (PI) for 20 min. Cytoplasmic proteins were obtained after centrifuge at 2500 rpm for 5 min at 4 °C. Cytoskeletal proteins were collected after pellet was incubated with Pellet Extraction Buffer (PEB) containing PI for 10 min at RT and centrifuged at 14,000 rpm. The extracts were subjected to Western blot analysis.

3. Results and Discussion- The functional relationship between Coro 1C and POPX2

3.1 Results

3.1.1 Screening for putative partners of POPX2 phosphatase

Previous findings from our laboratory show that cell motility and invasiveness of breast cancer cells are positively associated with the levels POPX2 in the cells (Susila et al., 2010). To understand how POPX2 contributes to enhanced cell migration and invasiveness in cancer cells, Flag-POPX2 pulldown-MS was conducted to explore new binding partners of POPX2 in HEK293 cells (Flag-POPX2 pulldown-MS was performed by Dr Weng Ting).

A total of 12 potential targets were co-precipitated with Flag-POPX2. In this experiment, Flag tag was used as a negative control. Coronin 1C (Coro 1C) is an interesting hit due to its role in actin cytoskeleton organization. Moreover, the number of Coro1C peptides is the highest amongst other potential targets (Fig 1 in the appendix). Therefore, we decided to further investigate the interaction between POPX2 and Coro 1C.

3.1.2 POPX2 interacts with Coro 1C

To validate the POPX2-pulldown-MS experiment and confirm the biological interaction between POPX2 and Coro 1C within the cells, Flag- and GST-pulldown assays were performed. Plasmid encoding Flag-Coro 1C was co-transfected with GST or GST-POPX2 into HEK293 cells. GST-POPX2 was detected in the complex obtained from Flag-Coro 1C pulldown (Fig 11A). Similarly, Flag-Coro 1C was observed in GST-POPX2 pulldown but not in the of GST pulldown (Fig 11B). Our results suggest that POPX2 and Coro 1C may form a complex in the cells. To test the binding of POPX2 to Coro 1C under physiological condition, endogenous POPX2 was isolated with anti-POPX2 and Coro 1C was found to co-immuno-precipitate with POPX2 (Fig 11C). Immuno-precipitation of Coro 1C using anti-Coro 1C also demonstrated that endogenous POPX2 forms a complex with Coro 1C in the cells (Fig 11D).

Figure 11. POPX2 interacts with Coro 1C.

(A) Plasmid encoding Flag-Coro 1C was co-expressed with GST or GST-POPX2 and cells were lysed 24 h post transfection. Flag-Coro 1C was isolated from cell lysates using Flag beads and subjected to Western blot analysis. (B) GST or GST-POPX2 cDNA constructs was co-expressed with Flag-Coro 1C and GST proteins were precipitated from cell lysates using Glutathione Sepharose beads 24 h after transfection. (C) Endogenous POPX2 was isolated with anti-POPX2 mouse antibody and co-precipitated proteins were analysed by Western blot. Coro 1C was found in POPX2 immuno-precipitated complex. (D) Endogenous Coro 1C was precipitated with Coro 1C rabbit antibody and POPX2 was detected in Coro 1C immuno-precipitated complex. Random IgG was used as control in (C) and (D).

3.1.3 POPX2 interacts with the coiled coil domain of Coro 1C

The binding of POPX2 to Coro 1C was validated using immunoprecipitation assays. Next, the binding regions of Coro 1C to POPX2 were investigated. Coro 1C consists of the N-terminus, WD40 repeats, conserved and unique linker and the coiled coil domain at the C-terminus (Fig 12A). Plasmids encoding various truncation mutants of Coro 1C were co-transfected with GST or GST-POPX2. Deletion of the coiled coil domain of Flag-Coro 1C¹⁻⁴⁴⁴ abolished the binding with POPX2, but deletion of the N-terminus of Flag-Coro 1C⁷²⁻⁴⁷⁴ did not affect its binding to POPX2, suggesting that the coiled coil domain of Coro 1C is crucial for its binding to POPX2 (Fig 12B-C).

To confirm that the coiled coil domain mediates Coro1C interaction with POPX2, constructs encoding the C-terminus with the coiled coil domain of Flag-Coro 1C³⁰⁰⁻⁴⁷⁴ and the C-terminus without the coiled coil domain of Flag-Coro 1C³⁰⁰⁻⁴⁴⁴ were used for co-precipitation assays. Comparison of Flag-Coro 1C³⁰⁰⁻⁴⁷⁴ and Flag-Coro 1C³⁰⁰⁻⁴⁴⁴ demonstrates that GST-POPX2 was co-precipitated with Flag-Coro 1C³⁰⁰⁻⁴⁷⁴, while GST-POPX2 was not observed in Flag-Coro 1C³⁰⁰⁻⁴⁴⁴ pulldown lysates (Fig 12D). Furthermore, the coiled coil domain of GST-Coro 1C⁴⁴⁴⁻⁴⁷⁴ was detected in the complex obtained from Flag-POPX2 pulldown (Fig 12E). Taken together, the results demonstrate that the coiled coil domain of Coro 1C is required for the interaction with POPX2.

A

B

C

Figure 12. POPX2 interacts with the coiled coil domain of Coro 1C.

(A) Schematic image of Coro 1C constructs that was used for co-precipitation assays. Coro 1C consists of the β -propeller domain including the N-terminus, WD40 repeats and the C-terminus containing conserved, unique linker and the coiled coil domain. Full-length and truncated constructs of Coro 1C were co-expressed with GST or GST-POPX2 in HEK293 cells. (B) Plasmids encoding Flag-Coro 1C⁷²⁻⁴⁷⁴ and Flag-Coro 1C¹⁻⁴⁴⁴ were co-expressed with GST or GST-POPX2. (C) Full length of Coro 1C and Flag-Coro 1C¹⁻⁴⁴⁴ were co-transfected together with GST or GST-POPX2. GST proteins were precipitated using Glutathione Sepharose beads and subjected to Western blot analysis. (D) GST or GST-POPX2 was co-expressed with Flag-Coro 1C³⁰⁰⁻⁴⁷⁴ and Flag-Coro 1C³⁰⁰⁻⁴⁴⁴. (E) GST-Coro 1C⁴⁴⁴⁻⁴⁷⁴ or GST was co-transfected with Flag-POPX2. The cell lysates were subjected to Flag pulldown and proteins isolated were analysed by Western blot in (D-E).

3.1.4 POPX2 directly interacts with Coro 1C

The interaction between POPX2 and the C-terminus of Coro 1C was validated using co-precipitation assays (Fig 12). We then investigated whether POPX2

directly interacts with Coro 1C or not. As full length of Coro 1C is poorly expressed in bacteria cell, the C-terminus of His-Coro 1C³⁴⁹⁻⁴⁷⁴ was used for the *in vitro* assays (small-scale expression screening of protein in *E.coli* and cloning were performed by Protein Production Platform (PPP) at NTU). Bacterially expressed recombinant GST-POPX2 protein or GST were incubated with bacterially expressed recombinant His-Coro 1C³⁴⁹⁻⁴⁷⁴. Proteins from the reaction mixture were isolated using Glutathione Sepharose beads and His-Coro 1C³⁴⁹⁻⁴⁷⁴ was pulled down together with GST-POPX2 (Fig 13). The results suggest that GST-POPX2 directly interacts with the C-terminus of His-Coro 1C³⁴⁹⁻⁴⁷⁴.

Figure 13. Direct interaction between POPX2 and the C-terminus of Coro 1C³⁴⁹⁻⁴⁷⁴

Bacterially expressed recombinant His-Coro 1C³⁴⁹⁻⁴⁷⁴ protein was purified using Ni Sepharose and GST-POPX2 and GST proteins were purified using Glutathione Sepharose beads. GST or GST-POPX2 was incubated with His-Coro 1C³⁴⁹⁻⁴⁷⁴ and isolated using Glutathione Sepharose beads to investigate the binding between POPX2 and the C-terminus of Coro 1C. Pulled down lysates were subjected to Western blot analysis.

3.1.5 POPX2 dephosphorylates Coro 1C

It has been reported that the coiled coil domain at the C-terminus of Coro 1C is important for the regulation of oligomerization and subcellular localization of Coro 1C to the plasma membrane (Spoerl et al., 2002). Moreover, the coiled coil domain contains a reported phosphorylation site at Ser463 (Xavier et al., 2012). We next tested whether Coro 1C is a substrate of POPX2 using *in vivo* and *in vitro* phosphatase assays.

Flag-Coro 1C was co-expressed with GST, GST-POPX2 or GST-POPX2M (phosphatase-dead mutant). As there is no commercially available phospho-Coro 1C antibody, Flag-Coro 1C was precipitated from cell lysates and phosphorylated Coro 1C was detected using phospho-Ser/Thr antibody. The results show that total amount of phospho-Coro 1C was attenuated by overexpression of GST-POPX2. As GST-POPX2M has reduced phosphatase activity due to the mutation within the catalytic domain, overexpression of GST or GST-POPX2M did not have significant effect on dephosphorylation of Coro 1C (Fig 14A). The relative ratio of phospho-Coro 1C/Coro 1C demonstrates that overexpression of POPX2 efficiently dephosphorylates Coro 1C *in vivo* (Fig 14B).

To study whether POPX2 is able to dephosphorylate Coro 1C directly, we performed *in vitro* phosphatase assays. Flag-Coro 1C was transfected into cells and treated with Calyculin A (CA) to inhibit dephosphorylation of Coro 1C by PP1 and PP2A phosphatases. Flag-Coro 1C was isolated from cell lysates using Flag beads and incubated with or without bacterially expressed recombinant GST-POPX2 protein in phosphatase buffer for *in vitro* reaction. The results show

that phospho-Coro 1C was reduced by GST-POPX2 (Fig 14C). The relative ratio of phospho-Coro 1C/Coro 1C demonstrates that phosphorylation of Coro 1C is significantly attenuated by incubation with GST-POPX2 *in vitro* (Fig 14D). Taken together, the results suggest that Coro 1C is dephosphorylated by POPX2 *in vivo* and *in vitro* and Coro 1C is a substrate of POPX2.

Figure 14. POPX2 dephosphorylates Coro 1C.

GST, GST-POPX2 or GST-POPX2M was co-expressed with Flag- Coro 1C in HeLa cells. Flag-Coro 1C was precipitated from cell lysates and subjected to Western blot analysis. Phospho-Coro 1C was detected using phospho-Ser/Thr antibody and Flag-Coro 1C was observed using Flag antibody. (B) Densitometry measurement was done using ImageJ for three independent experiments. (C) HeLa cells were transfected with Flag-Coro 1C and treated with DMSO (control) or CA to prevent dephosphorylation by PP1 and PP2A before cell lysis. Precipitated Flag-Coro 1C was incubated with or without bacterially expressed GST-POPX2 in phosphatase buffer at 30 °C for 45 min. The reaction mixtures were subjected to SDS-PAGE and Western blot. Input for Flag-Coro 1C with DMSO (lane 1), input for Flag-Coro 1C with CA (lane 2), precipitated Flag-Coro 1C (lane 3), precipitated Flag-Coro 1C with purified GST-POPX2 (lane 4), precipitated Flag-Coro 1C with CA treatment (lane 5) and precipitated Flag-Coro 1C with CA and incubated with purified GST-POPX2 (lane 6). (D) The analysis was done as mentioned in (B) for three independent experiments. Error bars represent standard deviation. * $p \leq 0.05$, as analysed by Student's t-test.

3.1.6 Coro 1C interacts with POPX2 and POPX2M

Since Coro 1C is a substrate of POPX2, we tested whether phosphorylation of Coro 1C affects its interaction with POPX2. Flag-Coro 1C was co-expressed with GST, GST-POPX2 or GST-POPX2M and co-precipitation assays were executed. GST-POPX2 and GST-POPX2M were detected in the complex obtained from Flag-Coro 1C pulldown. Interestingly, higher levels of GST-POPX2M were observed from Flag-Coro 1C pulldown than GST-POPX2 (Fig 15A).

Consistently, GST fusion proteins were precipitated from cell lysates and Flag-Coro 1C was observed in the complex obtained from GST-POPX2 and GST-POPX2M pulldown. Higher levels of Flag-Coro 1C was associated with GST-POPX2M than GST-POPX2 (Fig 15B). Fig 14A shows that overexpression of POPX2 reduced phosphorylated Coro 1C, while overexpression of POPX2M did not. Thus, these results suggest that POPX2 might interact with phosphorylated Coro 1C and that is why GST-POPX2M may have more complex with Flag-Coro 1C.

Figure 15. POPX2 and POPX2M interacts with Flag-Coro 1C.

(A) GST, GST-POPX2 or GST-POPX2M was co-expressed with Flag-Coro 1C in HEK293 cells. Flag-Coro 1C was isolated from cell lysates using Flag beads and subjected to SDS-PAGE and Western blot. (B) Plasmids were transfected as mentioned in (A) and GST-tagged proteins were precipitated using Glutathione Sepharose beads and analysed by Western blot.

3.1.7 Silencing POPX2 results in the loss of Coro 1C localization to the cell periphery

The C-terminus of Coro 1C is important for its subcellular localization (Spoerl et al., 2002). In this current study, we found out that POPX2 interacts with the C-terminus of Coro 1C and POPX2 dephosphorylates Coro 1C. Dephosphorylation of Coro 1C has been reported to promote its localization from the cytoplasm to the plasma membrane (Hasse et al., 2005; Spoerl et al., 2002). To verify the functional link of the interaction between POPX2 and Coro 1C, we analysed the subcellular localization of Coro 1C in control and POPX2-knockdown cells.

Interestingly, Coro 1C localizes to the cell periphery in wild type (WT) and control (siLuc) cells, while Coro 1C is found mainly in the cytoplasm in POPX2-knockdown (siX2) cells (Fig 16A-B, arrows). Quantification reveals that cells displaying Coro 1C at the cell periphery is reduced from around 80% to 40% by silencing POPX2 (Fig 16C). Western blot analysis shows that silencing POPX2 did not affect the overall levels of Coro 1C protein and POPX2-knockdown efficiency was validated using Western blot (Fig 16D).

Figure 16. Silencing POPX2 using siRNA reduces the localization of Coro 1C to the cell periphery.

(A) HeLa cells were transfected with luciferase siRNA (siLuc) and POPX2 siRNA (siX2) for 48 h. Cells were fixed and stained with Coro 1C antibody (green) and DAPI (blue) to visualize Coro 1C and the nucleus, respectively. Scale bar 20 μ m. (B) The images were cropped using Image J to show the cell periphery. (C) Percentage of cells displaying Coro 1C localization to the cell periphery in siLuc and siX2 cells. Error bars represent mean $\pm$ S.E. of three independent experiments (N=200). ** $p \leq 0.01$, as analysed by Student's t-test. (D) Protein levels of Coro 1C, POPX2 and actin were analysed by immunoblotting and actin was used as a loading control.

3.1.8 POPX2-knockout in MDA-MB-231 cells display diminished localization of Coro 1C and active Rac1 to the membrane protrusion

Knocking-down POPX2 by siRNA in HeLa cells leads to loss of Coro 1C localization to the cell periphery (Fig 16). In the following experiments, we have decided to use POPX2-knockout (KO) MDA-MB-231 cells to make sure that all cells visualized do not contain POPX2. MDA-MB-231 cells express high levels of POPX2 (Susila et al., 2010), the knock-out cell line was constructed using CRISPR. (POPX2-knockout in MDA-MB-231 cells using CRISPR was generated by Dr Zhang Songjing.)

MDA-MB-231 wild type (WT) cells display Coro 1C localization to lamellipodial-like extensions, while POPX2-KO cells exhibit reduced localization of Coro 1C to the cell periphery (Fig 17A). To observe the membrane protrusion and the localization of Coro 1C and F-actin, the images were enlarged and cropped (Fig 17A, rightmost panels). Fluorescence intensity of Coro 1C and F-actin were measured along the white dash lines as shown in Fig 17A. WT cells show increased fluorescence intensity of Coro 1C along with the lamellipodial-like extension compared with POPX2-KO cells (Fig 17B). Taken together, our findings suggest that POPX2 regulates localization of Coro 1C to the membrane protrusion in MDA-MB-231 cells.

Coro 1C interacts with GDP-Rac1 and redistributes Rac1 from the lateral membrane to the leading edge for Rac1 activation (Williamson et al., 2014). To study whether the loss of Coro 1C to the membrane protrusion in POPX2-KO cells affects its functional role in the cells, the localization of active Rac1 was investigated in WT and POPX2-KO cells. POPX2-KO cells exhibit reduced

localization of active Rac1 and Coro 1C to the membrane protrusion compared with WT cells (Fig 17C). WT cells show increased fluorescence intensity of active Rac1 and Coro 1C compared with POPX2-KO cells (Fig 17D). Western blot analysis validates absence of POPX2 expression in POPX2-KO cells, the levels of Coro 1C and total Rac1 are not affected. Taken together, our results suggest that POPX2 regulates the localization of Coro 1C and redistribution of Rac1 from the lateral membrane to the leading edge.

Figure 17. POPX2 regulates the localization of Coro 1C and active Rac1 to the membrane protrusion.

(A) MDA-MB-231 WT and POPX2-KO cells were fixed and stained using anti-Coro 1C antibody and phalloidin. White box refers the region of enlarged image. (B) Intensity profiles of the Coro 1C and F-actin were obtained across the white dash lines (from outside to inside of cell) in each corresponding image. Six individual cells from each cell type were analysed, representative profiles from one cell of each cell type are shown here. (C) Cells were fixed and stained with anti-active Rac1 antibody and anti-Coro 1C antibody. (D) Intensity profiles of the Coro 1C and active Rac1 were obtained across the white dash lines in each corresponding image (from outside to inside of cell). (E) Validation of POPX2-KO by Western blot and actin was used as a loading control. Coro 1C and total Rac1 antibodies were used to detect the amount of proteins in WT and POPX2-KO cells. Scale bar 10 μ m.

3.1.9 Constitutively active Rac1 promotes Coro 1C localization to the cell periphery

The localization of XCoronin (Xenopus Coronin) has been reported to be regulated by Rac1 activity in Swiss 3T3 fibroblasts. XCoronin was observed in the lamellipodia in constitutively active RacV12 overexpressing fibroblasts but not in case of RhoV14 overexpressing cells (Mishima & Nishida, 1999), suggesting that the localization of XCoronin to the lamellipodia is induced by active Rac.

We next examine the effect of Rac1 on the localization of Coro 1C to the membrane protrusions. The localization of Coro 1C was detected in cells overexpressing GFP (control), constitutively active GFP-RacV12 or dominant negative GFP-RacN17. Coro 1C localizes to the dominant membrane protrusion in GFP expressing cells. Coro 1C accumulates to lamellipodia and membrane ruffles in GFP-RacV12 overexpressing cells, whereas Coro 1C loses its localization at the cell edges in GFP-RacN17 overexpressing cells (Fig 18A). Fluorescence intensity profiles demonstrate that Coro 1C localizes at the cell edges in GFP and GFP-RacV12 overexpressing cells, while Coro 1C localizes to the cytosol in GFP-RacN17 overexpressing cells (Fig 18B). Therefore, we conclude that Rac1 activity regulates the localization of Coro 1C to the cell periphery.

Figure 18. Coro 1C localizes to the cell periphery in GFP-RacV12 overexpressing cells.

(A) Plasmids encoding GFP, GFP-RacV12 or GFP-RacN17 was transfected into HeLa cells and cells were fixed and stained with anti-Coro 1C antibody. (B) Intensity profiles of the Coro 1C and GFP were obtained across the white dash lines in each corresponding image (from outside to inside of cells). Scale bar 20 μm .

3.1.10 POPX2 enhances cell spreading on crossbow-shaped micropattern

We have established that high levels of POPX2 modulate the localization of Coro 1C to the cell periphery in two different cell lines (HeLa and MDA-MB-231). To evaluate the possible involvement of Coro 1C in actin-dependent extension of the cell periphery, we used NIH3T3 fibroblasts to examine the effect of cell spreading because NIH3T3 cells can spread well on substrates and have higher transfection efficiency. In this study, we used the crossbow-shaped micropattern to control the geometry of cell spreading (Fig 19A).

NIH3T3 cells overexpressing GFP, GFP-POPX2 or GFP-POPX2M were seeded onto fibronectin coated crossbow-shaped micropattern as shown in Fig 19A. Bright-field microscopy images show that overexpression of POPX2 in NIH3T3 fibroblasts increases the lamellipodial extension compared to control cells (Fig 19C). To quantify the extent of the lamellipodial extension, we measured the length and area of the lamellipodium as shown in Fig 19B. We defined the lamellipodium as the portion of the plasma membrane which showed a convex curvature, as illustrated by the red line. Green line drawn along the cell boundary indicates the distance between two adhesion sites.

The average length of lamellipodium per cell in GFP transfected cells is $\sim 30\ \mu\text{m}$, that in GFPX2 (cells overexpressing GFP-POPX2) transfected cells is $\sim 40\ \mu\text{m}$ and that in GFPX2M (cells overexpressing POPX2M, phosphatase-dead mutant) transfected cells is $\sim 30\ \mu\text{m}$. The average area of lamellipodium per cell in GFP transfected cells is $\sim 300\ \mu\text{m}^2$, that in GFPX2 cells is $\sim 400\ \mu\text{m}^2$ and that in GFPX2M cells is $\sim 300\ \mu\text{m}^2$ (Fig 19D). Our observations suggest that

overexpression of GFP-POPX2 in cells enhances the lamellipodial extension area and length. POPX2M overexpressing cells which have reduced POPX2 phosphatase activity resembled the control cells, suggesting that the catalytic activity of POPX2 may contribute to the lamellipodial extension at the leading edge.

Figure 19. POPX2 overexpression leads to wider lamellipodial extension.

(A) The image represents crossbow-shaped micropatterns on glass bottom dish. The schematic image indicates the leading edge and rear of the cell. (B) The lamellipodia area was measured by the region of a convex curvature (as indicated as red line) and the distance was measured by the length of two adhesion sites (as indicated as green line). (C) GFP, GFP-POPX2 and GFP-POPX2M overexpressing cells were plated on fibronectin-coated crossbow-shaped micropatterns and fixed after 1 h. Bright-field images of GFP (control) and POPX2 overexpressing fibroblasts. The images were enlarged and cropped using Image J. Scale bar 20 μm (D) Cells transfected with GFP, GFP-POPX2 or GFP-POPX2M were analysed as mentioned in (B) and the results were displayed as mean $\pm$ S.E. (error bar) from three individual experiments (N=35). ***p $\leq$ 0.001, as analysed by Student's t-test.

3.1.11 Coro 1C localizes to the lamellipodia in POPX2 overexpressing cells during cell spreading

In this study, we discovered that overexpression of POPX2 leads to wider lamellipodial extension. To study the possible involvement of Coro 1C in POPX2-mediated lamellipodial extension during cell spreading, live cell imaging analysis was performed. GFP-Coro 1C was co-transfected with mCherry, mCherry-POPX2 or mCherry-POPX2M into NIH3T3 fibroblasts and re-plated onto fibronectin coated dish to observe the localization of Coro 1C during the cell spreading.

GFP-Coro 1C accumulates in the lamellipodial extension in mCherry-POPX2 overexpressing cells during the cell spreading. Cells transfected with mCherry show the localization of GFP-Coro 1C to the actin filaments with no distinct localization to the lamellipodia. Cells overexpressing mCherry-POPX2M exhibit GFP-Coro 1C localization to the edges of cells but no distinct localization to lamellipodia (Fig 20A). After 30 min of cell spreading, cells were fixed and spreading areas were measured. Consistent with previous results (Fig 19D), POPX2 overexpression enhances cell spreading area (Fig 20B). Therefore, the results implicate that POPX2 contributes to enhanced cell spreading and Coro 1C is concentrated to the lamellipodial extension in POPX2 overexpressing cells.

Figure 20. POPX2 overexpressing NIH3T3 fibroblasts display Coro 1C accumulation at the lamellipodial extension.

(A) NIH3T3 fibroblasts were transfected with plasmids encoding mCherry + GFP-Coro 1C, mCherry-POPX2 + GFP-Coro 1C or mCherry-POPX2M + GFP-Coro 1C. The Cells were re-plated onto fibronectin coated dish 24 h post transfection. Time lapse images were taken with 60 s interval. Left panel is the time lapse images of GFP and mCherry and right panel is the merged images. Scale bar 20 μm . (B) Cells were fixed after 30 min of cell spreading and spreading areas were measured using Image J. The results were displayed as mean $\pm$ S.E. (error bar) from three individual experiments (N=25). * $p \leq 0.05$, *** $p \leq 0.001$, as analysed by Student's t-test.

3.1.12 POPX2 overexpressing cells show increased Coro 1C localized to cytoskeletal fraction compare to control cells

Immunofluorescence and live imaging results suggest that POPX2 regulates the localization of Coro 1C from the cytosol to the cell periphery (Fig 16-17, 20). To confirm our observation, we conducted subcellular protein fractionation assay to investigate the localization of Coro 1C in control and POPX2 overexpressing cells. GST-POPX2 overexpressing cells have increased Coro 1C localized to cytoskeletal fraction compare to control cells (Fig 21). This observation is consistent with our previous findings that POPX2 regulates the localization of Coro 1C.

Figure 21. POPX2 overexpressing cells have more cytoskeletal Coro 1C compared with control cells.

Plasmid encoding Flag-Coro 1C and GST or GST-POPX2 were transfected into HeLa cells and subcellular protein fractionation assay was performed and subjected to Western blot analysis (Left panel). NF-kB is a supernatant (cytoplasm) marker and vimentin is a pellet marker (cytoskeleton). The chart shows quantification of Coro 1C amount in the cytoskeleton (%) in GST and GST-POPX2 overexpressing cells and the results include three independent experiments. (S: supernatant, P: pellet) Error bars represent standard deviation.

3.1.13 POPX2 and Coro 1C increase cell motility

Actin-based protrusion is the first step in cell migration. The lamellipodium is a distinct region of the cell at the leading edge that facilitate cell motility involving cytoskeletal dynamics (Pollard & Borisy, 2003). We discovered that cells with high levels of POPX2 enhance the localization of Coro 1C to membrane protrusions and increase cell spreading. Next, we studied whether cells with high levels of POPX2 and/or Coro 1C exhibit higher cell migration ability in wound healing assays. Cells transfected with GFP-POPX2, GFP-Coro 1C or GFP-POPX2+Coro 1C migrate faster than control to similar extent (Fig 22A). Quantification of wound closure after 8 h reveal that around 20 ~25 % of wound was closed in cells overexpressing POPX2 and/or Coro 1C compared with around 10 % for control cells (Fig 22B).

Figure 22. Overexpression of POPX2 and/or Coro 1C enhance cell migration in would healing assays

(A) NIH3T3 fibroblasts were transfected with GFP, GFP-Coro 1C, GFP-POPX2 or GFP- POPX2+Coro 1C. Cells were re-plated onto culture-insert for wound healing assay 24 h after transfection. After cell density reached 100% confluent, the culture-insert was removed. Wound closure was evaluated at 0, 8 and 24 h. Scale bar 100 μ m. (B). Wound gap at 0 and 8 h were quantified using image J software. Error bars represent mean $\pm$ S.E. of three independent experiments. * $p \leq 0.05$, as analysed by Student's t-test.

3.1.14 Coro 1C regulates cell migration in a phosphorylation-dependent manner

Coro 1C has been reported to regulate cellular protrusion formation and this regulation is dependent on the phosphorylation status of Ser463 (Ziemann et al., 2013). In this work, we narrowed down the potential phosphorylation-sites of Coro 1C that might be dephosphorylated by POPX2. There are totally 6 identified serine/threonine phospho-sites with more than 2 mass spectrometry (MS) references from the online database as shown in Fig 23A (Hornbeck et al., 2004, 2012; Klammer et al., 2012; Olsen et al., 2010; Sharma et al., 2014; Shiromizu et al., 2013; Xavier et al., 2012; Ziemann et al., 2013). The schematic image shows that the unique linker region and the coiled coil domain have the phosphorylation-sites Thr415 and Ser463, respectively. The unique linker region is important due to its actin binding ability and phosphorylation of Coro 1C on Ser463 within the coiled coil domain regulates its interaction with Arp2/3 (Chan et al., 2012; Xavier et al., 2012). As we have earlier demonstrated that POPX2 interacts with the C-terminus of Coro 1C, we went on to generate the phospho-mimic (T415E, S463D) and phospho-dead (T415A, S463A) mutants to study the functional role of these two phospho-sites.

Cells transfected with phospho-mimic mutant T415E or S463D of Coro 1C display slower cell motility compared to the cells transfected with phospho-dead mutant T415A, S463A of Coro 1C or WT (Fig 23B-C). Taken together, the results suggest that Coro 1C may mediate cell motility in a phospho-Thr415 and -Ser463 dependent manner.

Figure 23. Coro 1C increases cell motility in a phospho-dependent manner.

(A) The schematic image of Coro 1C and its phospho-sites with more than 2 references from phospho-site plus® website. (B) Plasmid encoding GFP-Coro 1C WT, GFP-T415E, GFP-S463D, GFP-T415A or GFP-S463A was transfected into NIH3T3 fibroblasts. Wound closure was evaluated at 0, 8 and 24 h. Scale bar 100 μ m. (C) Wound gap at 0 and 8 h were quantified using image J software. Error bars represent mean $\pm$ S.E. of three independent experiments. * $p \leq 0.05$, as analysed by Student's t-test.

3.1.15 Flag-Coro 1C pulldown-MS with Calyculin A (PP1 and PP2A inhibitor) and PMA (PKC activator)

Since the localization and function of Coro 1C are dependent on its phosphorylation status, there is a possibility that Coro 1C might have different binding partners depending on its phosphorylation states. In order to determine the binding partners of Coro 1C and phosphorylated Coro 1C, we performed the following experiments. We conducted Flag-Coro 1C pulldown-MS with CA (Calyculin A), PMA (PKC activator) and compared with Flag-Coro 1C pulldown-MS. CA was used to inhibit dephosphorylation of Coro 1C by PP1 and PP2A phosphatases (Cohen, 1989) and PMA was added to induce phosphorylation of Coro 1C by PKC activation.

POPX2 (PPM1F) peptides were identified in Flag-Coro 1C pulldown-MS and the coverage of POPX2 peptides were around 25% in Flag-Coro 1C pulldown and 33% in Flag-Coro 1C+PMA+CA pulldown. The coverage of POPX2 and the number of unique POPX2 peptides in Flag-Coro 1C pulldown-MS was increased by treating with CA and PMA (Fig 24A). These observations suggest that POPX2 is a binding partner of Coro 1C and phosphorylated Coro 1C might bind better to POPX2. The Coro 1C pulldown-MS corroborate our earlier findings that POPX2 and Coro 1C are interacting partners (Fig 11) and phosphorylated Coro 1C shows increased binding affinity with POPX2 (Fig 15).

Furthermore, there were slight changes in the binding partners of Coro1C upon treatment with CA and PMA (Fig 24B). A total 86 proteins were commonly found in both Flag-Coro 1C pulldown (A) and Flag-Coro 1C+PMA+CA (B). There are 51 proteins only found in Flag-Coro 1C pulldown and 31 proteins only

found in Flag-Coro 1C+CA+PMA pulldown. The results suggest that these proteins have different binding affinity with Coro 1C depend on the phosphorylation status of Coro 1C.

To study abundant proteins that found in Flag-Coro 1C pulldown-MS, we filtered the proteins that have more than 10 identified peptides (Fig 24C). As expected, actin cytoskeletal proteins were co-precipitated with Coro 1C. Cytoplasmic actin, WD repeat-containing protein 1 were commonly found. Coro 1B was only discovered in Flag-Coro 1C pulldown.

In addition to identify binding partners of Coro 1C through pulldown-MS, we also tried to incubate Coro 1C pulled down (Flag-Coro 1C alone or Flag-Coro 1C +PMA +CA) together with POPX2, followed by MS to detect phospho-peptides of Coro 1C. The aim was to identify possible phosphorylation sites on Coro 1C that could be dephosphorylated by POPX2. However, there was no phospho-peptide of Coro 1C identified despite of CA and PMA treatment. One probably requires the enrichment of phospho-peptides before conducting MS.

Figure 24. Flag-Coro 1C pulldown-MS experiments.

HeLa cells were transfected with a plasmid encoding Flag-Coro 1C and treated with CA and PMA before cell lysis. Flag immuno-precipitation was performed. The pulldown mixtures were subjected to MS analysis. (A) Sum (coverage) of POPX2 (PPM1F) and Coro 1C peptides and the number of unique peptides found in pulldown MS. (B) Venn diagram showing the number of proteins that identified in Flag-Coro 1C pulldown (A) and Flag-Coro1C+CA+PMA pulldown (B). A total 86 proteins were commonly found in A and B. 51 proteins were

identified only in A and 31 proteins were discovered only in B. (C) Proteins that have at least 10 identified peptides in Flag-Coro 1C pulldown-MS.

3.2 Discussion

3.2.1 Elucidation of Coro 1C serine/threonine phosphatase

Phosphorylation of proteins is a major mechanism that has been adopted by many proteins in the regulation of their functions in the cells. Examples include actin related signalling pathways such as Rac/CDC42-PAK-LIMK and RhoA-ROCK-MLC, which are activated/inactivated by phosphorylation and dephosphorylation by kinases and phosphatases (Edwards et al., 1999; Totsukawa et al., 2000; G.-H. Xiao et al., 2002). Phosphorylation is also known to regulate the function of mammalian coronins. The role of Coro 1C in actin reorganization and the localization of Coro 1C to the leading edge have been reported to be regulated by phosphorylation (Spoerl et al., 2002; Xavier et al., 2012). Furthermore, the role of Coro 1C in malignancy of cancer is associated with its phosphorylation status (Hasse et al., 2005).

CK2 is a serine/threonine kinase that has been identified to phosphorylate Coro 1C at Ser463 (Xavier et al., 2012). PTP1B is a tyrosine phosphatase of Coro 1C, yet the specific dephosphorylation site is not known (Mondol et al., 2014). To date, there is no identified serine/threonine phosphatase of Coro 1C. In this study, we discovered POPX2 as a first serine/threonine phosphatase of Coro 1C.

Although decreased phosphorylation of Coro 1C protein was observed by overexpression of POPX2 *in vivo* and through *in vitro* phosphatase assays (Fig 14), the specific phosphorylation-sites of Coro 1C that are dephosphorylated by POPX2 remain unknown. Although POPX2 directly interacts with the C-

terminal of Coro 1C, it remains to be investigated whether POPX2 is able to dephosphorylate Coro 1C at Thr415 and/or Ser463.

3.2.2 Identification of phospho-sites of Coro 1C in Flag-Coro 1C pulldown-MS

In order to identify phospho-sites of Coro 1C, we used Flag-Coro 1C pulldown-MS with POPX2. The aim for this experiment was to identify phospho-sites of Coro 1C that might be dephosphorylated by POPX2.

Cells were treated with CA and PMA to increase phosphorylation of Coro 1C by inhibiting the activities of PP1 and PP2A phosphatases (Cohen, 1989) and activating PKC. Since PKC phosphorylates Coro 1A and Coro 1B, we thought that it might also phosphorylate Coro 1C (Cai et al., 2005; Oku et al., 2012). Cells treated with CA show reduced dephosphorylation of Coro 1C in Western blot analysis (Fig 14C-D), suggesting that PP1 and PP2A may dephosphorylate Coro 1C or its upstream kinases. Although we observed increase in phosphorylation of Coro 1C with CA treatment in Western blot analysis, we could not identify phospho-peptides of Coro 1C in MS experiments. It is likely due to the low amount of phosphorylated Coro 1C in our MS samples because we have not carried out the enrichment step for phosphoproteome such as the use of IMAC (Immobilized metal affinity chromatography). However, phospho-peptide of Coro 1B at Ser2 was found in MS results, suggesting that PMA activated PKC and induced phosphorylation of Coro 1B (Cai et al., 2005). Alternatively, our results might suggest that Coro 1C may not be phosphorylated by PKC. Phospho-peptides of Coro 1C have been previously reported in phosphoproteome MS by overexpressing upstream kinase (CK2) and through the

enrichment of phospho-peptides in HeLa cells (Sharma et al., 2014; Xavier et al., 2012). Therefore, for further investigation on dephosphorylation of Coro 1C by POPX2, we can study the phosphoproteome and phospho-peptide enrichment after triggering phosphorylation of Coro 1C by CK2 instead.

3.2.3 Phosphorylation and actin binding sites of Coro 1C regulate its subcellular localization

The two reported mechanisms that are responsible for the localization of Coro 1C to the cell periphery are actin binding and phosphorylation. There are two actin binding sites on Coro 1C: Arg28 at the N-terminus and four lysine residues at the C-terminus. Phosphorylation at the C-terminus of Coro 1C have also been reported.

Since the N-terminus and the C-terminus of Coro 1C are crucial for its localization to the plasma membrane (Spoerl et al., 2002), it suggests that two actin binding sites might be required. Coro 1C at the leading edge can bind to F-actin and regulate actin reorganization. Therefore, Coro 1C can modulate membrane protrusions and ruffling through its localization to the plasma membrane. Similarly, the actin binding ability of Coro 1A and Coro 1B are important in the regulation of their function. Arg29 of Coro 1A and Arg30 of Coro 1B are required for binding to F-actin (Cai et al., 2007; Oku et al., 2003). Coro 1A R29D and Coro 1B R30D mutants show loss of binding to F-actin and diminished Coro 1A- and Coro 1B -mediated membrane ruffling (Cai et al., 2007; Ojeda et al., 2014). These observations suggest that the actin binding sites of coronins are required for their function in membrane ruffling.

It has been found that phosphorylation can regulate Coro 1C localization and its interaction with Arp2/3. Phosphorylated Coro 1C is observed in the cytoplasm, while dephosphorylated Coro 1C is found in the membrane fraction (Hasse et al., 2005; Spoerl et al., 2002), suggesting that phosphorylation of Coro 1C regulates its localization between the cytoplasm and the plasma membrane. In addition, phosphorylation of Coro 1C at Ser463 by CK2 leads to weaker interaction between Coro 1C and Arp2/3 (Xavier et al., 2012). As Coro 1C and Arp2/3 cooperatively regulate cell motility in gastric cancer cells (Y. Sun et al., 2014), it is likely that the interaction between Arp2/3 and Coro 1C is required for cell migration.

It is still unclear about the relation between phosphorylation and actin binding property of Coro 1C. A possible mechanism is that phosphorylation at S463 abolishes the binding of Coro 1C to Arp2/3 as well as binding affinity to F-actin. Another possible mechanism is that phosphorylation at the C-terminus hinders the binding of the four lysine residues to F-actin, leading to translocation of Coro 1C to the cytoplasm.

Since POPX2 interacts with the C-terminus of Coro 1C and dephosphorylates Coro 1C, we hypothesize that POPX2 may dephosphorylate phospho-Ser/Thr residues at the C-terminus of Coro 1C. Coro 1C has two reported phosphorylation sites at the C-terminus, Thr415 and Ser463. Wound healing assays using phospho-mutants demonstrate that dephosphorylation of Coro 1C at Thr415 or Ser463 increases cell motility (Fig 23). Taken into account that POPX2

overexpressing cells also demonstrate higher cell motility, there might be a possibility that POPX2 acts upstream of Coro 1C and enhances cell motility.

3.2.4 POPX2 regulates the localization of Coro 1C

To study the effects of POPX2 on Coro 1C localization in different cell lines, we have used HeLa, MDA-MB-231 and NIH3T3 fibroblasts cells. Since HeLa and MDA-MB-231 cells express relatively high levels of POPX2 (Susila et al., 2010), we used siRNA or CRISPR to knockdown or knockout POPX2, respectively. As NIH3T3 fibroblasts display low levels of POPX2, GFP-POPX2 overexpression was adopted for experiments using this cell type. Although these three cell lines have slightly different morphology, similar observations were obtained. Coro 1C localizes to the cell periphery with high levels of POPX2, whereas Coro 1C is mainly found in the cytoplasm when POPX2 levels are low (Fig16-17, 20).

Coro 1C is observed in perinuclear region, cytoplasm, endosome, plasma membrane and submembranous cytoskeleton (Rosentreter et al., 2007), which may pose a problem to identify the exact location of Coro 1C. Due to the highly dynamic changes of Coro 1C localization in the cells, we performed live cell imaging to observe the localization of Coro 1C during the cell spreading. POPX2 overexpressing cells are found to display wider spreading area with accumulated Coro 1C at the membrane ruffling compared with control cells (Fig 20). These observations suggest that Coro 1C may participate in the regulation of the actin cytoskeleton leading to larger lamellipodia in POPX2 overexpressing cells. Indeed, we do observe POPX2 overexpressing cells to be larger and more spread.

3.2.5 The relationship between Rac1 and Coro 1C

Coro 1C interacts with GDP-Rac1 and modulate trafficking of Rac1 from the lateral membrane to the leading edge (Williamson et al., 2014). In this study, we show that silencing POPX2 in MDA-MB-231 cells reduces the localization of Coro 1C to the membrane protrusions and consequently it leads to the loss of active Rac1 at the membrane protrusions (Fig 17C-D). Although we did not directly address whether relative ratio of GTP-Rac1/GDP-Rac1 is affected in POPX2-KO cells, it is likely that loss of Coro 1C localization at the cell periphery in POPX2-KO cells can further inhibit its role in Rac1 trafficking.

Previous study shows that XCoronin (Xenopus Coronin) localizes to the leading edge in a Rac1 activity-depend manner (Mishima & Nishida, 1999). Similarly, we observe that Coro 1C localizes to the periphery of GFP-RacV12 overexpressing cells, while Coro 1C localizes to the cytoplasm in GST-RacN17 overexpressing cells (Fig 18). Thus, this observation suggests that Rac1 activity can regulate the localization of Coro 1C in the cells.

It is not known if Rac1 regulates the localization of Coro 1C first or Coro 1C regulates the localization of Rac1 first in cells. One possible model arising from this study is that Coro 1C may redistribute GDP-Rac1 from the lateral membrane to the leading edge first, which in turn leads to Rac1 activation by GEF. Subsequently, GTP-Rac1 can activate downstream effectors such as PAK1. Rac1- β PIX (GEF)-PAK1-POPX2 pathway may then regulate Coro 1C localization to the membrane protrusions through the interaction between POPX2 and Coro 1C.

3.2.6 POPX2 acts as a regulator of Coro 1C

In this study, we propose that POPX2 regulates the function of Coro 1C in cell spreading and cell motility through their interaction and dephosphorylation of Coro 1C (Fig 25). When the levels of POPX2 are high, Coro 1C becomes dephosphorylated and translocates from the cytoplasm to the leading edge (Fig 14, 16-17). Subsequently, Coro 1C at the leading edge binds to actin filaments and promotes actin cross-linking and bundling. Actin reorganization by Coro 1C leads to lamellipodia formation and increased cell motility. One of the supporting evidences came from the experiment which shows that cells transfected with phospho-dead mutants (T415A, S463A) of Coro 1C or WT have higher cell motility compared with the cells transfected with phospho-mimic mutants (T415E, S463D) of Coro 1C (Fig 23), suggesting that Coro 1C regulates cell motility in a phospho-dependent manner.

Low levels of POPX2 in the cells would correspond to less dephosphorylation of Coro 1C and more Coro 1C in the cytoplasm. Reduced amount of Coro 1C at the leading edge can result in diminished Coro 1C-mediated actin cross-linking and bundling. Therefore, POPX2 can act as a regulator of Coro 1C in cell spreading and cell motility.

Figure 25. Proposed working model of POPX2 in the regulation of Coro 1C.

When POPX2 levels in the cell are high, Coro 1C becomes dephosphorylated and gets translocated to the leading edge of the cell. Coro 1C can then cross-link and bundle actin filaments leading to enhanced lamellipodial formation and cell migration. Low POPX2 levels may result in higher Coro 1C phosphorylation and the localization of Coro 1C from the leading edge to the cytoplasm.

4. Results and Discussion- The role of POPX2 phosphatase in DNA damage pathway

4.1 Results

4.1.1 Prediction of POPX2 interacting partners using bioinformatic analysis

We conducted a two-pronged bioinformatic analysis to identify substrates of POPX2 phosphatase as illustrated in Fig 26. The first part of the analysis is to identify POPX2 substrates by relation to known substrates of POPX2 and PP2C domain (blue arrows in Fig 26) and the second part of the analysis is to discover POPX2 substrates by curation of known substrates of proteins sharing homology to POPX2 (purple arrows in Fig 26). (The bioinformatic analysis 5.1-5.3 were done in collaboration with Dr Koon Yen Ling and Dr Raphael Tze Chuen Lee.)

Figure 26. Prediction of POPX2 substrates using bioinformatic analysis.

Schematic of the bioinformatic analysis. The analysis is split into two parts. (1) Identification of potential substrates of POPX2 by domain-domain interaction and phylogenetic analysis (blue arrows). (2) Curation of known interactors of POPX2 homologs (purple arrows). Possible substrates of POPX2 are found from both parts of bioinformatic analysis (red arrow). (The image was created by Dr Koon Yen Ling)

4.1.2 Prediction of POPX2 substrates from Domain-Domain Interactions (DDIs) database

As proteins evolve via shuffling of functional domains, the same domain can be found in various dissimilar proteins (Shoemaker et al., 2006). These domains mediate protein-protein interactions (PPIs) and many domain pairs are maintained in evolution across different organisms (Itzhaki et al., 2006). Thus, we used domain-domain interactions (DDIs) database to find reliable protein-protein interactions (Rao et al., 2014; Wojcik & Schächter, 2001). NCBI conserved domain database shows that POPX2 contains a PP2C domain, PF00481, between amino acids 155 to 406 (Fig 27A) (Marchler-Bauer & Bryant, 2004). The PP2C domain is found to interact with the Pkinase domain as deduced from 3UJG using iPfam, which is a database of domain interactions based on known 3D structures that are found in the Protein Data Bank (PDB) (Finn et al., 2014). The 3UJG structure is a co-complex of a plant PP2C phosphatase (HAB1) and SnRK2 kinase domain (SnRK2.6) (Soon et al., 2012). Structural similarity examination of 3UJG and PP2C domain of POPX2 shows possibility of similar DDI between PP2C (HAB1 and POPX2) and Pkinase (SnRK2.6). The activation loop of the Pkinase domain docks into the binding pocket of PP2C domain, resulting in the dephosphorylation of the serine residue within the activation loop of the Pkinase domain (Fig 27B-C) (Soon et al., 2012).

The amino acid sequence alignment between the PP2C domain of POPX2 (here and so forth termed as PP2C_{POPX2}) and the PP2C domain in 3UJG (here and so forth termed as PP2C_{3UJG}) shows that active sites between PP2C_{POPX2} and PP2C_{3UJG} are well conserved (Fig 27A). It suggests that PP2C_{POPX2} may also interact with other Pkinase domains via a similar mechanism. This is further

supported by the fact that both PAK1 and CaMKII contain the Pkinase domain and are dephosphorylated by POPX2 at Thr423 and Thr286 within the activation loop region, respectively (Ishida et al., 1998; Koh et al., 2002). Thus, we next search for potential substrates of POPX2 containing the Pkinase domain from POPX2 proteomic data.

Figure 27. Prediction of POPX2 substrates using Domain-Domain Interactions (DDIs) database.

(A) PP2C domain of POPX2 is highlighted as yellow (amino acid residues 155 and 406). The amino acid sequence alignment between PP2C_{3UJG} and PP2C_{POPX2} are shown below. The red stars indicate the residues at the active site including histidine and aspartic acid which are likely to be responsible for catalysis. (B) Structure of Pkinase-PP2C complex with the dotted portion expanded in (C). The

PDB structure of PP2C_{3UJG} (green) and PP2C_{POPX2} (red) are superimposed and active site residues of PP2C_{3UJG} and PP2C_{POPX2} are represented in cyan and magenta, respectively. Magnesium ion is depicted by the yellow balls and the residues labelled I to V are Arg12 (Arg10), Asp250 (Asp303), Asp206 (Asp243), Asp44 (Asp54) and His46 (His56) of PP2C_{POPX2} (PP2C_{3UJG}), respectively. The homology model of PP2C_{POPX2} resembles PP2C_{3UJG} with an RMSD of 0.69Å. The activation loop of Pkinase domain (blue) docks into the binding pocket of PP2C. (The image was created by Dr Koon Yen Ling and Dr Raphael Tze Chuen Lee)

4.1.3 Prediction of POPX2 substrates using phylogenetic analysis

To reduce the pool of proteins for initial screening, we used previous POPX2-SILAC (Stable Isotope Labeling by/with Amino acids in Cell culture)-MS (mass spectrometry) proteomic data. SILAC-MS was performed on control and POPX2-knockdown MDA-MB-231 breast cancer cells and proteins that exhibit prominently different expression levels following POPX2-knockdown were identified (Zhang et al., 2013). There are two criteria for protein identification: (1) The interactors of POPX2 should maintain reasonable levels in control cells to elicit downstream effects of POPX2 and (2) proteins showing either up-regulated or down-regulated following POPX2-knockdown are likely to be involved in POPX2 regulated pathways.

A total of 2,146 proteins are identified from POPX2-SILAC proteomic data and 46 proteins containing the Pkinase domain are discovered. Phylogenetic analysis is then performed on these 46 proteins to identify their relation with known substrates of POPX2 including the Pkinase in 3UJG, PAK1 and CaMKII α (CaMKII α is encoded by the CaMKIIA gene in human). Phylogenetic analysis reveals that the Pkinase domain of Chk1 (Chk1 is encoded by the CHEK1 gene

in human) is closely related to CaMKII α and Pkinase in 3UJG (Fig 28). Chk1 is an essential component of the DNA damage pathway and regulates G2 checkpoint activation (Q. Liu et al., 2000; Sanchez et al., 1997). Furthermore, a number of proteins that differentially expressed in POPX2-knockdown cells were found to be related to the cell cycle pathways (Zhang et al., 2013). These observations strongly suggest that Chk1 might be a substrate of POPX2.

Figure 28. Prediction of POPX2 substrates by phylogenetic analysis.

Phylogenetic tree built with 46 proteins containing the Pkinase domain together with PAK1, CaMKIIA(CaMKII α), 3UJG_Pkinase (magenta) and CHEK1 (blue). PAK1 and CaMKII α are known substrates of POPX2 and 3UJG_Pkinase is a substrate of 3UJG_PP2C domain. CHEK1 shows similar phylogeny with CaMKII α and 3UJG_Pkinase. (The image was created by Dr Koon Yen Ling)

4.1.4 Prediction of POPX2 substrates using homologs of POPX2

In the second part of the analysis, we identify potential substrates of POPX2 by consolidating known substrates of POPX2 homologs. Proteins with close sequence similarity to POPX2 (PPM1F) are identified from BLAST. As expected, PP2C family phosphatases (PPM1A to PPM1M) are found as similar proteins of POPX2. Subsequently, we identify the substrates of PP2C family phosphatases using STRING (Szklarczyk et al., 2015). Since homologous proteins are likely to share similar interactions, it is possible to identify proteins performing similar functions and their interactors through sequence similarity (Espadaler et al., 2005). This implies that substrates of PP2C family phosphatases may also be substrates of POPX2. Table 5 shows the proteins containing the Pkinase domain that are predicted or known interactors of POPX2 homologs. Chk1 has previously been reported as a substrate of PPM1D and the binding of PPM1D to Chk1 leads to dephosphorylation of Chk1 at Ser345 (Lu et al., 2005). In addition, MAP3K7 (also known as TAK1) is also a substrate of POPX2 (Weng & Koh, 2017) and phosphatases from the same family, PPM1B and PPM1L (Hanada et al., 2001; M. G. Li et al., 2003).

As a result, Chk1 is identified by domain-domain interactions (DDIs) and phylogenetic analysis as a substrate of PP2C phosphatases. Therefore, Chk1 appears to be a plausible substrate of POPX2 and experimental validation of the interaction between Chk1 and POPX2 was conducted.

Substrates	POPX2 Homolog
CDK9	PPM1A
MAP2K3	PPM1A
MAP2K4	PPM1A
MAP2K6	PPM1A
MAP2K7	PPM1A
PRKAA1	PPM1A
PRKAA2	PPM1A
MAP3K7	PPM1B
ABL1	PPM1B
PAK4	PPM1B
CHUK	PPM1B
HIPK2	PPM1D
CHEK2	PPM1D
CHEK1	PPM1D
CAMK1	PPM1F
MAP3K7	PPM1L

Table 5. Known or predicted substrates of POPX2 homologs using STRING.

Known or predicted PPM1 family's substrates containing the Pkinase domain. MAP3K7 (also known as TAK1) is highlighted in purple due to its interaction with PPM1B and PPM1L. Chk1 (Chek1) is highlighted in blue and identified as a substrate of PPM1D.

4.1.5 Biological validation of the interaction between Chk1 and POPX2

In this section, we performed GST- and Flag-pulldown assays to confirm the biological interaction between POPX2 and Chk1 that was predicted using bioinformatic analysis. Chk1-Flag and GST-POPX2 constructs were transfected into HEK293 cells. Chk1-Flag was detected in the complex obtained from GST-POPX2 pulldown but not GST pulldown (Fig 29A). Next, the interaction between POPX2 and activated Chk1 was verified. After cells were transfected with Chk1-Flag and GST or GST-POPX2, cells were treated with Etoposide (VP-

16), a topoisomerase II inhibitor, to induce DNA damage. The results demonstrate that GST-POPX2 could associate with Chk1-Flag regardless of VP-16 treatment, implying that Chk1 interacts with POPX2 in the absence of DNA damage as well (Fig 29B). To confirm that POPX2 interacts with Chk1 under physiological condition, endogenous Chk1 was precipitated using anti-Chk1 antibody and POPX2 was detected in the immunoprecipitated complex (Fig 29C). Consistently, endogenous Chk1 was isolated together with precipitated POPX2 using anti-POPX2 antibody. Taken together, these findings suggest that POPX2 and Chk1 exist as a complex within the cells (Fig 29D).

Figure 29. POPX2 interacts with Chk1.

(A) GST or GST-POPX2 cDNA constructs was co-transfected with Chk1-Flag construct into HEK293 cells. GST proteins were pulldown from cell lysates using Glutathione Sepharose beads 24 h post transfection and were subjected to SDS-PAGE and Western blot analysis. (B) Plasmid encoding Chk1-Flag was co-transfected with GST or GST-POPX2 into HEK293 cells and subjected to DMSO or VP-16 treatment. About 20 min after the addition of VP-16, cells were lysed and Chk1-Flag was isolated using Flag beads. Precipitated proteins were separated by SDS-PAGE and subjected to Western blot analysis. * indicates non-specific band. (C) Endogenous Chk1 was isolated using anti-Chk1 antibody and proteins that co-precipitated together with Chk1 were subjected to Western analysis using POPX2 antibody. (D) Endogenous POPX2 was precipitated with anti-POPX2 mouse antibody and Chk1 was identified from POPX2 immunoprecipitated complex. Random IgG was used as control in (C) and (D).

4.1.6 Chk1 is dephosphorylated by POPX2

After the interaction between POPX2 and Chk1 was confirmed, we next investigated the functional link between Chk1 and POPX2 in response to genotoxic stress. To study whether Chk1 is a substrate of POPX2, *in vitro* and *in vivo* phosphatase assays were performed. Chk1-Flag was co-transfected with GST, GST-POPX2 or GST-PP2A into HEK293 cells followed by VP-16 treatment at indicated time points. We observed more dephosphorylated Chk1-Ser317 and -Ser345 in cells co-transfected with GST-POPX2 but not in the case when co-transfected with GST alone. For cells co-transfected with GST-PP2A, we could not consistently observe decrease in phosphorylated Chk1-Ser317 and -Ser345 levels (Fig 30A). The relative ratio of phospho-Chk1/Chk1 was markedly reduced by overexpression of GST-POPX2 compare to that with GST after 20 min of VP-16 treatment (Fig 30B). These observations suggest that POPX2 might be a specific phosphatase for Chk1 during early stages of VP-16 induced DNA damage. However, under prolonged VP-16 treatment, there was no observable difference in phospho-Ser317 and phospho-Ser345 levels, suggesting that the activity of POPX2 might not be required during later stages of VP-16 induced DNA damage response.

In vitro phosphatase assay was performed to observe if POPX2 exerts an effect on dephosphorylation of Chk1. Cells were transfected with Chk1-Flag and treated with DMSO or VP-16 24 h after transfection. VP-16 was added to induce DNA damage and increase phosphorylation of Chk1. Subsequently, cells were lysed and Chk1-Flag was isolated for *in vitro* reaction. Precipitated Chk1-Flag was incubated with or without bacterially expressed recombinant GST-POPX2

protein for *in vitro* phosphatase assay. The results show that phosphorylated Chk1-Ser317 and -Ser345 were efficiently dephosphorylated by GST-POPX2 (Fig 30D). The relative ratio of phospho-Chk1/Chk1 was remarkably decreased in the presence of GST-POPX2, suggesting that Chk1 is a substrate of POPX2 (Fig 30E).

Figure 30. POPX2 dephosphorylates Chk1.

(A) GST, GST-POPX2 or GST-PP2A was co-expressed with Chk1-Flag in HEK293 cells, followed by treatment with DMSO (control) or VP-16 (40 μ g/ml) at indicated time points. Phosphorylation of Chk1 was detected using anti-pChk1 Ser317 and Ser345 antibodies. (B) Densitometry measurement was done using ImageJ for three independent experiments. (C) HEK293 cells were transfected with plasmid encoding Chk1-Flag and treated with DMSO or VP-16. Isolated Chk1-Flag was incubated with or without bacterially expressed GST-POPX2 protein at 30 °C for 45 min

in phosphatase buffer and the results were analysed by immunoblotting. (D) Relative fold change of pChk1/Chk1 was analysed as mention in (B). Error bars represent standard deviation. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, as analysed by Student's t-test.

4.1.7 POPX2 does not affect the degradation of CDC25A phosphatase

CDC25 phosphatases (CDC25A/B/C) activate CDKs through dephosphorylation and induce cell cycle progression. In response to DNA damage, CDC25 phosphatases undergo degradation by the SCF complex (Donzelli et al., 2002; Uchida et al., 2011). Replication stress activates Chk1, which in turn induces degradation of CDC25A and arrest cells at G2 phase (Z. Xiao et al., 2003). Since POPX2 acts as a negative regulator of Chk1 through dephosphorylation, we investigate whether POPX2 regulates the downstream target of Chk1, CDC25A phosphatase, through modulation of Chk1 activity. GST-POPX2 and GST-POPX2M (phosphatase dead mutant) overexpressing cells show slightly increased CDC25A compared with GST (control) in normal conditions, however there was no significant difference in CDC25A levels in GST and GST-POPX2 overexpressing cells after VP-16 stimulation (Fig 31A-B).

Figure 31. POPX2 does not affect the degradation of CDC25A phosphatase.

(A) Plasmid encoding GST, GST-POPX2 or GST-POPX2M (phosphatase dead mutant) was transfected into HEK293 cells, followed by treatment with DMSO (control) or VP-16 (40 µg/ml) for 20 min. CDC25A was detected using anti-CDC25A antibody and actin was used as a loading control. (B) Densitometry measurement was done using ImageJ for three independent experiments. Error bar represents standard deviation.

4.1.8 POPX2 regulates cell cycle progression in response to DNA damage

Since POPX2 dephosphorylates Chk1 at Ser317 and Ser345, which are crucial phosphorylation sites that activate DNA damage induced checkpoints, we next determine the role of POPX2 in VP-16 mediated cell cycle arrest or delay. Cell cycle analysis using FACS shows that there was no significant difference in cell cycle progression in POPX2-knockdown (siX2) and control (siLuc) U-2OS cells under normal conditions (Fig 32A). Interestingly, silencing POPX2 in VP-16 treated cells (siX2+VP-16) led to an increased population at G1 phase after 20 h of VP-16 treatment compared to that in control cells (Fig 32B). Quantification of cell cycle stages in control and POPX2-knockdown cells revealed that siX2+VP-16 cells have higher number of cells at G1 phase compared to that of control cells (Fig 32B).

We then overexpressed POPX2 to determine the effects of high POPX2 levels on cell cycle progression when the cells suffer DNA damage. Plasmids encoding GST and GST-POPX2 were transfected into U-2OS cells and cell cycle analysis was performed. We found no difference in cell cycle progression in POPX2 overexpressing cells and control cells under normal conditions (Fig. 32C). When cells are exposed to DNA damaging agents, POPX2 overexpressing cells have significantly increased S phase population compared to control cells (Fig. 32D). Quantification of cell cycle stages in GST and GST-POPX2 overexpressing cells shows that more GST-POPX2+VP-16 cells were at S phase and lower numbers were found at G1 phase compared to GST overexpressing cells (Fig. 32D). Overall, our observations suggest that POPX2-knockdown cells encounter delay in G1 to S transition when the cells suffered DNA damage. On the other hand, POPX2 overexpressing cells can proceed to S phase in response to DNA damage.

Figure 32. POPX2 regulates G1-S cell cycle transition in response to DNA damage.

Cell cycle progression of siLuc and siX2 cells treated with DMSO (A) and VP-16 in (B). U-2OS cells were transfected with siRNA targeting Luciferase (siLuc) or POPX2 (siX2) for 48 hr, followed by treatment with DMSO or VP-16 for 20 hr. Cells were fixed and stained with PI/RNase dye for 1 hr before cell sorting.

PI stained cells were analysed using FACS machine and FlowJo software was used for data analysis. U-2OS cells were transfected with GST or GST-POPX2 plasmid, followed by treatment with DMSO or VP-16 for 20 hr. Cell cycle progression in GST and GST-POPX2 overexpressing cells treated with DMSO (C) and VP-16 (D). The charts on the right represent the percentage of cells in different cell cycle phases. Error bars represent mean $\pm$ S.E. of three independent experiments in (A-D). * $p \leq 0.05$, as analysed by Student's t-test.

4.2 Discussion

4.2.1 Application of bioinformatic analysis in protein-protein interactions (PPIs) prediction

Majority of proteins in the cells interact with other molecules to fulfil their function. Protein-protein interactions (PPIs) come together to construct signalling pathways (Berggård et al., 2007). In order to understand the protein interactome, computational methods for PPIs can use biological data including amino acid sequence, evolution, gene or protein expression and protein structure based data (Keskin et al., 2016). Although many PPI tools are available as open source websites, they might not provide comprehensive coverage. For example, both InterPreTS (Aloy & Russell, 2003) and Interactome3D are not able to search for the interaction between POPX2 and its known substrates, PAK1 and CaMKII.

In this work, we attempt to uncover interactors of POPX2 using two strategies that are guided by evolutionary principles. Firstly, domain-domain interactions that mediate protein interactions have been found to be maintained across evolution (Itzhaki et al., 2006). Secondly, homologous proteins of POPX2, PPM1 family members are likely to share similar interactions (Espadaler et al., 2005). As an example, Chk1 and TAK1 are also substrates of other PPM1 family proteins. PPM1D binds and dephosphorylates Chk1 (Lu et al., 2005). TAK1 is also a substrate of PPM1B and PPM1L (Hanada et al., 2001; M. G. Li et al., 2003). For this study, we have discovered Chk1 as a potential substrate of POPX2 using bioinformatic analysis and confirmed it through biological validation.

4.2.2 The interaction of POPX2 and Chk1 in DNA damage response

Transcriptomic analysis in control vs POPX2-knockdown in MDA-MB-231 breast cancer cells reveals that POPX2 might participate in a myriad of signaling pathways, especially those related to focal adhesion and cell cycle regulation are remarkably enriched (Zhang et al., 2013). Interestingly, DNA damage response relevant genes, such as NEK1, BRIP1 and BRCA2 are also found to be significantly up-regulated in POPX2-knockdown cells. Both BRIP1 (BRCA1 interacting protein C-terminal helicase1, BACH1) and BRCA2 are involved in the repair of double-stranded breaks (Cantor et al., 2001; Liu and West, 2002). Although Chk1 and Chk2 are also found from the transcriptome data, it is not significantly down- or up-regulated in POPX2-knockdown cells, suggesting that POPX2 might regulate Chk1 through interaction or post-translation modifications.

Phosphorylation of Chk1-Ser345 has been reported to be responsible for the increase in Chk1 kinase activity (Q. Liu et al., 2000). In this study, we found that POPX2 dephosphorylates Chk1 at Ser317 and Ser345, suggesting that POPX2 acts as a negative regulator of Chk1's activity. Chk1 activates G1/S and G2 checkpoints via CDC25 phosphatases (CDC25A/B/C). Upon phosphorylation of CDC25A and CDC25C by Chk1, they are sequestered into the cytoplasm by 14-3-3 or degraded, leading to inactivation of CDK/cyclin complexes (Donzelli et al., 2002; Sanchez et al., 1997; Uchida et al., 2011). We investigated the levels of CDC25A protein in control and POPX2 overexpressing cells in response to DNA damage, however there was no significant difference in CDC25A levels after VP-16 stimulation (Fig 30). We still need further study to investigate

whether POPX2 has a role in other CDC25 phosphatases such as CDC25B and CDC25C.

In this study, we found that POPX2 can dephosphorylate Chk1 at Ser317 and Ser345. As activation of Chk1 is important in the regulation of DNA damage checkpoint response, we analysed cell cycle progression with or without a DNA damaging agent in POPX2-knockdown cells and POPX2 overexpressing cells. Silencing POPX2 leads to increased G1 population and reduced S population compared to control cells in response to DNA damage. On the other hand, POPX2 overexpressing cells display increased S population and decreased G1 population compared to control cells in response to DNA damage. Overall, our observations suggest a possible role for POPX2 in the regulation of cell cycle checkpoint at G1-S in response to DNA damage. As Chk1's kinase activity is important for its function (Patil et al., 2013; Z. Xiao et al., 2003), it is possible that POPX2 regulates DNA damage response through dephosphorylating Chk1, leading to inactivation of Chk1's downstream targets and G1-S checkpoint.

Figure 33. Proposed working model in control and POPX2-overexpressing cells (POPX2 O/E).

Proposed working model of DNA damage response in control and POPX2 overexpressing cells. Chk1 is phosphorylated at Ser317 and Ser345 by ATM/ATR kinases in response to DNA damage and translocate from the nucleus to the cytoplasm. Active Chk1 induces the activation of checkpoint at G1-S transition to prevent damaged DNA from being replicated. However, POPX2 overexpressing cells have reduced Chk1 phosphorylation at Ser317 and Ser345 in response to VP-16. As a result, inactivation of Chk1 by POPX2 leads to impaired G1-S checkpoint activation and cells can proceed from G1 to S phase despite of DNA damage.

4.2.3 The implication of the interaction between Chk1 and POPX2 in cancer therapy.

Chk1 is in the first line of defense against DNA damage and regulation of Chk1 is crucial in cancer progression. Thus, deregulation of Chk1 in DNA damage response can contribute to tumorigenesis. There is emerging evidence which suggests that Chk1 is associated with cancer therapy resistance. Loss of Chk1 in mice reduces tumorigenicity driven by carcinogen exposure and decreases resistance to anti-cancer therapies (Tho et al., 2012). The role of Chk1 in resistance seems to be regulated by its activation. Increase in phosphorylation of Chk1 at Ser317 correlated with elevated radio-resistance in metastatic brain and lung cancer patients (Seol et al., 2011). Therefore, Chk1 inhibitors can be used to sensitize the chemotherapy and chemosensitizing effects of Chk1 inhibitors in cisplatin-resistant and radio-resistant cancers have been extensively studied (Aasland et al., 2019; Dinkelborg et al., 2019; Gadhikar et al., 2013; Koppenhafer et al., 2018; C.-C. Li et al., 2016).

Here, we report the interaction between POPX2 and Chk1 and POPX2 act as a negative regulator of Chk1 by dephosphorylating it at Ser317 and Ser345. In our study, cells with low levels of POPX2 exhibit increased population at G1 and cells with high levels of POPX2 show increased population at S phase, suggesting the role of POPX2 at G1-S transition in response to DNA damage.

TAK1 is a substrate of POPX2 that regulate apoptosis via the TAK1-IKK-NF- κ B pathway in response to DNA damage. In a previous study from our lab, POPX2-knockdown cells were found to have higher phospho-TAK1, resulting in increased anti-apoptotic gene expression and less cell death through NF- κ B

mediated transcription in response to VP-16 treatment. These findings suggest that POPX2 has a role in modulating/responding to sensitivity of VP-16 (Etoposide), which is a chemotherapy drug used for the treatment of a number of cancer types (Weng & Koh, 2017). As POPX2 acts as a negative regulator of Chk1 by dephosphorylating it at Ser317 and Ser345, it is possible that high levels of POPX2 with chemotherapy (etoposide) might lead to enhanced sensitivity of chemotherapy in similar manner as Chk1 inhibitors.

Figure 34. Potential role of POPX2 in chemotherapy.

A combination of Chk1 inhibitor and chemotherapy such as gemcitabine and temozolomide shows increase in sensitivity of chemotherapy for cancer cells (Aasland et al., 2019; Koppenhafer et al., 2018). As POPX2 acts as a negative regulator of Chk1 by dephosphorylating it at Ser317 and Ser345, it is possible that high levels of POPX2 with chemotherapy (etoposide) might lead to enhanced sensitivity of chemotherapy in similar manner as Chk1 inhibitors.

5. Conclusion and Perspective

5.1 Conclusion

In this current work, we have used different approaches to identify POPX2 interacting proteins and then decipher what these interactions might be implicated in the biological function of POPX2. Coro 1C and Chk1 are found to be novel interacting partners of POPX2. The key findings are summarised below.

Study 1. The functional relationship between Coro 1C and POPX2

In this study, we demonstrated that POPX2 phosphatase interacts with Coro 1C using pulldown-MS and immunoprecipitation assays. POPX2 dephosphorylates Coro 1C *in vivo* and *in vitro*. We also found that the levels of POPX2 in the cells can affect the localization of Coro 1C to the cell periphery and cell protrusions. POPX2 overexpressing cells show increased localization of Coro 1C to the leading edge, which lead to enhanced cell spreading and migration.

Study 2. The role of POPX2 phosphatase in DNA damage pathway

In this study, we identified Chk1 as a potential target of POPX2 from a combination of bioinformatic analysis and POPX2-SILAC proteomic data. We discovered that POPX2 interacts with Chk1 using immunoprecipitation assays. Chk1 is dephosphorylated at Ser317 and Ser345 by POPX2 *in vivo* and *in vitro*, suggestion that POPX2 is a negative regulator of Chk1.

5.2 Perspective

The metastasis cascade starts with epithelial-mesenchymal transition (EMT), dissemination and intravasation followed by circulation, extravasation and colonization (Valastyan & Weinberg, 2011). My current work and previous findings on POPX2 suggest that POPX2 is involved in the regulation of cancer progression through its various interacting partners and signaling pathways. Here, we propose a working model of POPX2 in cancer progression (Fig 35).

In early stages of metastasis, high levels of POPX2 promote metastasis through interacting with KIF3A, Coro 1C and LATS1. High levels of POPX2 in the cells can lead to impaired N-cadherin and β -catenin transport to the cell periphery through KIF3 (Phang et al., 2014). Loss of N-cadherin to the cell periphery in turn results in the loss of proper cell-cell contacts, which causes cell dissociation from the epithelial layer. In addition, inhibited β -catenin pathway induces loss of contact inhibition and uncontrolled tumour growth (Ozawa, 2015). Cells with high levels of POPX2 display increased cell motility and invasiveness (Susila et al., 2010). It is likely that POPX2 promotes cell motility through the interaction with Coro 1C by translocating Coro 1C to the leading edge of migrating cells. Moreover, impaired N-cadherin transport to the cell periphery in POPX2 overexpressing cells also contributes to random migration (Hoon et al., 2014), which allow tumor cells to move to blood vessels for invasion and intravasation (Shestakova et al., 2001). Cells with high levels of POPX2 have suppressed LATS-mediated the Hippo pathway and acquire resistance to anoikis, resulting in increased cell survival without cell-cell contacts (Rahmat et al., 2019).

At later stages of metastasis, low levels of POPX2 in the cells appear to contribute to cell proliferation for cancer colonies at secondary sites through promoting angiogenesis and by inhibiting cell death through interacting with TAK1 and Chk1. Cells with low levels of POPX2 promote angiogenesis by up-regulating the secretion of exosomes and pro-angiogenic cytokines (Zhang et al., 2017). Low levels of POPX2 can also favour inhibition of apoptosis and reduced Chk1 inhibition leading to induced checkpoint activation in response to DNA damage, resulting in increased cell viability (Weng and Koh, 2017 and this study).

The mechanism of switching POPX2 activity in early and late stages of cancer progression still remains unknown. To date, there are only few studies about the regulation of POPX2 expression and activity. Protocadherin (Pcdh)- γ C5 has been identified as an endogenous regulator of POPX2. The C-terminal cytoplasmic fragment of Pcdh- γ C5 are generated by intracellular processing and interacts with POPX2. Binding of Pcdh- γ C5 to POPX2 has been reported to enhance POPX2's activity on CAMKI (Onouchi et al., 2015). There are two microRNAs that have been reported to target POPX2. Both miRNA-200c and miRNA-149 inhibit cancer metastasis through repressing POPX2 expression (Jurmeister et al., 2012; Luo et al., 2015).

These studies suggest that POPX2 might be a multi-faceted regulator of cancer metastasis through the modulation of various biological processes including N-cadherin cargo transport, cell migration, cell polarity, anoikis, exosome and cytokine secretion, cell cycle arrest and apoptosis as well as exhibiting differing roles at different stages of cancer progression.

Figure 35. Schematic illustration of POPX2-mediated cancer progression.

High levels of POPX2 at early stages of metastasis favour cancer cell invasion and migration. However, low levels of POPX2 for cancer cells which have established at metastatic sites favour angiogenesis, which enhances cell proliferation. Meanwhile, lower levels of POPX2 also reduce TAK1-IKK-NF- κ B mediated apoptosis and activation of checkpoint, which in turn allow the cancer cells to proliferate in response to DNA damage.

Appendix

Putative binding partners of POPX2 identified in HEK293				
Protein name	Gene name	Protein accession number	No. of peptide identified	Function
Cytoskeleton related				
Coronin, Actin Binding Protein, 1C	CORO1C	Q9ULV4	27	Actin filament binding
Kinesin Family Member 11	KIF11	P52732	20	Microtubule motor activity; protein kinase binding
T-Complex Protein 1 Subunit Gamma	CCT3	P49368	6	Molecular chaperon, assist protein folding
Drebrin 1	DBN1	Q16643	5	Actin binding; profilin binding
Vimentin	VIM	P08670	4	Structural constituent of cytoskeleton
CAP-GLY Domain Containing Linker Protein	CLIP1	P30622	2	Microtubule binding; protein homodimerization activity
Spectrin, Beta, Non-Erythrocytic 1	SPTBN1	Q01082	2	Phospholipid binding; calmodulin binding
Others				
TGF-Beta Activated Kinase 1/MAP3K7 Binding Protein 1	TAB1	Q15750	2	Kinase activator activity
Serine/Threonine Kinase 38	STK38	Q15208	2	Protein serine/threonine kinase activity
BTB/POZ Domain-Containing Protein KCTD5	KCTD5	Q9NXV2	2	Substrate adapter in E3 ligase complex
Methylosome subunit PICln	CLNS1A	P54105	2	poly(A) RNA binding
MOB Kinase Activator 1B	MOB1B	Q7L9L4	2	Kinase activator activity

Figure 1. List of potential POPX2 interacting proteins found in Flag-POPX2 pulldown-MS experiments.

A total of 12 proteins were identified in Flag-POPX2 pulldown-MS experiments and the results were subdivided into the actin cytoskeleton related group and others. Coro 1C was identified as a possible binding partner of POPX2. TAB1 was found in Flag-POPX2 pulldown-MS and TAB1-TAK1 complex was validated as a binding partner of POPX2. Figure 1 is from PhD thesis of Weng Ting (2014) from A/P Koh Cheng Gee's laboratory at NTU.

Figure 2. Original membrane images of Figure 29D.

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