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RESEARCH PAPER



# Phosphatase POPX2 interferes with cell cycle by interacting with Chk1

Pu Rum Kim[a], Yen Ling Koon[b,c], Raphael Tze Chuen Lee [c], Farouq Azizan [a], Dylan Hong Zheng Koh [a], Keng-Hwee Chiam[c], and Cheng-Gee Koh[a]

[a]School of Biological Sciences, Nanyang Technological University, Singapore, Singapore; [b]Interdisciplinary Graduate School, Nanyang Technological University, Singapore, Singapore; [c]ASTAR, Biopolis, Bioinformatics Institute, Singapore, Singapore

**ABSTRACT**

Protein–protein interaction network analysis plays critical roles in predicting the functions of target proteins. In this study, we used a combination of SILAC-MS proteomics and bioinformatic approaches to identify Checkpoint Kinase 1 (Chk1) as a possible POPX2 phosphatase interacting protein. POPX2 is a PP2C phosphatase that has been implicated in cancer cell invasion and migration. From the Domain–Domain Interaction (DDI) database, we first determined that the PP2C phosphatase domain interacts with Pkinase domain. Subsequently, 46 proteins with Pkinase domain were identified from POPX2 SILAC-MS data. We then narrowed down the leads and confirmed the biological interaction between Chk1 and POPX2. We also found that Chk1 is a substrate of POPX2. Chk1 is a key regulator of the cell cycle and is activated when the cell suffers DNA damage. Our approach has led us to identify POPX2 as a regulator of Chk1 and can interfere with the normal function of Chk1 at G1-S transition of the cell cycle in response to DNA damage.

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## Introduction

POPX2 (Partner of PIX 2) is a phosphatase belonging to the protein phosphatase 2C (PP2C) family, one of the four main categories of serine/threonine-specific protein phosphatases in mammals [1,2]. PP2C, in turn, belongs to the $Mg^{2+}/Mn^{2+}$-dependent phosphatase PPM family. POPX2 shares extensive homology with POPX1 with approximately 66% protein sequence similarity within the core phosphatase domain [3]. POPX1 is mainly expressed in the brain and testis, whereas POPX2 is ubiquitously expressed in most human tissues. POPX2's role as a phosphatase is exemplified in its regulation of p21-activated kinase 1 (PAK1) and calcium/calmodulin-dependent protein kinase II (CAMK2) [3,4]. POPX2 has been found to be a negative regulator of PAK1 by dephosphorylating PAK1 at Thr423, a crucial regulatory site within the kinase activation loop [3,5]. In agreement with the negative regulation of PAK1, overexpression of POPX2 leads to inhibition of actin stress fiber breakdown [3]. POPX2 has also been identified as a phosphatase which dephosphorylates CAMK2 at its autophosphorylation site at Thr286 leading to the inactivation of the kinase [4,6].

The activities and levels of POXP2 in the cells have been implicated in cancer metastasis. The invasiveness of breast cancer cells has been found to be positively correlated with POPX2 levels in the cells. POPX2 levels are high in invasive MDA-MB-231 cells while noninvasive MCF7 breast cancer cells express low amounts of POPX2 [7]. In addition, POPX2 overexpression leads to an increase in motility of MDA-MB-231 and MCF7 cells, possibly by modulating mitogen-activated protein kinase (MAPK) signaling [7,8]. In this context, POPX2 can enhance tumor progression via the promotion of cell motility and invasiveness. However, this is contrasted during late stages of metastasis where larger and more numerous tumor nodules are observed at metastatic sites in mice injected with POPX2-knockdown MDA-MB-231 cells [9]. Silencing POPX2 increases angiogenesis and consequently metastasis by increasing exosome secretion leading to the induction of pro-angiogenic cytokines [9]. This is further supported by recent findings that show that POPX2-knockdown cells undergo less apoptosis due to increased activity of transforming growth factor beta-activated kinase 1 (TAK1) resulting in upregulation of anti-apoptotic gene expression [10]. These studies suggest that

**CONTACT** Keng-Hwee Chiam chiamkh@bii.a-star.edu.sg; Cheng-Gee Koh cgkoh@ntu.edu.sg

POPX2 might be a multi-faceted regulator of cancer metastasis modulating various signaling pathways including MAPK signaling, exosome and cytokine secretion, apoptosis, as well as exhibiting differing roles at different stages of cancer progression.

In this study, we seek to further unravel POPX2's role in cancer by identifying other substrates of POPX2 through bioinformatics. Prospective candidates identified are validated through experimentation. Phosphatases are typically promiscuous, having multiple substrates [11]. The PP2C family of phosphatases is no exception and is characterized by broad substrate specificity [12]. Therefore, it is highly likely that there might be other unknown substrates, which are regulated by POPX2. Bioinformatics will allow us to make a more informed conjecture about POPX2 interactors and narrow down the pool of possible substrates. The predicted substrates can then be tested experimentally using co-immunoprecipitation and *in vitro* and *in vivo* phosphatase assays.

We now report a novel interaction between POPX2 and Checkpoint Kinase 1 (Chk1). Chk1 is a serine/threonine kinase encoded by the CHEK1 gene in human. It executes a crucial role in the coordination of DNA damage response and cell cycle progression [13]. When the cells are under genotoxic stress, Chk1 is phosphorylated at Ser317 and Ser345 and becomes activated [14–16]. Chk1 activation mediates G1/S transition, S and G2 arrest through its target proteins [15,17,18]. Chk1 is also involved in the activation of DNA repair pathways as well as the induction of apoptosis under severe DNA damage [19–22]. Initially, Chk1 was considered to be a tumor suppressor as it halts replication when DNA is damaged. However, recent work has suggested that Chk1 can promote tumor progression [23]. Chk1 has been linked to chemotherapy resistance as tumor cells can tolerate higher levels of DNA damage under increased Chk1 levels [23,24]. Chk1 inhibitors have been demonstrated to sensitize cancer cells for chemotherapy and radiotherapy [25–28]. Our discovery of POPX2 and Chk1 interaction could help to explain the different roles of Chk1 in the regulation of tumor cell biology. Our study also implicates POPX2 in the DNA damage response pathway through its interaction with Chk1.

## Materials and methods

### Bioinformatic analysis

The identification of protein domains is performed using NCBI conserved domain search [29]. Domain–domain interaction search is implemented using iPfam database [30]. Alignment between $PP2C_{POPX2}$ and $PP2C_{3UJG}$ is done using the L-INS setting in MAFFT [31]. Phylogenetic analysis is conducted using Randomized Axelerated Maximum Likelihood (RAxML) with PROTGAMMA and BLOSUM62 [32]. The homology model of $PP2C_{POPX2}$ is built using Modeller version 9.19 [33]. STRING database is used for substrate identification where a minimum interaction score of 0.7 is required [34,35]. All active interaction sources are used for the interaction score calculation in STRING [34]. Proteins with close sequence similarity to POPX2 are identified from BLAST using the non-redundant protein sequences database for Homo sapiens and BLOSUM62 as the scoring matrix. Only the top 100 Blast hits with a minimum expected threshold of 10 are considered.

### Cell culture and Western blot

U-2OS and HEK293 cells were cultured in DMEM containing 4.5 g/L glucose supplemented with 3.7 g/L sodium bicarbonate and 10% FBS. All mammalian cell lines were incubated at 37°C with 5% $CO_2$. Cells were washed with 1x PBS and lysed with protein 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% Triton X-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 was determined with the Bio-Rad protein assay. An equal amount of protein lysates in the final 1x SDS sample buffer were heated at 100°C for 10 min and loaded into each well on an SDS-PAGE gel. The proteins were transferred onto nitrocellulose membranes and the blocking step was carried out for 1 h in 5% skimmed milk or 3% BSA for phospho-proteins. 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).

Chemiluminescent signals were detected on X-ray film (Kodak) using Amersham ECL (GE healthcare).

### Plasmid and siRNA transfections

Cells were seeded at 80–90% confluence and transfected with 1–3 μg of plasmid DNA using Lipofectamine 2000™ and Opti-MEM™ according to the manufacturer's instructions. Cells were seeded at 80% confluence and transfected with 120 pmol of Stealth siRNA (Invitrogen) using Lipofectamine 2000™ and Opti-MEM™ according to the manufacturer's protocol. The transfection efficiency was validated using Western blot analysis and experiments were carried out 24 to 48 h post transfection.

### Glutathione s-transferase (GST) and flag pull-down assays

HEK293 cells were transfected with plasmid DNA and lysed with Co-IP buffer (20 mM Tris-Cl, 150 mM NaCl, 1% Triton X-100 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 pulldown proteins were eluted with 1x SDS sample buffer by heating at 100°C for 10 min. Proteins were loaded into each well on SDS-PAGE gel and Western blot was carried out as described earlier.

### Co-immunoprecipitation assay

HEK293 cells were lysed with Co-IP buffer and centrifuged at 14,000 rpm for 10 min. 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 either antibody or IgG at 4°C overnight. The 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 bound proteins were eluted with 1x SDS sample buffer by boiling at 100°C for 10 min. The eluted sample was analyzed by SDS-PAGE and Western blot was carried out as described earlier.

### *In vivo* and *in vitro* phosphatase assays

For *in vivo* phosphatase assay, plasmids encoding GST, GST-POPX2, or GST-PP2A were co-transfected with Chk1-Flag into HEK293 cells. Etoposide (VP-16, 40 μg/ml) was added to induce DNA damage and phosphorylation of Chk1. Subsequently, cells were lysed 24 h post transfection and Western blot was carried out as described earlier. HEK293 cells were transfected with siLuc or siX2 and *in vivo* phosphatase assay was performed as described earlier.

For *in vitro* phosphatase assay, HEK293 cells were transfected with Chk1-Flag and lysed 24 h post transfection. VP-16 was added before the cell lysis step. Cell lysates were added into ANTI-FLAG® Affinity gel at 4°C overnight. The beads were washed thrice with Co-IP buffer and incubated with bacterially expressed GST-POPX2 protein in phosphatase buffer (50 mM Tris-Cl pH 8.0, 300 mM NaCl, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM DTT) for 45 min at 30°C. The reaction was stopped by adding SDS sample buffer and boiled at 100°C for 10 min. Western blot was carried out as described earlier.

### Gst-tagged protein purification

The pGEX-6P1-POPX2 plasmid was transformed into *E.coli* strain BL21. Upon reaching $OD_{600}$ value at 0.4–0.6, 1 mM IPTG was added and incubated at 160 rpm, 16°C overnight. Bacterial culture was centrifuged at 4000 rpm for 30 min at 4°C, and the bacterial pellet was 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. Sonication was performed at 25% to 30% power for 1 min 5 times with 1-min interval. The clear lysate was loaded to Glutathione Sepharose™ 4B 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. PD-10 desalting column (GE Healthcare) was used for protein storage buffer exchange and Centricon® centrifugal filter units (Millipore) were used to concentrate purified protein.

### Fluorescence-activated cell sorting (FACS)

U-2OS cells were transfected with Luciferase (siLuc) or POPX2 (siX2) siRNA and fixed with ice-cold 70% ethanol at 4°C overnight, 48 h post transfection.

Cells were centrifuged for 5 min and washed five times with ice-cold 1x PBS. Fixed cells were stained with Propidium Iodide/RNase for 1 h at room temperature. X-20 Fortessa (BD Biosciences) with FACS Diva software was used to analyze stained cell samples and the results were obtained using FlowJo software. U-2OS cells were transfected with plasmid encoding GST or GST-POPX2 and cell sorting was performed as described earlier.

## Results

### *Bioinformatic analysis to search for potential POPX2 substrates*

We conduct a two-pronged bioinformatic analysis to uncover novel POPX2 substrates. False-positive rate is kept low since only substrates that are picked up by both strategies are used as leads for experimental validation. The two-pronged approach for prediction of POPX2 substrates is illustrated in Figure 1 and described below:

(1) Prediction of POPX2 substrates by relation to known substrates of POPX2 and PP2C phosphatase domain (Blue arrows in Figure 1).
(2) Prediction of POPX2 substrates by the curation of known substrates of proteins sharing homology with POPX2 (Purple arrows in Figure 1).

### *Prediction of POPX2 substrates by relation to known substrates of POPX2 and PP2C phosphatase domain*

Proteins evolve via shuffling of functional domains; the same domain can be observed in various dissimilar proteins [36]. These domains mediate protein–protein interaction (PPI) and many domain pairs are maintained in evolution across different organisms [37]. Thus, information about domain–domain interaction (DDI) can be used to infer reliable protein interactions [38,39].

Using NCBI Conserved Domain search, POPX2 is found to contain a PP2C domain, PF00481, between amino acids 155 to 406 (Figure 2(a)) [29]. Using iPfam, the PP2C domain is found to interact with the Pkinase domain as deduced from the protein data bank (PDB) structure, 3UJG [30]. The 3UJG

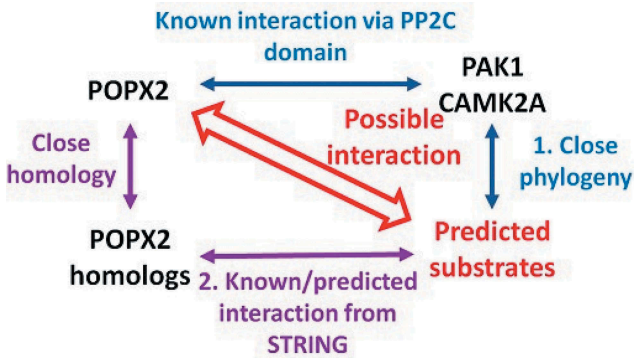


**Figure 1.** Prediction of POPX2 substrates using bioinformatic analysis.

Schematic of the bioinformatic analysis. The analysis is split into two parts. The first part involves the identification of potential substrates by relation to known substrates of POPX2 and PP2C phosphatase domain (shown by the blue arrows). The second part involves the curation of known interactors of POPX2 homologs (indicated by the purple arrows).

structure is a co-complex of a plant PP2C phosphatase (HAB1) and SnRK2 kinase domain (SnRK2.6) [40]. Examination of 3UJG in Figure 2(b and c) suggests that the DDI between PP2C (3UJG and POPX2) and Pkinase is through docking of the kinase activation loop into the active site of PP2C. Following this, the serine within the activation loop of the Pkinase domain is dephosphorylated [40].

An alignment is performed 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 $PP2C_{3UJG}$). As observed in Figure 2(a), active site residues between $PP2C_{POPX2}$ and $PP2C_{3UJG}$ are well conserved suggesting that $PP2C_{POPX2}$ may also interact with other Pkinase domains via a similar mechanism. This is further substantiated by the fact that known substrates of POPX2, namely, PAK1 and CAMK2 do contain the Pkinase domain and PAK1 has been reported to be dephosphorylated by POPX2 at the activation loop region [3]. In addition, Pkinase-containing protein, STK38 (NDR1), has been found to be a POPX2-interacting protein [41]. Thus, we narrowed our search for potential substrates of POPX2 by only concentrating on proteins containing the Pkinase domain.

There are approximately 20,000 protein-coding genes in the human genome [42,43]. To reduce the pool of proteins for initial screening, we leverage on previous proteomic data performed on POPX2-

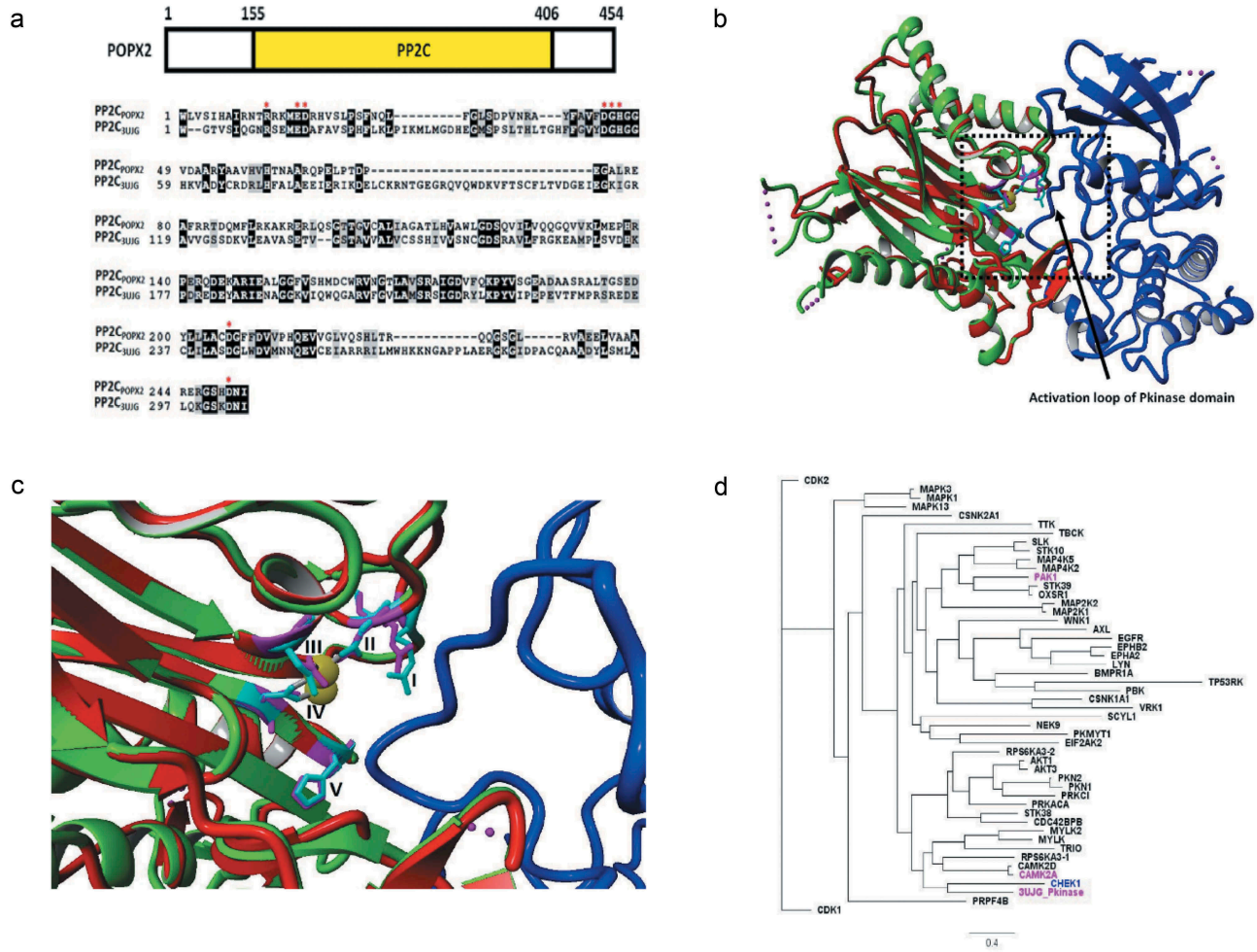


**Figure 2.** Prediction of POPX2 substrates using domain–domain interaction (DDI) and phylogenetic analysis.

(a) Schematics of POPX2 domains and alignment between $PP2C_{3UJG}$ and $PP2C_{POPX2}$. POPX2 contains PP2C domain between amino acid residues 155 and 406. 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 3UJG PDB structure is shown where the blue and green chain represents the Pkinase domain and PP2C domain in 3UJG, respectively. The homology model of $PP2C_{POPX2}$ is superimposed onto $PP2C_{3UJG}$ and represented in red. Active site residues of $PP2C_{3UJG}$ and $PP2C_{POPX2}$ are represented in cyan and magenta, respectively. Magnesium ion is depicted by the yellow balls. The residues labeled 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 (Root Mean Square Deviation) of 0.69A. In the figure, the activation loop of the Pkinase domain docks onto the binding pocket of $PP2C_{3UJG}$ and $PP2C_{POPX2}$. (d) Phylogenetic tree built with 46 Pkinase proteins together with PAK1, CAMK2A, 3UJG_Pkinase and CHEK1 (Chk1). PAK1 and CAMK2A are known substrates of POPX2 and 3UJG_Pkinase is a substrate of the 3UJG_PP2C domain.

knockdown MDA-MB-231 breast cancer cells [8]. In the study by Zhang et al., proteomes of POPX2-knockdown and control cells are identified using SILAC (Stable Isotope Labeling by/with Amino acids in Cell culture) mass spectrometry. Proteins that exhibit markedly different expression levels following POPX2-knockdown can then be determined. Our assumption is that interactors of POPX2 should maintain reasonable levels in control cells to elicit effects downstream of POPX2 and that proteins which show differential levels in control vs POPX2-knockdown cells are likely to be involved in POPX2 regulated pathways.

Proteins identified in the study by Zhang et al. are filtered for Pkinase domain. Out of the 2146 proteins, only 46 proteins contain the Pkinase domain. Phylogenetic analysis is then performed on the Pkinase domains of these 46 proteins to identify their relationship with known substrates of PP2C including the Pkinase in 3UJG, PAK1, and CAMK2α (CAMK2α is encoded by the CAMK2A gene in human). From phylogenetic analysis

(Figure 2(d)), the Pkinase domain of Chk1 is found to be closely related to CAMK2α and Pkinase in 3UJG. Chk1 is a regulator of cell cycle and apoptosis during DNA damage. In addition, proteins differentially expressed in POPX2-knockdown cells were found to be highly enriched in the cell cycle pathway [8]. These observations strongly suggest Chk1 as a likely candidate for POPX2 interaction.

### Prediction of POPX2 substrates by the curation of known substrates of phosphatases sharing homology to POPX2

In the second part of the analysis, we identify potential substrates of POPX2 by consolidating known substrates of POPX2 homologs and PP2C family phosphatases. Proteins with close sequence similarity to POPX2 are identified from BLAST. Unsurprisingly, isolated proteins are members of the PP2C family: PPM1A to PPM1M.

For each of the PP2C protein identified above, we identify their substrates using STRING [34]. Homologous proteins are likely to share similar interactions. Therefore, it is possible to identify proteins performing similar functions and their interactors through sequence similarity and protein–protein interactions [44]. This implies that substrates of POPX2's homologs may also be substrates of POPX2. Table 1 summarizes the proteins containing the Pkinase domain that are predicted or known to interact with PP2C members. Once again, Chk1 appears in the list where it was previously reported to interact with PPM1D. The binding of PPM1D to Chk1 leads to the dephosphorylation of Chk1-Ser345 and reduces checkpoint activity [45].

Based on the bioinformatic analysis detailed above, Chk1 is identified independently by both analyses and appears to be a plausible substrate capable of interacting with POPX2. In the ensuing section below, we show the experimental validation of the interaction and dephosphorylation of Chk1 by POPX2.

**Table 1.** Known or predicted substrates of PP2C members identified using STRING.

| 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 |

Only substrates that contain the Pkinase domain are shown for clarity. MAP3K7 (also known as TAK1) is highlighted in purple due to its interaction with PPM1B and PPM1L as well as being implicated in upregulating anti-apoptotic activity in POPX2-knockdown cells [10]. Once again, Chk1 (CHEK1, blue font) is picked up due to Chk1 being a substrate of PPM1D.

### Experimental validation of the interaction between POPX2 and Chk1

In this section, we seek to confirm the prediction from the bioinformatic analysis by validating the biological interaction between POPX2 and Chk1 using co-immunoprecipitation assays. Glutathione S-transferase (GST) and Flag pulldown assays were performed to demonstrate the interaction between Chk1-Flag and GST-POPX2. Both constructs were transfected into HEK293 cells, Chk1-Flag was detected in the complex obtained from GST-POPX2 pulldown but not GST pulldown (Figure 3(a)). Next, to investigate if the binding between the two proteins was enhanced during DNA damage response, transfected cells were treated with Etoposide (VP-16), a topoisomerase II inhibitor, to induce DNA damage. We found that GST-POPX2 could associate with Chk1-Flag in DMSO (control) and VP-16 treated cells (Figure 3(b)). The relative amounts of GST-POPX2 pulled down with Chk1-Flag are similar in DMSO and VP-16 treated cells, suggesting that POPX2 can interact with Chk1 in control cells and cells suffering DNA damage (Figure 3(c)). Furthermore, 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 (Figure 3(d)). Consistently, endogenous Chk1 was isolated together with immunoprecipitated POPX2, indicating that POPX2 and Chk1 exist as a complex within the cells (Figure 3(e)).

### Chk1 is dephosphorylated by POPX2 phosphatase

To investigate the functional link between Chk1 kinase and POPX2 phosphatase in response to

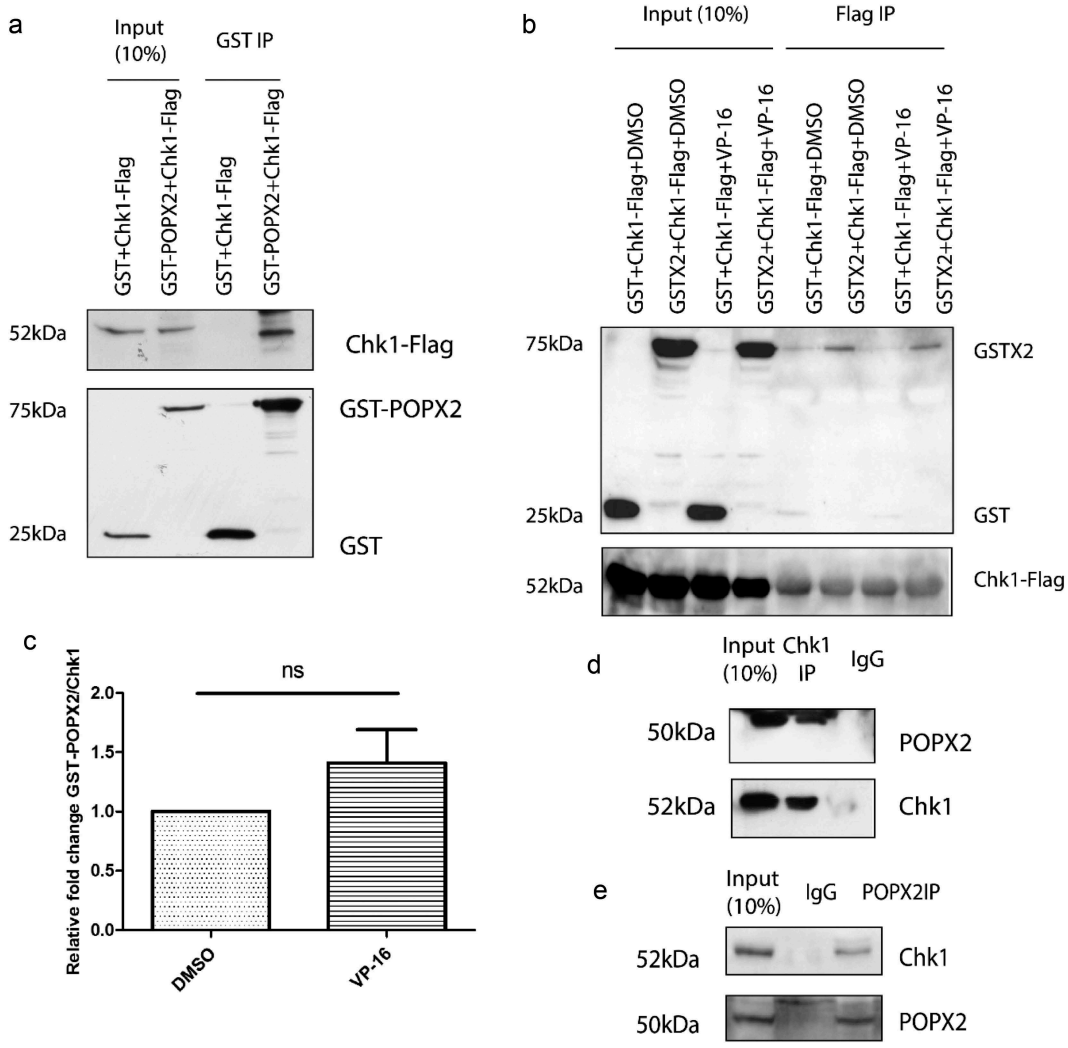


**Figure 3.** POPX2 interacts with Chk1 regardless of DNA damage.

(a) GST or GST-POPX2 cDNA construct 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, followed by treatment with DMSO (control) or VP-16 (40 µg/ml). 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. (c) Relative fold change of GST-POPX2/Chk1 was measured from the amounts of GST-POPX2 pulled down together with Chk1-Flag in cells treated with DMSO or VP-16 in (B). Error bar represents mean ± S.E. of four independent experiments. (d) Endogenous Chk1 was isolated using anti-Chk1 mouse antibody and proteins that co-immunoprecipitated together with Chk1 were subjected to Western analysis using POPX2 antibody. (e) Endogenous POPX2 was immuno-precipitated with anti-POPX2 mouse antibody and Chk1 was identified from POPX2 immunoprecipitated complex. Random IgG-mouse was used as a control in (d) and (e).

genotoxic stress, *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. PP2A was included as it has earlier been reported to downregulate phosphorylation of Chk1 [46]. However, there is also a report which suggests that PP2A does not dephosphorylate Chk1 [45]. In our western analysis, we did not see a consistent trend for PP2A in our triplicates. Therefore, PP2A's role in the regulation of Chk1 is still not conclusive. We observed more dephosphorylated Chk1-Ser317 and -Ser345 in cells co-transfected with GST-POPX2 but not in the case of GST alone. For cells co-transfected with GST-PP2A, we could not consistently observe a reduction in Ser317 and Ser345 phosphorylation levels. Our observations suggest that POPX2 might be

a specific phosphatase for Chk1 during the early stages of VP-16 induced DNA damage (Figure 4(a)). However, under prolonged VP-16 treatment, there was no observable difference in phospho-Ser317 and phospho-Ser345 levels for control and POPX2 or PP2A co-expressed cells, suggesting that the activity of POPX2 might not be required during later stages of VP-16 induced DNA damage response. The relative ratio of phospho-Chk1/Chk1 was significantly reduced by overexpression of GST-POPX2 compare to GST alone after 20 min of VP-16 addition (Figure 4(b)).

We next examined whether POPX2 dephosphorylates Chk1 *in vitro*. Cells were transfected with Chk1-Flag and treated with DMSO or VP-16 to induce Chk1 phosphorylation. Chk1-Flag pulldown was incubated with or without bacterially expressed GST-POPX2 for the *in vitro* phosphatase assays. We found that phosphorylated Chk1-Ser317 and -Ser345 were efficiently dephosphorylated by GST-POPX2 *in vitro* (Figure 4(c)). The relative ratio of phospho-Chk1/Chk1 was significantly decreased in the presence of GST-POPX2 (Figure 4(d)). These observations suggest that Chk1 is a possible substrate of POPX2.

### Silencing POPX2 does not significantly affect Chk1 phosphorylation

Since we have found that high levels of POPX2 affect the phosphorylation status of Chk1-Ser317 and Ser345, we proceeded to determine if silencing POPX2 would lead to changes of Chk1 phosphorylation in the cells. siRNA targeting luciferase (siLuc) and two different siRNAs targeting POPX2 (siX2#1 or siX2#2) were transfected into HEK293 cells, followed by treatment with DMSO or VP-16. The cells were lysed and POPX2-knockdown was validated using Western blot analysis (Figure 5(a)). POPX2-knockdown cells did not show significant differences in Chk1 phosphorylation at Ser317 and Ser345 compared to control cells after treated with VP-16 (Figure 5(b)). As Chk1 phosphorylation is already increased significantly in response to VP-16, it is possible that we might not observe a further increase in Chk1 phosphorylation level with POPX2-knockdown. As for the cells without VP-16 treatment, we also did not observe significant changes in Chk1 phosphorylation levels in POPX2-knockdown cells. One possible reason is that other phosphatases such as PP1 and PPM1D could be maintaining the levels of Chk1 phosphorylation status in the unperturbed situation.

### POPX2 regulates cell cycle progression in response to DNA damage

As Chk1 executes a significant role in cell cycle arrest and activation of checkpoints, we next determine if POPX2 plays any role in VP-16 mediated cell cycle delay or arrest. In response to DNA damage, Chk1 activates G1/S or G2/M checkpoints via CDC25 proteins for DNA damage repair or apoptosis [17,18,22]. Our previous study reported that POPX2 could promote apoptosis through dephosphorylating TAK1 in response to DNA damage [10]. In this study, we focused on cell cycle progression under replication stress and whether POPX2 plays any role in cell cycle control. Cell cycle analysis using FACS showed that there was no significant difference in cell cycle progression in POPX2-knockdown (siX2) and control (siLuc) U-2OS cells under normal conditions (Figure 6(a)). Interestingly, silencing POPX2 in VP-16 treated cells (siX2+ VP-16) led to a reduced cell population at G2. Accumulation at G1 phase was observed after 20 h of VP-16 treatment instead, whereas a higher number of VP-16 treated control cells (siLuc+VP-16) were arrested at the G2 checkpoint (Figure 6(b)). Quantification of cell cycle stages in control and POPX2-knockdown cells shows that a higher number of siX2+ VP-16 cells were at G1 and less were found at G2 phase compared to control cells (Figure 6(b)).

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 (Figure 6(c)). When cells are exposed to DNA-damaging agents, POPX2-overexpressing cells have significantly increased the S phase population compared to control cells (Figure 6(d)). 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

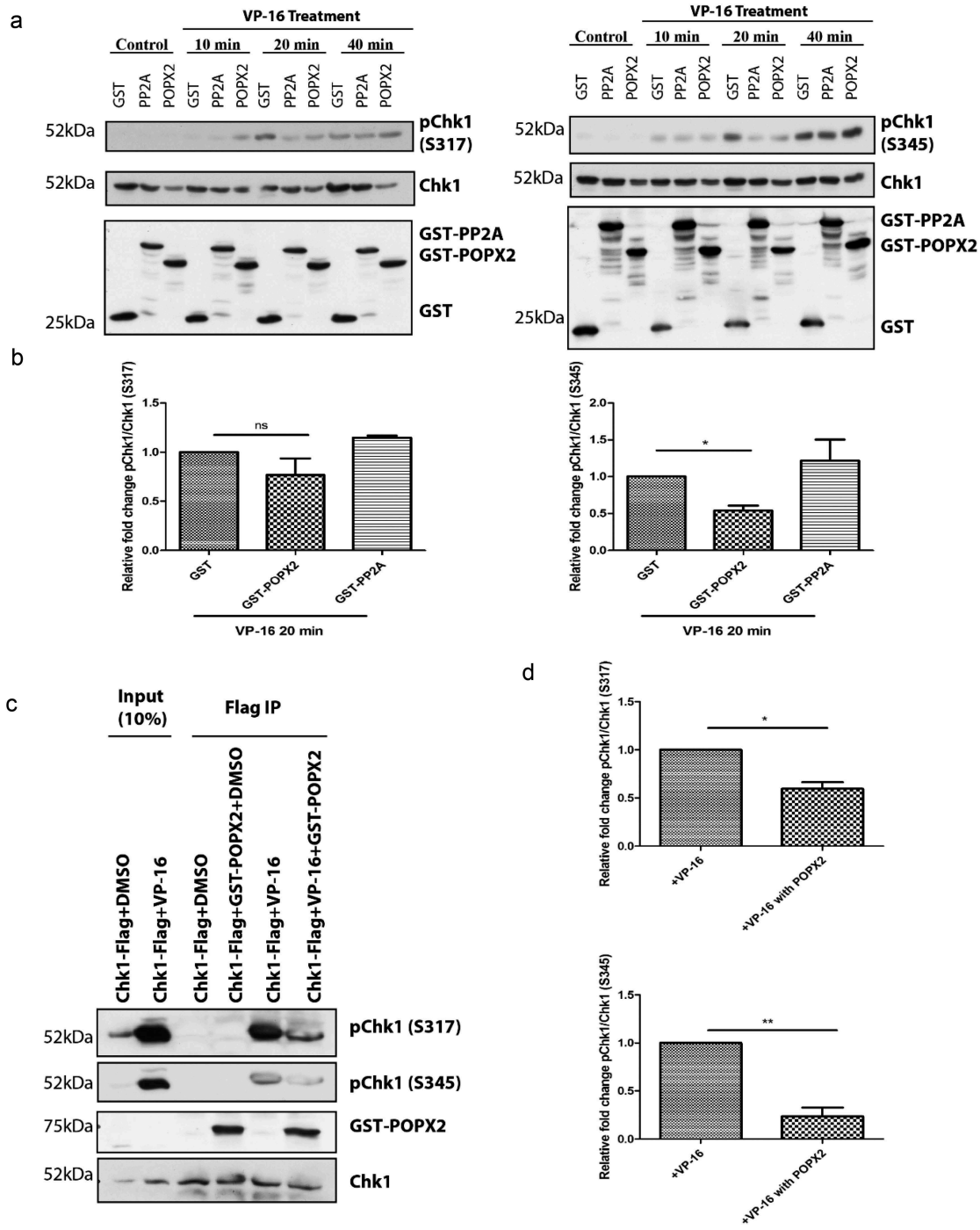


**Figure 4.** Chk1 is dephosphorylated by POPX2.

(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 μg/ml) at indicated time points. Phosphorylation of Chk1 was detected using anti-pChk1 Ser317 and Ser345 antibodies. (b) Densitometry measurement for (a) was done using ImageJ for three independent experiments. The ratio of pChk1/Chk1 was measured. (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 analyzed by immunoblotting. (d) The ratio of pChk1/Chk1 was analyzed as in (b). Error bars represent mean ± S.E. of three independent experiments in (b and d). *p ≤ 0.05, **p ≤ 0.01, as analyzed by Student's t-test.

phase compared to GST overexpressing cells (Figure 6(d)). Overall, our observations suggest that POPX2-knockdown cells encounter a 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.

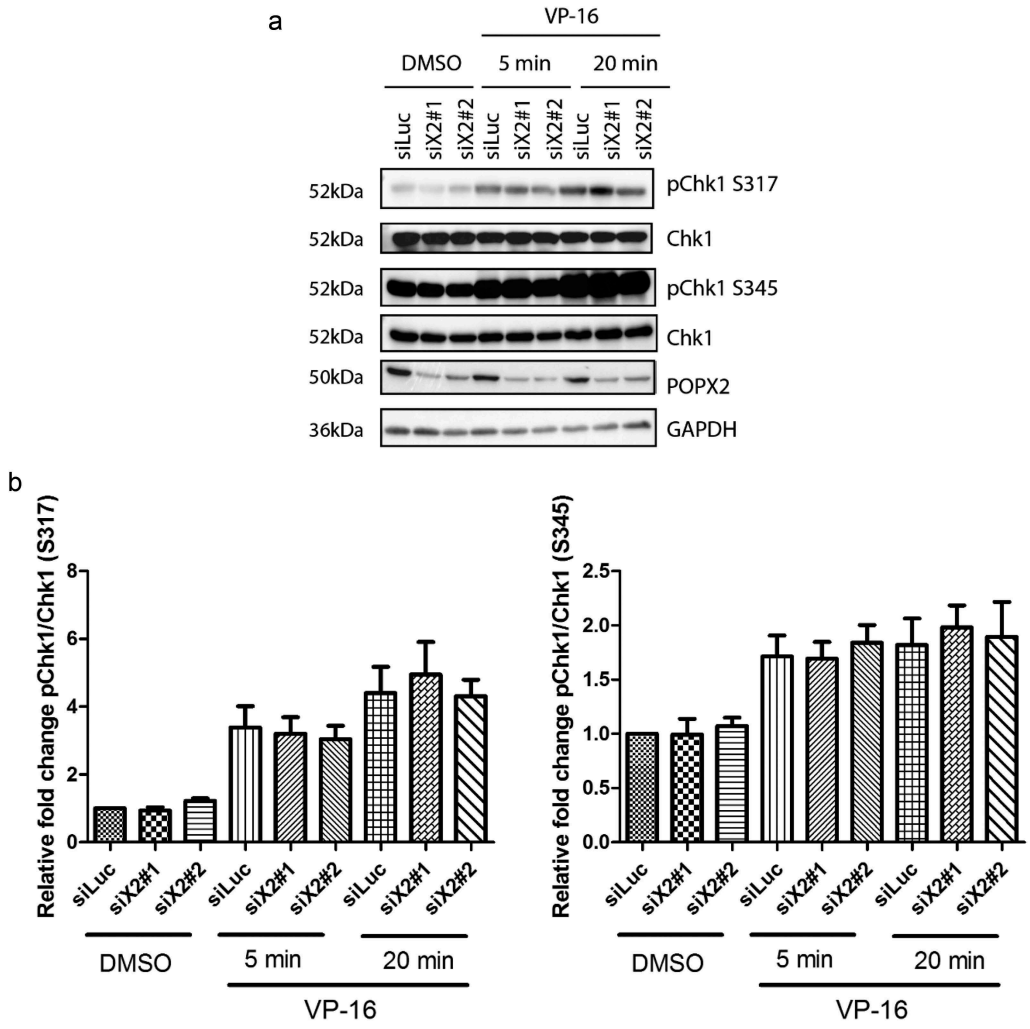


**Figure 5.** Chk1 phosphorylation in control and POPX2-knockdown cells.

HEK293 cells were transfected with siLuc (control) or siRNAs targeting POPX2 (siX2#1 or siX2#2). Transfected cells were treated with DMSO or VP-16 48 h post transfection. The cells were lysed at indicated time points and subjected to Western blot analysis. (a) Phosphorylation of Chk1 was detected using anti-pChk1 -Ser317 and -Ser345 antibodies. POPX2 was detected using anti-POPX2 antibodies and GAPDH was used as a loading control. (b) Densitometry measurement was done using Image Lab™ software. The ratio of pChk1/Chk1 in each experimental setup was computed. Error bars represent mean ± S.E. of three independent experiments.

Taken together, our observation suggests that POPX2 is involved in G1-S transition in response to DNA damage (Figure 6(e)).

## Discussion

Identifying protein–protein interactions (PPIs) can provide information on the roles and functions played by proteins in the cells [47]. Although many PPI tools are available as open-source websites, they might not provide comprehensive coverage. For example, both InterPreTS [48] as well as Interactome3D [49] are unable to deduce the interaction between POPX2 and its known substrates such as PAK1 and CaMK2. Therefore, in this study, we attempt to uncover the interactors of POPX2 using a two-pronged strategy. In the first strategy, potential POPX2 substrates are chosen based on curated domain–domain interactions as well as relation to known POPX2 substrates. At the same time, we also use the SILAC proteomic approach to identify a pool of differentially expressed proteins so that we can narrow down the total proteins that we need to screen. The second strategy involves discovering substrates of POPX2 (also known as PPM1F) from known substrates of the PPM1 family. Both strategies are guided by evolutionary principles. Domain–domain interactions

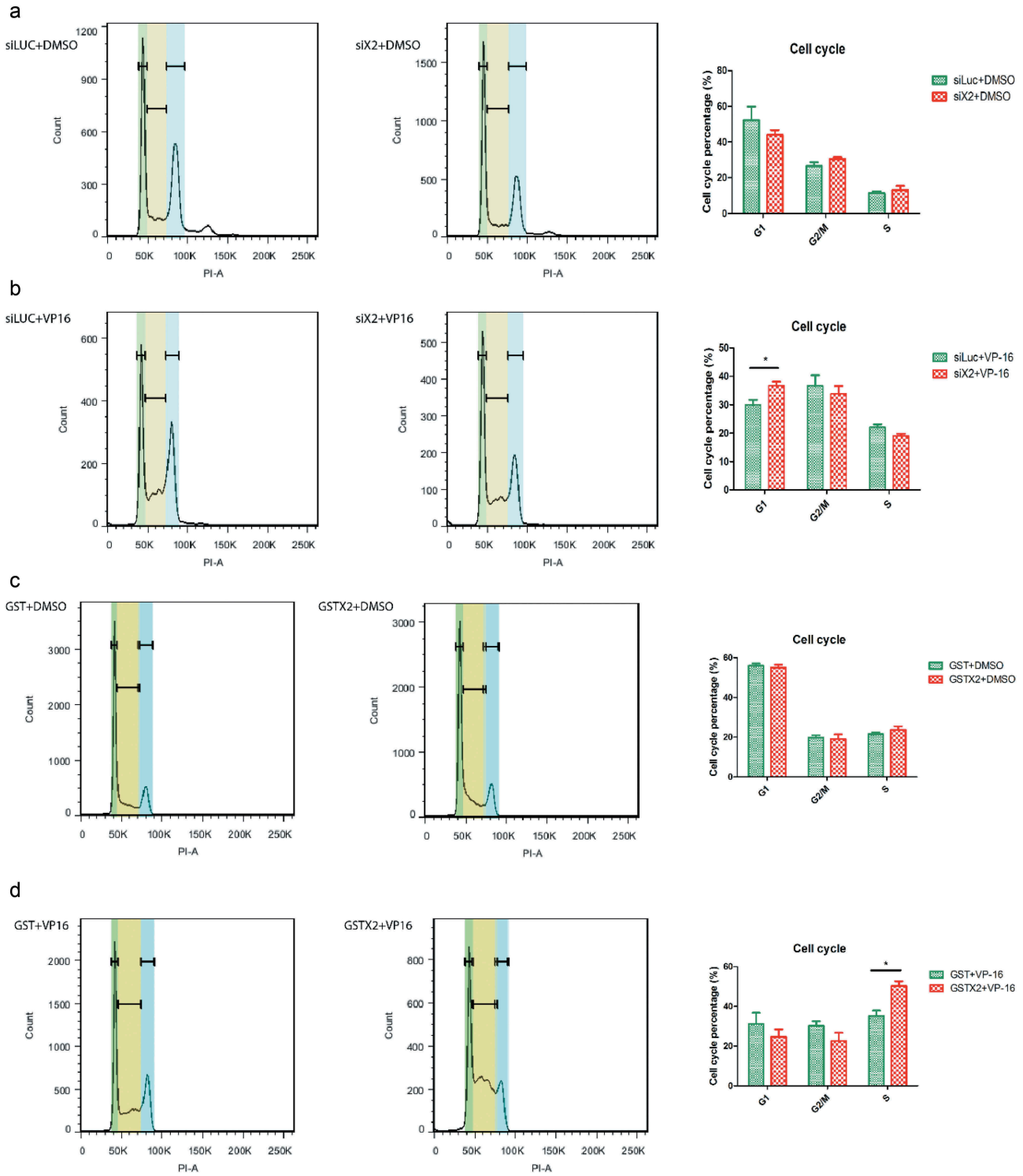


**Figure 6.** POPX2 regulates G1-S cell cycle transition in response to DNA damage.
Cell cycle progression of siLuc and siX2 cells treated with DMSO (a) and VP-16 in (b). U-2OS cells were transfected with siRNA targeting Luciferase (siLuc) or POPX2 (siX2) for 48 h, followed by treatment with DMSO or VP-16 for 20 h. Cells were fixed and stained with PI/RNase dye for 1 h before cell sorting. PI stained cells were analyzed 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 h. Cell cycle progression in GST and GST-POPX2 overexpressing cells treated with DMSO (c) and VP-16 (d). The bar charts on the right represent the percentage of cells in different cell cycle phases. Error bars represent mean ± S.E. of three independent experiments in (A-D). *$p \leq 0.05$, as analyzed by Student's t-test. (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 translocates from the nucleus to the cytoplasm. Active Chk1 induces the activation of the 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 are able to proceed from G1 to S phase despite DNA damage.

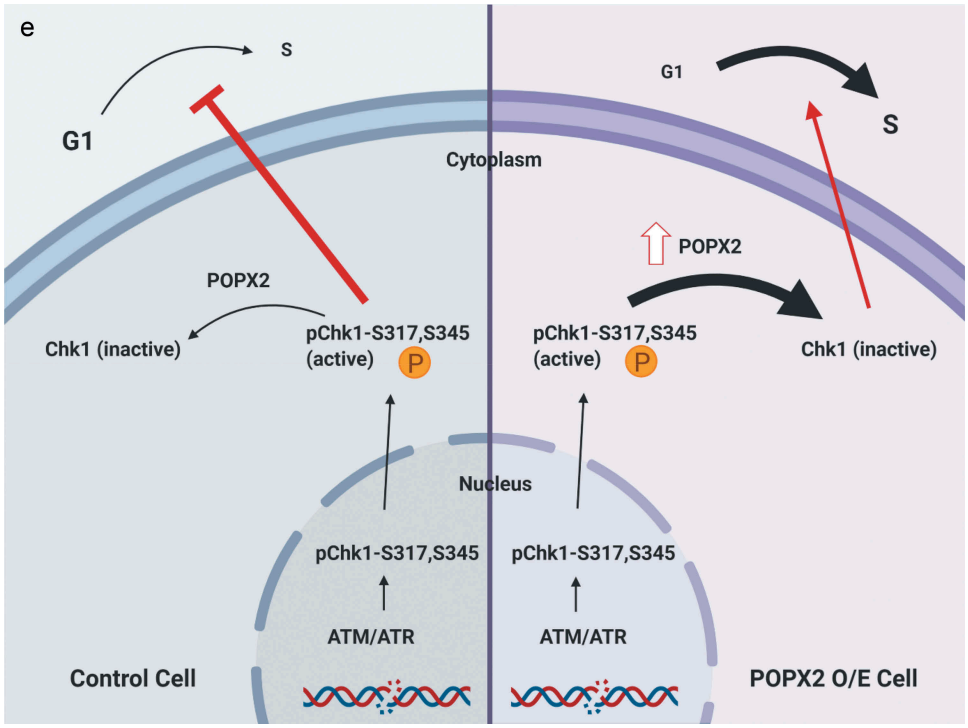


**Figure 6.** (Continued).

that mediate protein interactions have been found to be maintained across evolution [37]. Homologous proteins are also likely to share similar interactions [44]. As an example, Chk1 and TAK1 are also substrates of other PPM1 family proteins. PPM1D binds and dephosphorylates Chk1 [45] and TAK1 is also a substrate of PPM1B and PPM1L [50,51].

Phosphorylation of Chk1-Ser345 has been reported to be responsible for the increase in Chk1 kinase activity [14,16]. In this study, we found that POPX2 is able to dephosphorylate Chk1 at Ser317 and Ser345. As activation of Chk1 is important in the regulation of DNA damage checkpoint response, we analyzed cell cycle progression with or without a DNA-damaging agent in POPX2-knockdown cells and POPX2-overexpressing cells. In the presence of DNA damage, silencing POPX2 leads to an increased G1 population and reduced S population compared to control cells. On the other hand, POPX2 overexpressing cells display an increased S population and decreased G1 population compared to control cells when cells suffered 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 [15,18], it is possible that POPX2 regulates DNA damage response through dephosphorylating Chk1, leading to inactivation of Chk1's downstream targets and G1-S checkpoint.

Chemosensitizing effects of Chk1 inhibitors in cisplatin-resistant cancers and radio-resistant cancers have been extensively studied [25–28]. Here, we report that Chk1 is a substrate of POPX2 and that its activity can be negatively regulated by POPX2. Our previous studies showed that POPX2 negatively regulates TAK1 and silencing POPX2 leads to increased metastasis [9,10]. Taken together, we propose that POPX2 could regulate the sensitivity of cancer cells toward anti-cancer drug (VP-16) through modulation of apoptosis and cell cycle checkpoint through TAK1 and Chk1, respectively. It is tempting to speculate that combinations of chemo-therapeutic drugs together with modulation of POPX2 activity might contribute to sensitizing cancer cells for chemotherapy and inhibition of cancer metastasis in a specific context.

## Conclusion

Here, we propose an innovative approach to investigate protein–protein interactions using a combination

of bioinformatics and proteomics. A two-pronged bioinformatic analysis was performed to predict possible substrates of POPX2 by analyzing (1) known substrates of POPX2 and PP2C domain, and (2) known substrates of proteins sharing homology with POPX2. As a result, Chk1 with Pkinase domain was identified as a potential target of POPX2. Chk1 is a key regulator of the DNA damage pathway and activation of Chk1 induces the G1/S and G2/M checkpoints for DNA repair and apoptosis. In this study, we found and confirmed that Chk1 interacts with POPX2 as predicted from bioinformatic analysis. We also determined that POPX2 can dephosphorylate Chk1-Ser317 and -Ser345 and is a potential regulator of Chk1 function in the cell.

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## Author contributions

Conceptualization and methodology, P.R.K., Y.L.K., R.T.C.L., F.A., and D.H.Z.K.; Writing – Original Draft, P.R.K., Y.L.K., and R.T.C.L.; Writing – Review & Editing, P.R.K., Y.L.K., R.T.C.L., D.H.Z.K, K.H.C., and C.G.K.; Funding Acquisition, C.G.K, and K.H.C.; Supervision, C.G.K, and K.H.C. Data analysis: all authors.

## Disclosure statement

No potential conflict of interest was reported by the authors.

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## ORCID

Raphael Tze Chuen Lee http://orcid.org/0000-0001-5634-9154
Farouq Azizan http://orcid.org/0000-0002-9350-0981
Dylan Hong Zheng Koh http://orcid.org/0000-0002-5252-6309

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