4.1 Introduction

PPM1G is an Mg2+/Mn2+ dependent serine/threonine human protein phosphatase that belongs to the PP2C family of serine/threonine phosphatases. PPM family of phosphatases is Mg2+ or Mn2+ dependent protein phosphatases and are insensitive to phosphatase inhibitors such as okadaic acid and microcystin. There are 22 members in the PPM family of Protein Ser/Thr phosphatases. PPM family of phosphatases participate in several cellular processes such as cell differentiation, survival, growth, apoptosis, stress signaling, and metabolism [317].

PPM1G gene localizes at 2p23.3 position on the chromosome and is highly conserved among eukaryotes. PPM1G is expressed ubiquitously in all the tissues and shows elevated expression in testis, skeletal muscle, and heart [318].

4.1.1 PPM1G protein structure

PPM1G was first identified by Travis et. al. in 1997 [318]. The group identified a protein that was 34% identical to PP2Cα and PP2Cβ, hence, it was named as PP2Cγ. PPM1G is a 546 amino acid protein and has a molecular weight of 59 kDa. The size of PPM1G is larger than PPM1A and PPM1B, and this larger size of PPM1G is due to the presence of additional domain in PPM1G. PPM1G protein has a modular structure that includes two phosphatase domains (at N-terminus and C-terminus respectively), and one acidic domain (at the centre) (Fig. 4.1). The acidic domain (200 aa) is a unique domain found in PPM1G and it is highly rich in acidic amino acids. Although PPM1G is a protein with molecular weight of 59 kDa, it appears at a molecular mass of 75 kDa in the SDS-gel, the anomalous mobility of PPM1G is attributed to the presence of acidic region presumably because of weak binding with SDS [319]. The acidic domain in PPM1G contains conserved sequences for phosphorylation by casein kinase II, and it plays a role in substrate identification and recruitment [318]. Besides,
PPM1G contains three PEST motifs present between amino acid 172-197, 197-209 and 255-320 that might determine the stability of PPM1G protein.

Figure 4.1. PPM1G domain architecture. PPM1G has a modular structure. It contains two phosphatase domains, one at N-terminus, and another at C-terminus. Also, it contains a central acidic domain. The acidic domain is unique to PPM1G and plays a role in substrate recognition and binding. {The purple lines indicate the amino acid residues (D441, D491, and D496) that are required for the phosphatase activity of PPM1G}

4.1.2 PPM1G protein function

PPM1G regulates several cellular processes such as splicing, cell cycle, DNA damage, histone exchange, transcription elongation, and translation initiation. PPM1G controls splicing of specific mRNAs by auto-phosphorylation restricted interaction with the spliceosome-associated factor, YB-1. YB-1 is a spliceosome associated factor, and it interacts with the acidic domain in PPM1G. PPM1G in association with YB-1 affects the alternative splicing of CD44 [320].

PPM1G arrests the cells in S-phase of the cell cycle. The cell-cycle arrest in S-phase induced by PPM1G overexpression is surprisingly via downregulation of p21. P21 is a cell cycle regulatory protein and it is degraded via proteasome upon PPM1G expression. However, the machinery that promotes PPM1G induced proteasomal degradation of p21 remains to be identified [321]. Also, PPM1G participates in the DNA damage response by dephosphorylating and subsequently downregulating USP7 during ionizing radiation-induced cell stress. USP7 plays a crucial role in regulating the genome stability and cancer. However, the regulation of USP7 was not known previously. In normal cells, USP7 is phosphorylated by CK2, phosphorylated USP7
stabilizes Mdm2 and leads to downregulation of p53. In contrast, upon DNA damage by ionizing radiation, USP7 is dephosphorylated by PPM1G that leads to destabilization of Mdm2 and stabilization of p53. Hence, PPM1G induces p53-mediated cell sensitivity towards ionizing radiation [322].

PPM1G also functions as histone chaperone i.e. PPM1G promotes the histone exchange by binding and dephosphorylating H2A and H2B. Histone exchange regulates chromatin functions such as gene expression and genome integrity both in resting cells and stressed cells [323].

PPM1G participates in the transcription elongation by relieving the transcription pause. Transcription elongation after transcription initiation is a crucial transition process. NELF and DSIF are negative regulators of elongation and pause the RNA polymerase II downstream the transcription start site. P-TEFb kinase antagonizes NELF and DSIF mediated pause of RNA polymerase II. P-TEFb is sequestered to 7SK snRNP through Hexim1 and 7SK RNA. Further, 7SK snRNAP occupies the promoters and prevents the transcription pause release by making p-TEFb unavailable. The transcription factors such as NF-KB and Tat recruits PPM1G to the target promoters causing the release of p-TEFb from 7SK snRNP complex. Released p-TEFb relieves the transcription pause, and transcription elongation ensues [324].

In addition, PPM1G negatively regulates the cap-dependent translation. 4E-BP1 is an important regulator of protein translation and phosphorylation of 4E-BP1 plays a crucial role in regulating the 4E-BP1 function in protein translation. 4E-BP1 binds to translation initiation factor 4E and inhibits the translation (cap-dependent translation). The mTORC phosphorylates the 4E-BP1 and promotes the translation initiation. PPM1G dephosphorylates 4E-BP1 and thus inhibits translation. Further,
knockdown of PPM1G results in the increased cap-dependent translation, cell size, and protein content suggesting that PPM1G functions as an important negative regulator of translation by dephosphorylating 4E-BP1 [325].

4.1.3 PPM1G regulation

There are very limited studies on identifying the regulators of PPM1G. However, it has been identified that PPM1G in its acidic domain contains consensus sequences for phosphorylation by CKII. Hence, the role of CKII in PPM1G regulation may be important [322]. Also, as the acidic domain is exclusively present only in PPM1G, the acidic domain may regulate PPM1G localization and function by recruiting regulatory proteins [318]. In addition, a potential myristoylation site has been identified in PPM1G that may regulate the localization of PPM1G by targeting it to the membrane and thus may regulate its function. Also, PEST sequence present in PPM1G may regulate the PPM1G protein stability. Cadmium regulates the activity of PPM1G [318]. Cadmium coordinates with the negative charge of D441 residue in PPM1G and inhibits the phosphatase activity of PPM1G that is reflected in the cadmium-mediated inhibition of PPM1G regulated AKT signaling [326].

4.2 Results

4.2.1 PPM1G is a novel WWP2 associated protein

As our results (Chapter 3) suggested that the interplay between the monomeric and heterodimeric WWP2 states is critical in regulating the p73 and ΔNp73 levels, we hypothesized that a molecular switch might exist in the cells that may alter WWP2 in these two states under different cellular conditions. To test this hypothesis, we again analyzed the list of WWP2 associated proteins identified by Mass Spectrometry. After
testing various listed WWP2 associated proteins, we found that a phosphatase, PPM1G specifically interacts with WWP2 (Fig. 4.2a, Fig. 4.2b).

**Figure 4.2. PPM1G specifically interacts with WWP2.** (a) SFB-tagged WWP2, WWP1, HACE1, and E6AP along with Myc-PPM1G were expressed in 293T cells, and their association was tested by pull-down with SBP beads, followed by immunoblotting with an anti-Myc antibody. (b) HEK293T cell lysate expressing SFB-WWP2, SFB-WWP1, SFB-HACE1 or SFB-E6AP was added to GST-protein or GST-PPM1G immobilized on glutathione sepharose beads. The interaction of E3 ligases with PPM1G was assessed by immunoblotting with an anti-Flag antibody after pull-down with GST beads. The expression of GST-protein and GST-PPM1G was shown by Coomassie staining.

Further, we examined the region in PPM1G essential for the interaction with WWP2. The various PPM1G deletion constructs (Fig. 4.3a) were transfected along with full-length WWP2 and we found that the acidic domain in PPM1G is required for interaction with WWP2 (Fig. 4.3b).
Figure 4.3. Acidic domain in PPM1G is required for interaction with WWP2. (a) Schematic representation of full-length SFB-PPM1G and its various SFB-tagged deletion mutants (D1 to D6). (b) HEK293T cells were transfected with full-length SFB-PPM1G and its deletion constructs along with Myc-WWP2. The region of interaction was determined by pull-down using SBP beads followed by immunoblotting with an anti-Myc antibody.

In a reverse mapping experiment, we transfected various WWP2 deletion constructs (Fig. 4.4a) along with full-length PPM1G, and we found that the WW1 domain in WWP2 is essential for interaction with PPM1G (Fig. 4.4b).
Figure 4.4. WW1 domain in WWP2 is required for interaction with PPM1G. (a) Schematic representation of full-length Myc-WWP2 and its various deletion mutants (D1 to D5) (b) HEK293T cells were transfected with full-length Myc-WWP2 and its Myc-tagged deletion mutants along with SFB-tagged PPM1G. The region of interaction was determined by immunoblotting with anti-Myc antibody after pull-down using SBP beads.

4.2.2 PPM1G and WWP2 do not regulate the stability of each other

Since we found PPM1G as a novel WWP2 associated protein, we were interested to find the functional significance of WWP2-PPM1G interaction. We first tested if WWP2 regulates PPM1G stability. We tested the PPM1G protein levels in the presence of WWP2 (gradient expression). We found that WWP2 has no effect on PPM1G protein levels (Fig. 4.5a). Further, we tested the WWP2 protein stability by
overexpression of PPM1G WT or PPM1G D3A3 (phosphatase-inactive) mutant. We found that PPM1G has no effect on WWP2 protein stability (Fig. 4.5b).

**Figure 4.5.** PPM1G and WWP2 do not affect the protein stability of each other (a) HEK293T cells were transfected with PPM1G along with the increasing concentrations of WWP2, and the protein levels were detected by immunoblotting with respective antibodies. Actin was used as a loading control. (b) HEK293T cells were transfected with SFB-WWP2 alone or with Myc-PPM1G WT or with Myc-PPM1G D3A3. At 24 hrs. post-transfection cells were treated with cycloheximide. Cells were harvested at indicated time points, and protein levels were determined by using indicated antibodies.

As in our study (Chapter 3), we identified that WWP2 exists in two states [monomeric and heterodimeric (with WWP1) state]. We hypothesized that PPM1G might regulate the interplay of WWP2 between these two distinct states in cells.

### 4.2.3 PPM1G regulates the WWP2-WWP1 heterodimerization

We tested the possibility of PPM1G as a molecular switch for monomeric versus heterodimeric WWP2 states, we depleted PPM1G in the cells and examined the WWP2-WWP1 interaction. As shown in Fig. 4.6a, depletion of PPM1G severely
affected the WWP2-WWP1 complex formation. PPM1G activity is required for the assembly of WWP2-WWP1 complex as inhibition of PPM1G activity by cadmium chloride reduced the WWP2-WWP1 interaction in cells (Fig. 4.6b and Fig. 4.6c).

![Diagram](image)

**Figure 4.6.** PPM1G regulates the WWP2-WWP1 heterodimerization. (a) HeLa cells were transfected with control siRNA or two individual PPM1G siRNAs. The presence of WWP2-WWP1 heterodimeric complex in these cells was analyzed by immunoprecipitation with WWP2 antibody followed by immunoblotting with WWP1 antibody. (b) Cells transfected with SFB-WWP2 and Myc-WWP1, were either mock treated with buffer or treated with CdCl2 (1.5µM) and the association of WWP2-WWP1 in these cells was analyzed by immunoblotting with anti-Myc antibody after pull-down with SBP-beads. (c) Cells were either mock treated with buffer or treated with CdCl2 (1.5µM), and the association of WWP2-WWP1 in these cells was
analyzed by immunoprecipitation with WWP2 antibody followed by immunoblotting with WWP1 antibody.

Since PPM1G is essential for the assembly of WWP2-WWP1 complex, we tested PPM1G effect on the protein levels of p73 and ΔNp73. We observed that p73 ubiquitination is enhanced (Fig. 4.7a and Fig. 4.7b), whereas ΔNp73 ubiquitination is significantly reduced upon inhibition of PPM1G activity (Fig. 4.7c and Fig. 4.7d).

**Figure 4.7.** PPM1G regulates the ubiquitination of p73 and ΔNp73 in cells. (a) HEK293T cells were transfected with p73 and WWP2, and 24 hrs. later they were either mock treated or treated with CdCl2. WWP2-mediated p73 ubiquitination was detected by immunoblotting with anti-HA antibody after immunoprecipitation with an anti-HA antibody. (b) HEK293T cells were transfected with p73 and WWP2, and 24 hrs. later they were either mock treated or treated with CdCl2. WWP2-mediated p73
ubiquitination was detected by immunoblotting with anti-Ub antibody after immunoprecipitation with an anti-HA antibody. (c) Cells were transfected with ΔNp73 and WWP1, and 24 hrs. post transfection, cells were either mock treated or treated with CdCl2. WWP1-mediated ΔNp73 ubiquitination was detected by immunoblotting with anti-HA antibody after immunoprecipitation with an anti-HA antibody. (d) Cells were transfected with ΔNp73 and WWP1, and 24 hrs. post transfection, cells were either mock treated or treated with CdCl2. WWP1-mediated ΔNp73 ubiquitination was detected by immunoblotting with an anti-Ub antibody after immunoprecipitation with an anti-HA antibody.

Since, PPM1G affects the WWP2, and WWP1 mediated ubiquitination of p73, and ΔNp73 respectively, we tested the effect of PPM1G on protein half-life of p73 and ΔNp73. Our cycloheximide-chase experiment revealed that PPM1G affects the protein stability p73 and ΔNp73 as inhibition of PPM1G reduced the protein half-life of p73, whereas ΔNp73 was stabilized (Fig. 4.8a). The finding suggests that PPM1G might inhibit WWP2 activity and thus stabilize p73, at the same time, inactive WWP2 complexes with WWP1 leading to enhanced degradation of ΔNp73.

![Diagram](image.png)

**Figure 4.8.** PPM1G regulates the stability of p73 and ΔNp73 in cells. (a) HEK293T cells transfected with p73 or ΔNp73 were either mock treated or treated with CdCl2. At 24 hrs. post transfection, cells were treated with cycloheximide (CHX) and were collected at the indicated time points. Protein levels of p73 and ΔNp73 were determined by immunoblotting with an anti-HA antibody.
We tested the effect of PPM1G on E3 ligase activity of WWP2. Our *in vivo* ubiquitination experiment performed by treating the cells with the cadmium chloride (PPM1G inhibitor) revealed that PPM1G inactivates WWP2, as the inhibition of PPM1G resulted in enhanced auto-ubiquitination of WWP2 (Fig. 4.9a and Fig. 4.9b). On the other hand, PPM1G did not show any significant effect on the auto-ubiquitination activity of WWP1 (Fig. 4.9c and Fig. 4.9d).

**Figure 4.9.** PPM1G regulates the activity of WWP2 in cells. (a) Cells expressing SFB-WWP2 were either mock treated or treated with CdCl2. After 6 hrs. of MG132 treatment, WWP2 activity was assayed by detecting its auto-ubiquitination levels using anti-Flag antibody after pull-down with SBP beads. (b) Cells expressing SFB-WWP2 were either mock treated or treated with CdCl2. After 6 hrs. of MG132 treatment, WWP2 activity was assayed by detecting its auto-ubiquitination levels using anti-Ub antibody after pull-down with SBP beads. (c) Cells expressing SFB-WWP1 were either mock treated or treated with CdCl2. After 6 hrs. of MG132 treatment, WWP1 activity was assayed by detecting its auto-ubiquitination levels using anti-Flag antibody after pull-down with SBP beads. (d) Cells expressing SFB-
WWP1 were either mock treated or treated with CdCl2. After 6 hrs. of MG132 treatment, WWP1 activity was assayed by detecting its auto-ubiquitination levels using anti-Ub antibody after pull-down with SBP beads.

4.2.4 PPM1G regulates the apoptosis-controlling ability of WWP2

We examined the PPM1G effect on WWP2 function by testing the PPM1G effect on WWP2-controlled p73-mediated apoptosis. We found that WWP2 reduced the p73-induced cellular apoptosis, which is rescued by co-expression of PPM1G. The rescue effect of PPM1G is dependent on its catalytic activity as cadmium chloride treatment or transfection of a catalytically inactive PPM1G mutant reversed the apoptotic phenotype (Fig. 4.10a). Further, the depletion of PPM1G by siRNA reduced the ability of p73 to induce apoptosis (Fig. 4.10b). Together, these results suggest that PPM1G acts as a functional molecular switch that promotes the assembly of WWP2-WWP1 heterodimeric complex, and thus controls the balance between p73 and ΔNp73.
Figure 4.10. PPM1G regulates the WWP2 controlled apoptosis. (a) HeLa cells were transfected with various constructs as indicated, and the percentage of apoptosis was determined by propidium iodide staining followed by sub-G1 peak analysis using flow cytometry. Error bars indicate standard deviations (n=3, P < 0.01; Student’s t-test). (b) HeLa cells were transfected with either control siRNA or PPM1G siRNA. 24 hours later, cells were transfected with control vector, or p73 alone or p73 in along with WWP2 WT. The percentage of apoptosis was determined by Propidium Iodide staining followed by sub-G1 peak analysis by using flow cytometry. Error bars indicate standard deviation (n=3), P<0.01; students t-test.

As WWP2-WWP1 E3 ligase complex regulates the p73/ΔNp73 ratio during cisplatin-induced cellular stress, we examined PPM1G role in stress-induced cell death. In fact, cells became severely resistant to cisplatin-induced apoptosis with either pretreatment with the PPM1G inhibitor cadmium chloride (Fig. 4.11a) or siRNA-mediated depletion of PPM1G (Fig. 4.11b). Taken together, these results indicate that the inactivation of WWP2 by PPM1G during cellular stress leads to specific accumulation of p73, and inactive WWP2, with the help of PPM1G, heterodimerizes with WWP1 and destabilizes ΔNp73.
Figure 4.11. PPM1G regulates the WWP2 controlled apoptosis upon cellular stress. (a) HeLa cells were either mock treated or treated with cisplatin alone or together with CdCl₂, and the percentage of apoptosis was determined by Propidium Iodide staining followed by sub-G1 peak analysis by using flow cytometry. Error bars indicate standard deviation (n=3), P<0.01; students t-test. (b) HeLa cells were transfected with either control siRNA or two individual PPM1G siRNA. 48 hours later cells were left untreated or treated with cisplatin, and the percentage of apoptosis was determined by propidium iodide staining, followed by sub-G1 peak analysis.
using flow cytometry. Error bars indicate standard deviations (n=3, \( P<0.01 \); Student’s \( t \)-test).

In conclusion, we identified that WWP2 exists in monomeric and WWP2-WWP1 heterodimeric states under normal cellular conditions. However, upon cisplatin-induced cellular stress, WWP2 becomes inactive, thereby p73 is stabilized. Inactive WWP2 heterodimerizes with WWP1 and regulates \( \Delta \text{Np73} \). PPM1G serves as a molecular switch for WWP2 monomeric and WWP2-WWP1 heterodimeric states both under normal and cisplatin-induced stress conditions (Fig. 4.12).

**Figure 4.12. Proposed Model.** A proposed model to show the role of monomeric WWP2 and PPM1G-promoted WWP2-WWP1 heterodimeric complex in regulating p73 and \( \Delta \text{Np73} \) levels under normal and cisplatin-induced stress conditions.

**4.3 Conclusion and Discussion**

In our previous study (chapter 3), we found that WWP2 in monomeric form regulates the p73 protein stability and function, in contrast WWP2 in complex with WWP1 regulates the \( \Delta \text{Np73} \) protein stability and function. Also, the equilibrium between WWP2 monomeric and WWP2-WWP1 heterodimeric states is altered to stabilize p73.
upon cisplatin-induced DNA damage. Thus, the equilibrium between WWP2 monomeric and heterodimeric states is critical in regulating the p73/ΔNp73 ratio both in the normal and damage cells. However, the molecular mechanism that regulates WWP2 in these two states was not identified. We analyzed the WWP2 associated proteins obtained through mass spectrometry, we found PPM1G as a novel WWP2 associated protein. PPM1G is a Ser/Thr phosphatase that is known to regulate several cellular processes such as nucleosome assembly, cell survival control, mRNA splicing, and DNA damage response [322, 323, 326], but PPM1G role in tumorigenesis has not been reported so far. In our study, we found that PPM1G regulates the p73/ΔNp73 ratio in the cells by regulating the equilibrium of WWP2 in two distinct states; monomeric WWP2 and WWP2-WWP1 heterodimeric states. PPM1G inactivates WWP2 as evident by the enhanced auto-ubiquitination activity of WWP2 in the presence of PPM1G inhibitor. Interestingly, inactive WWP2 remains available in the cells to complex with WWP1. WWP2-WWP1 heterodimeric complex interacts, ubiquitinates and degrades ΔNp73. Also, we found that PPM1G regulates the cisplatin-induced apoptosis, as PPM1G knockdown confers resistance to cisplatin-induced cell death. The results suggest that PPM1G is required for p73-mediated apoptosis both in the normal and stress conditions. Since, our study identifies PPM1G as a potential tumor suppressor that functions by inhibiting the E3 ligase activity of WWP2, it would be interesting to identify the mechanism of regulation of E3 ligase activity of WWP2 by PPM1G. As PPM1G is a Ser/Thr phosphatase, it might serve as a phosphatase for WWP2. There are several potential Ser/Thr phosphorylation sites in WWP2 that may be tested as potential dephosphorylation sites for PPM1G (Phosphosite.org). Further, inactivation of E3 ubiquitin ligase activity of WWP2 by PPM1G suggests that PPM1G might serve as a strong tumor suppressor by stabilizing
the tumor suppressor substrates of WWP2 (PTEN, Smads and p73). Therefore, the effect of PPM1G is proposed to be cumulative effect of stabilization of WWP2 substrates in the cell. Also, PPM1G might affect other cellular functions by regulating the substrates of WWP2 in these processes. For example, WWP2 regulates the craniofacial development by monoubiquitinating the transcription factor Goosecoid [217]. The effect of PPM1G might be tested in regulating the WWP2 controlled cellular function and vice-versa. Also, PPM1G might serve exclusive functions in the cells, therefore, identification and characterization of PPM1G associated proteins in the cells would be an interesting work to pursue. For therapeutic purposes, it would be intriguing to screen the patient tumors harboring mutations in the PPM1G gene or protein and studying the correlation with other proteins such as WWP2 in such tumors to establish the molecular mechanisms of tumor development.