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Nuclear protein phosphatases in DNA damage response and other cellular functions

Význam jaderných fosfatáz v kontrole buněčné odpovědi na poškození DNA a další buněčné funkce

Bachelor´s thesis

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Declaration of authorship

I hereby declare that I am the sole author of this thesis and that I have mentioned all of the sources used. This thesis, neither its substantial part, has not been submitted in order to obtain any other academic title. During the writing process, artificial intelligence ChatGPT from OpenAI was used for aid with finding informational sources.

Prague, 29. 4. 2025 Jana Kuběnková

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Abstrakt

Buňky jsou neustále pod náporem genotoxických činidel pocházejících z různých zdrojů. Ty způsobují poškození DNA, které musí být odstraněno, aby byla zachována integrita genomu. Z toho důvodu jsou v buňkách aktivovány mechanismy opravy DNA, které jsou součástí komplexní sítě nazvané odpověď na poškození DNA (DDR), spolu se zapnutím kontrolních bodů buněčného cyklu a dalších procesů. Fosforylace proteinů je nejdůležitějším mechanismem, skrze který DDR kinázy zahajují a udržují odpověď aktivní. Nicméně, když je poškození opraveno, tak musí být DDR utlumeno, aby mohl být obnoven normální průběh buněčného cyklu. Zde přicházejí na řadu proteinfosfatázy, které potlačují fosforylaci a zabraňují přenosu signálu.

Tato práce se soustředí na roli Ser/Thr fosfatáz z PPM rodiny, konkrétně na její 2 zástupce, PPM1D a PPM1G, v DDR. U PPM1D bylo prokázáno, že řídí zastavení DDR signalizace skrze defosforylaci hlavní ATM kinázy, kináz kontrolního bodu a p53. To vede k inhibici na p53 závislé expresi a obnovení buněčného cyklu. Působení PPM1G bylo, na druhou stranu, navrhnuto jak v aktivaci DDR prostřednictvím stabilizace p53, tak v ukončení pomocí defosforylace γH2AX. Nicméně, její role v těchto procesech nebyla dosud objasněna či potvrzena v žádné nové studii. Kromě toho PPM1G byly i přisouzeny další funkce mimo DDR, a dokonce v cytoplazmě. Tato práce proto především shrnuje a diskutuje její navrhnuté role v DDR a z části také mimo ni.

Klíčová slova: fosfatáza, PPM1G, odpověď na poškození DNA, p53, chromatin

Abstract

Cells are constantly under the strain of genotoxic agents coming from various sources. They cause DNA lesions, which must be eliminated to preserve genome integrity. Cells, thus, induce DNA repair mechanisms, which are part of a complex network called DNA damage response (DDR), together with activation of cell cycle checkpoints. Protein phosphorylation is the most important mechanism through which DDR kinases initiate and maintain the DDR active. However, when damage is resolved, the DDR must be attenuated to restore the normal cell cycle. There comes the role of protein phosphatases, which counteract the phosphorylation and inhibit signalling.

This work focuses on the role of Ser/Thr phosphatases of the PPM family, specifically its 2 members, PPM1D and PPM1G, in the DDR. PPM1D has been shown to direct attenuation of the DDR signalling, regarding dephosphorylation of master ATM kinase, checkpoint kinases or p53. This leads to abrogation of p53-dependent expression and of cell cycle arrest. PPM1G, on the other hand, has been suggested to take part both in activating the DDR, through stabilising p53, and downregulating it by dephosphorylating γH2AX. However, its role in these processes has not yet been elucidated or confirmed in any newer study. Moreover, different roles outside the DDR and even in the cytoplasm were proposed. This work thus reviews and discusses primarily its proposed roles in DDR and briefly the other ones.

Keywords: phosphatase, PPM1G, DNA damage response, p53, chromatin

Abbreviations

3D – three-dimensional

4E-BP1 – 4E binding protein 1

53BP1 – p53-binding protein 1

Å – Ångström

ARF-BP1 – ARF-binding protein 1

ATM – ataxia telangiectasia mutated

ATR – ATM and Rad3-related

ATRIP – ATR-interacting protein

BARD1 – BRCA1-associated RING domain protein 1

BER – base excision repair

BRCA1 – breast cancer type 1 susceptibility protein

BRCA2 – breast cancer type 2 susceptibility protein

CDK1 – cyclin-dependent kinase 1

CDK2 – cyclin-dependent kinase 2

CDK9 – cyclin-dependent kinase 9

Chk1 – checkpoint kinase 1

Chk2 – checkpoint kinase 2

CK2 – casein kinase 2

cNHEJ – classical non-homologous end-joining

DDR – DNA damage response

DNA – deoxyribonucleic acid

DNA-PK – DNA-dependent protein kinase

DNA-PKcs – DNA-PK catalytic subunit

DSB – double-strand break

DSIF – 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole sensitivity-inducing factor

eIF4E – eukaryotic translation initiation factor 4E

Exo1 – exonuclease 1

Fig. – figure

GOF – gain-of-function

HCC – hepatocellular carcinoma

HEXIM1 – hexamethylene bisacetamide-inducible protein 1

HEXIM2 – hexamethylene bisacetamide-inducible protein 2

HIV-1 – human immunodeficiency virus 1

HR – homologous recombination

HUWE1 – HECT, UBA and WWE domain-containing E3 ubiquitin-protein ligase 1

IR – ionising radiation

KAP1 – KRAB-associated protein 1

LARP7 – La-related protein 7

LIG4 – DNA ligase IV

MAPK – mitogen-activated protein kinase

MDM2 – mouse double minute 2 homolog

MePCE – 7SK snRNA methylphosphate capping enzyme

MRE11 – meiotic recombination 11 homolog 1

MRN – MRE11-RAD50-NBS1

mRNA – messenger RNA

Mule – Mcl-1 ubiquitin ligase E3

NAP1L1 – nucleosome assembly protein 1-like 1

NAP1L4 – nucleosome assembly protein 1-like 4

NELF – negative elongation factor

NER – nucleotide excision repair

NF-κB – nuclear factor κB

NLS – nuclear localisation signal

p53 – tumour protein 53

PDP – pyruvate dehydrogenase phosphatase

PIKK – phosphatidylinositol 3-kinase-related kinase

Pol II – RNA polymerase II

PP2A – protein phosphatase 2A

PPM – metal-dependent protein phosphatase

PPM1A – protein phosphatase magnesium-dependent 1A

PPM1D – protein phosphatase magnesium-dependent 1D

PPM1G – protein phosphatase magnesium-dependent 1G

PPM1K – protein phosphatase magnesium-dependent 1K

PPM1L – protein phosphatase magnesium-dependent 1L

PPP – phosphoprotein phosphatase

pre-mRNA – precursor mRNA

pS and **pSer** – phosphoserine

pT and **pThr** – phosphothreonine

P-TEFb – positive transcription elongation factor b

PTM – post-translational modification

pY and **pTyr** – phosphotyrosine

RNA – ribonucleic acid

ROS – reactive oxygen species

RPA – replication protein A

SDS – sodium dodecyl sulphate

SDSA – synthesis-dependent strand annealing

siRNA – small interfering RNA

snRNP – small nuclear ribonucleoprotein particle

SRSF3 – serine/arginine-rich splicing factor 3

ssDNA – single-strand DNA

TBL1X – transducing β-like protein 1X

USP7 – ubiquitin-specific-processing protease 7

UTR – untranslated region

UV – ultraviolet

Wip1 – wild-type p53-induced phosphatase

XLF – XRCC4-like factor

XRCC4 – X-ray repair cross-complementing protein

XRCC6/5 – X-ray repair cross-complementing protein 6/5

YB-1 – Y-box binding protein 1

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1. Introduction

Post-translational modifications (PTMs) of proteins play a crucial role in the signalling and controlling cellular responses. Phosphorylation is one of the most common PTMs, which mediates signal transduction. Other frequent and important types of PTMs include acetylation, methylation, hydroxylation, glycosylation, ubiquitination and SUMOylation (Khoury et al., 2011). They also participate in the regulation of a wide variety of signalling pathways and influence protein activity, folding and stability.

Phosphorylation usually occurs on serine, threonine, tyrosine and histidine amino acid residues, from which serine and threonine comprise over 98 % of all phosphorylated sites (Olsen *et al.*, 2006). In the minority, lysine, arginine, cysteine, or amino acids with acidic side chains can be phosphorylated as well (Hardman *et al.*, 2019) but their function has not yet been well examined. Phosphorylation is catalysed by kinases. There are more than 500 genes in the human genome for protein kinases, suggesting their importance and substrate specificity (Manning et al., 2002). Protein phosphatases work as counteracting enzymes, removing the phosphate group. Together, protein kinases and phosphatases provide the fine-tuning of cellular responses.

Phosphorylation is essential for the DNA damage response (DDR), since it is initiated by three master protein kinases (Falck et al., 2005). They phosphorylate dozens of downstream effectors, leading to an effective response to the genotoxic stress. The DDR features DNA repair mechanisms, cell cycle checkpoints, damage tolerance processes and tumour-suppressing mechanisms like senescence or apoptosis. However, when DNA lesions are resolved, the DDR signalling has to be switched off through dephosphorylation by protein phosphatases and other attenuating processes, like proteasomal degradation.

Over the years, many phosphatases have been found to participate in the DDR. This work examines two nuclear metal-dependent protein phosphatases, PPM1D and PPM1G, and their role in the DDR. PPM1D is well known for its functions in attenuating master kinases' activities, p53-dependent transcription and cell cycle arrest. Its role in the DDR has been well established and reviewed several times. Because of that, the main focus of this work will be dedicated to PPM1G, which was suggested to participate in a couple of processes connected to the DDR and other cellular mechanisms outside of the DDR, as well.

2. DNA damage response

Every day, cells have to tackle the problem of genomic instability because of tens of thousands DNA lesions that arise from either extrinsic, such as ionising radiation (IR), ultraviolet (UV) light and genotoxic chemicals, or intrinsic causes, including reactive oxygen species (ROS) coming from respiration, spontaneous hydrolysis of nucleotides and even metabolic processes of DNA itself (Lindahl and Nyberg, 1972). For normal cell functions, these genomic insults must be cleared away. Otherwise, replication over damaged sites will cause mutations. Eventually, these mutations can accumulate and cause either loss or gain of function in affected proteins. If damage sets in and impairs the function of tumour suppressor genes or protooncogenes, it may result in a cancerous growth. To detect and resolve DNA lesions, our cells have evolved a global signalling network named the DNA damage response (DDR).

According to the damage type, different repair mechanisms can be set into action. Minor chemical modifications of DNA's bases are targeted by the base excision repair system (BER). The nucleotide excision repair (NER) is reserved for single-strand defects that cause a more severe disruption of the DNA structure. In both instances, lesions can be removed flawlessly with the help of a complementary strand, which serves as a template. If, however, damage affects both strands, for example, the double-strand breaks (DSBs) caused by IR, more effective mechanisms must be exploited as these lesions are highly cytotoxic and harder to repair. Usually, one of the most common repair mechanisms for DSBs, the homologous recombination (HR) or the classical non-homologous end-joining (cNHEJ), would be chosen (depicted in **Fig. 1**).

2.1. DNA double-strand break signalling

In response to the DSBs, the DDR cascade is directed by three serine/threonine (Ser/Thr) protein kinases from the phosphatidylinositol 3-kinase-related kinase (PIKK) family (see **Fig. 2**) (Falck et al., 2005). Recognition of the DSBs is facilitated through the MRE11-RAD50-NBS1 (MRN) protein complex. This complex then recruits and activates ataxia telangiectasia mutated (ATM) protein kinase at the damaged sites (Lee and Paull, 2005). When the DSBs are processed by resection of the DNA ends or because of the stalled replication forks on the DNA-damaged sites, single-strand DNA (ssDNA) sections appear

(Byun *et al.*, 2005; Jazayeri *et al.*, 2006). ssDNA is then coated by replication proteins A (RPAs). The ssDNA-RPA complex associates with ATR-interacting protein (ATRIP) and recruits the second important protein kinase, the ATM and Rad3-related (ATR) kinase (Zou and Elledge, 2003). The last kinase from the PIKK family that responds to the DSBs is DNA-dependent protein kinase (DNA-PK). It is a holoenzyme of DNA-PK catalytic subunit (DNA-PKcs), which is recruited by the Ku70/80 heterodimer (Gottlieb and Jackson, 1993).

Moreover, recognition of the DNA ends at the DSBs by Ku70/80 (also known as X-ray repair cross-complementing protein 6/5 [XRCC6/5]) and assembly of DNA-PK is an initiating step in the cNHEJ repair pathway (see **Fig. 1A**). It tethers the ends together (Graham et al., 2016) and facilitates the recruitment of end-processing enzymes, like Artemis nuclease (Ma *et al.*, 2002), and the ligation complex XRCC4-XLF-LIG4, comprised of DNA ligase IV (LIG4) and scaffolding subunits X-ray repair cross-complementing protein 4 (XRCC4) and XRCC4-like factor (XLF), which will reseal the break (Nick McElhinny et al., 2000; Ahnesorg et al., 2006). The cNHEJ can be quickly utilised throughout the cell cycle, however, during S or G2 phase, when sister chromatids are synthesised, a more error-free HR pathway may be chosen (Beucher *et al.*, 2009; Karanam *et al.*, 2012).

The HR starts with meiotic recombination 11 homolog 1 (MRE11) nuclease of the MRN complex and exonuclease 1 (Exo1) executing the resection of DNA ends, which displaces Ku70/80 and creates long 3' ssDNA tails (Garcia *et al.*, 2011; Langerak *et al.*, 2011). ssDNA is immediately covered by the RPA proteins that protect DNA and hinder the formation of secondary structures (Chen et al., 2013). However, it also blocks the formation of Rad51 nucleoprotein filament, which is important for the pairing of homologous chromatids and the creation of the displacement loop (Mazina and Mazin, 2004). Because of that, it then has to be replaced by recombinase Rad51 through binding of breast cancer type 2 susceptibility protein (BRCA2) (Jensen et al., 2010). Possible outcomes of the HR are shown in **Fig. 1B**, with non-crossover synthesis-dependent strand annealing (SDSA) being the most common one.

Whether the break is repaired by the cNHEJ or the HR depends not only on the cell cycle phase but also on the DNA ends' structure, time and proteins present (reviewed in Scully et al., 2019). An important regulator is, for example, p53-binding protein 1 (53BP1), since it promotes the cNHEJ and inhibits the HR by suppressing end resection (Bunting *et al.*,

2010). The antagonising role has a complex of breast cancer type 1 susceptibility protein (BRCA1) and BRCA1-associated RING domain protein 1 (BARD1), as it supports DNA repair by the HR. BRCA1-BARD1 ubiquitinates histone H2A, which leads to repositioning of the 53BP1 factor from the DSB sites and enables resection of the ends (Densham *et al.*, 2016). The BRCA1-BARD1 complex is also involved in other subsequent steps of the HR, for example, in the homology search of Rad51-ssDNA (see **Fig. 1B**) (Zhao *et al.*, 2017).

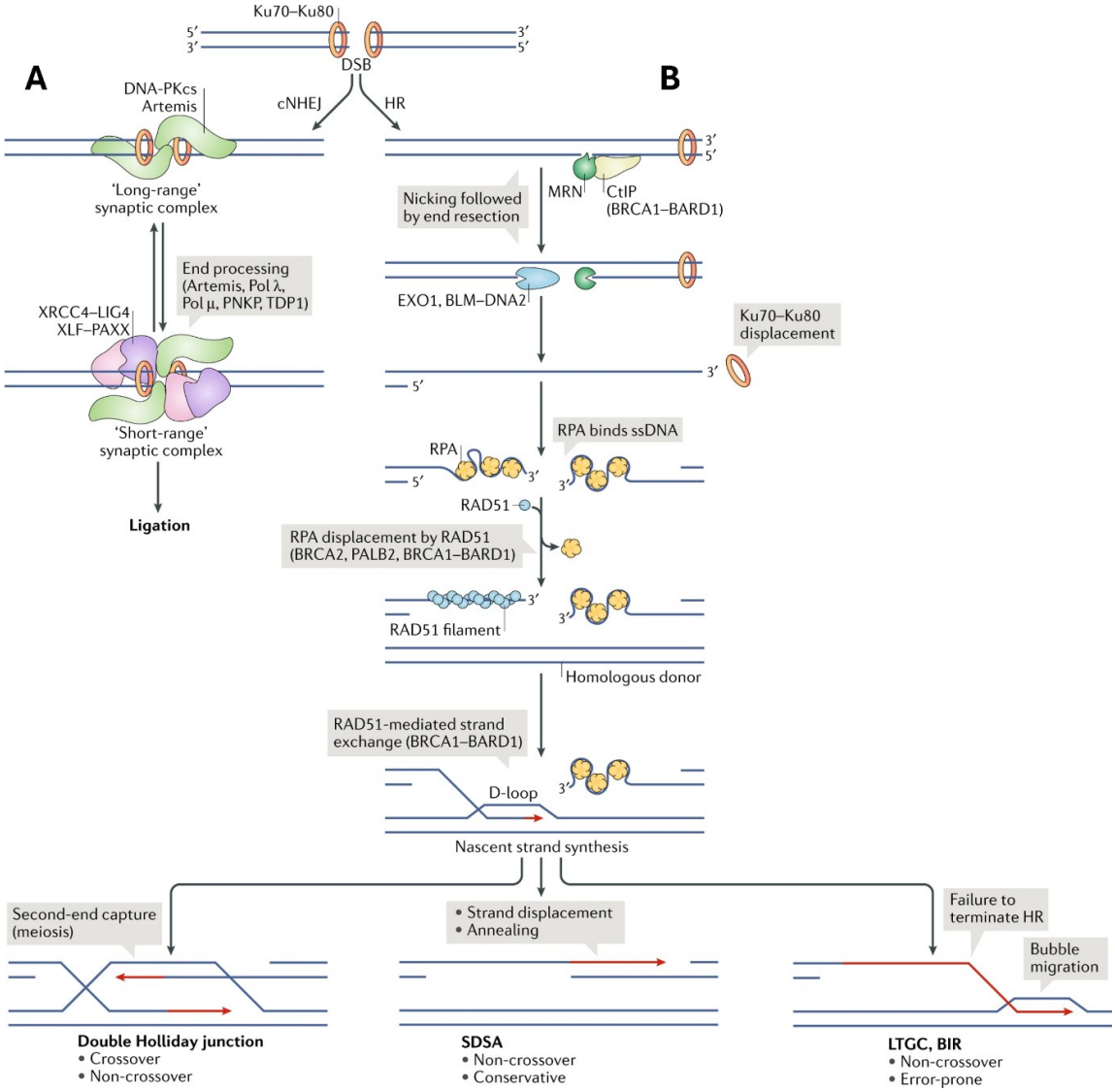


Figure 1. The two main pathways of DNA double-strand break repair – the classical non-homologous end joining (A) and the homologous recombination (B). Individual steps proteins' roles are described in the text. Adapted from Scully et al., 2019, edited.

At the site of DSBs, ATM, ATR and DNA-PK rapidly phosphorylate a histone H2AX, a rare variant of a histone H2A, at Ser139 (referred to as γH2AX) (Ward and Chen, 2001; Stiff *et al.*, 2004). This phosphorylation stretches a few megabases around the DNA lesion (Rogakou *et al.*, 1999). Because of that, γH2AX foci are generally used as DNA damage

markers. γH2AX facilitates the recruitment of some of the proteins involved in repair and checkpoint signalling to the DSB sites (Celeste *et al.*, 2003). Each of the kinases conducts signal further via phosphorylation of their downstream effectors, which can be either chromatin-bound or soluble, so they can diffuse throughout the nucleus.

ATM and ATR can together phosphorylate over 700 proteins (Matsuoka *et al.*, 2007), mostly at their SQ and TQ consensus sites (Kim *et al.*, 1999). Among their substrates are both proteins involved in the DSB repair, like 53BP1 or BRCA1, and the checkpoint activation (Matsuoka *et al.*, 2007), which simultaneously ensures the cell cycle arrest to provide enough time for the repair mechanisms. Besides, if the damage cannot be cleared or is too extensive, induction of senescence or even apoptosis will be set off to prevent tumorigenesis.

The cell cycle arrest is mainly mediated by three DDR effectors, checkpoint kinase 1 (Chk1), checkpoint kinase 2 (Chk2) and p21 (see **Fig. 2**). Chk1 is one of the well-known substrates of ATR, which phosphorylates it at Ser317 and Ser345 (Zhao and Piwnica-Worms, 2001). Active Chk1 can induce cell cycle arrest through several different pathways. It phosphorylates dual specificity phosphatase Cdc25A (Zhao et al., 2002), which targets it for the proteasomal degradation and thereby prevents cyclin-dependent kinase 2 (CDK2) activity and G1/S phase transition (Mailand *et al.*, 2000). Likewise, Chk1 phosphorylates dual specificity phosphatase Cdc25C, which leads to its nuclear export facilitated by 14-3-3 protein. Because of that, cyclin-dependent kinase 1 (CDK1) cannot be activated and the cell arrests at the G2 checkpoint (Peng *et al.*, 1997).

Chk2 is activated through phosphorylation at Thr68 by ATM (Ahn *et al.*, 2000; Matsuoka *et al.*, 2000). Phosphorylated Chk2 seems to promote G1/S phase arrest in the same ways as Chk1, through the phosphorylation of Cdc25A (Falck *et al.*, 2001). However, even though it targets many of the Chk1 phosphorylation sites, it cannot support Cdc25A degradation on its own (Jin *et al.*, 2008). Chk2 also phosphorylates Cdc25C and thereby prevents transition into mitosis (Matsuoka et al., 1998).

Altogether, ATM, Chk1 and Chk2 also influence the activity of one of the best-known tumour suppressors, the transcription factor tumour protein 53 (p53) (shown in **Fig. 2**). ATM-dependent phosphorylation at Ser15 and Ser20 (Banin *et al.*, 1998; Chehab *et al.*,

2000) reduces p53 interaction with mouse double minute 2 homolog (MDM2), the E3 ubiquitin-protein ligase (Shieh *et al.*, 1997; Unger *et al.*, 1999), which expression is stimulated by p53 (Barak *et al.*, 1993). Because of that, pSer15 and pSer20 impair the negative feedback loop between p53 and MDM2 and abrogate p53 ubiquitination and the proteasomal degradation. Checkpoint kinases can also phosphorylate p53 on multiple sites, including these two serines, and regulate its stability and activity through that (Shieh *et al.*, 2000; Ou *et al.*, 2005).

When p53 accumulates, it can activate transcription of its dependent genes. One of them is the *CDKN1A* gene coding a checkpoint regulator p21, also known as the cyclin-dependent kinase inhibitor 1 (El-Deiry *et al.*, 1994). p21 prevents cells from transitioning to the S phase through inhibition of the corresponding CDKs (Harper *et al.*, 1993). p53 and p21 are critical for a long-term arrest in the G2 phase, even though they are not necessary for activation of the G2/M checkpoint (Bunz *et al.*, 1998). If repair pathways fail, p53 can also induce apoptosis (Chipuk *et al.*, 2004).

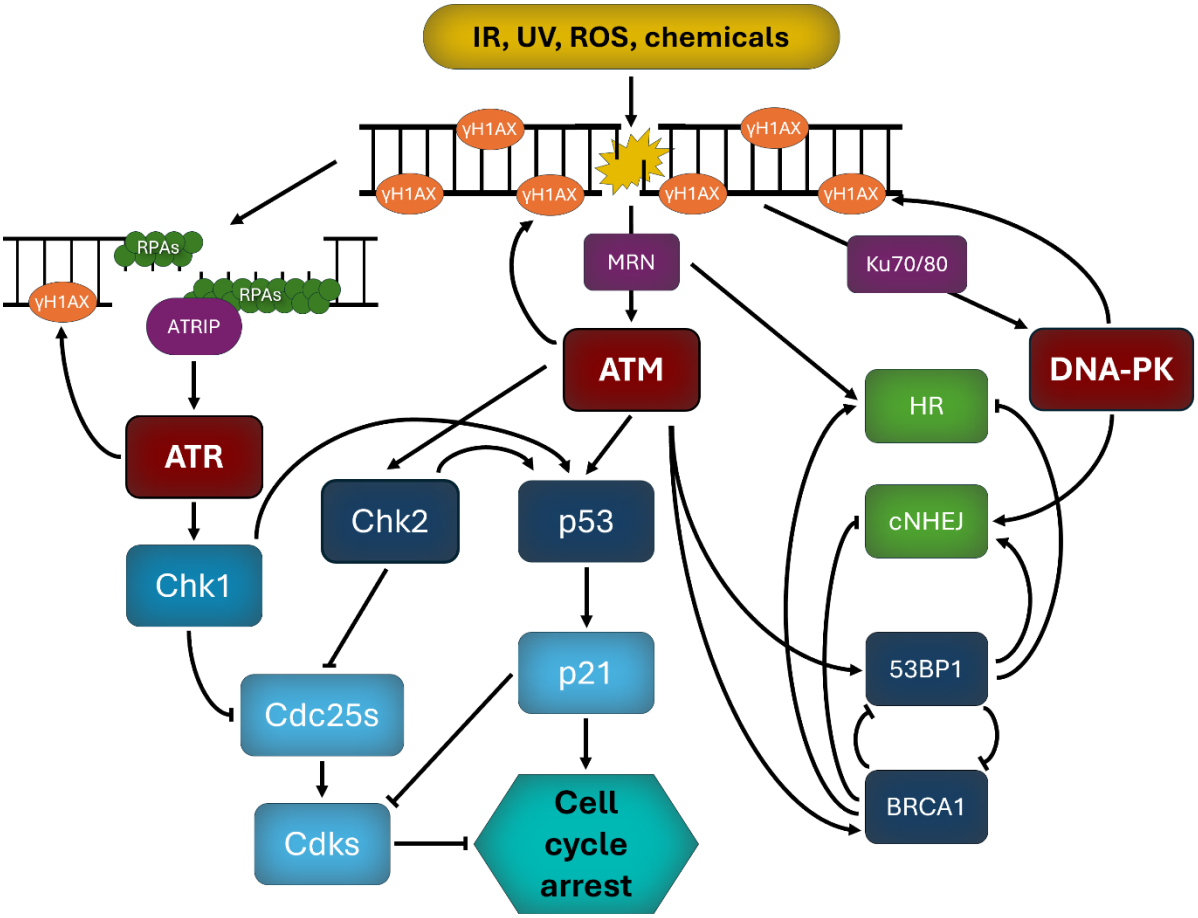


Figure 2. The basics of the DNA damage response signalling network to the double-strand breaks. A more detailed description is available in the text.

3. Protein phosphatases

In the past, the main focus was on the research of protein kinases' structure and functions. Nevertheless, more attention has recently been paid to protein phosphatases since their function extends beyond simply switching off phosphorylation events, but they significantly influence the duration and dynamics of signal transduction (Heinrich et al., 2002). In contrast to more than 500 genes for protein kinases, there are only 189 genes in the human genome for protein phosphatases (Chen et al., 2017). Out of them, only about 40 encode protein phosphatases that dephosphorylate phosphoserines (pS) or phosphothreonines (pT), although the vast majority of phosphorylated sites are at these amino acids.

Human Ser/Thr protein phosphatases are constituted primarily of two families, distinguished by the primary structure and catalytic mechanism: phosphoprotein phosphatases (PPPs), consisting of 13 members, and metal-dependent protein phosphatases (PPMs), which are composed of 20 enzymes (Chen et al., 2017). Despite the low number of unique catalytic subunits of phosphatases, the substrate specificity can be achieved in a different way. The PPPs can create protein complexes with scaffold and regulatory subunits, determining their subcellular localisation and substrate recognition (Brautigan and Shenolikar, 2018). Whereas phosphatases of the PPM family utilise regulatory and targeting domains in their structure.

3.1. Metal-dependent protein phosphatases

Phosphatases of the PPM family have a highly conserved core domain with residues essential for catalysis. The structural study of human PPM1A showed that the catalytic core consists of two anti-parallel β-sheets, creating a β-sandwich, and two pairs of anti-parallel α-helices surrounding them. β-sheets and α-helices are joined together by loops with phosphatase-specific features (Das *et al.*, 1996). Three divalent metal-binding sites represented by glutamic and aspartic acid residues are part of this core. Out of them, the third one binds with a lower affinity (Tanoue *et al.*, 2013). Bound metals then coordinate with water molecules, and the outer layer electrons of metals act through increasing the negative charge of the water and promoting S_N2 nucleophilic attack on the phosphorus of the phosphorylated protein. That is why the activity of PPMs is metal-dependent.

Binding sites chelate Mg^{2+}, Mn^{2+} and other divalent metal ions, like Fe^{2+}, Ni^{2+} or Co^{2+}, which efficiently promote PPMs' catalytic activity. On the other hand, some metals can work as inhibitors. This is especially the case of Ca^{2+} and Cd^{2+}, which share the same binding sites with activatory metals and work through the mechanism of competitive inhibition (Pan *et al.*, 2013; Debnath *et al.*, 2018). Apart from these conserved metal-chelating residues, several glycine, one glutamic acid and two arginine residues are conserved in almost all human PPM. They lie in the vicinity of the catalytic domain and are supposed to be important in the stabilisation of the core domain structure (Kamada et al., 2020).

The PPMs, except for pyruvate dehydrogenase phosphatases (PDPs), function as single-subunit enzymes in contrast to the PPPs (Yan et al., 1996). However, each phosphatase contains unique regulatory and targeting sequences in its structure in the N- and C-terminal parts or catalytic loops, to be specific. These domains are important for their regulation, subcellular localisation and substrate specificity. PPM1K and phosphates of the PDP subfamily are localised in the mitochondria with a mitochondrial targeting sequence at their N-terminal (Huang *et al.*, 1998; G. Lu *et al.*, 2007). PPM1L have a transmembrane domain in its N-terminal region, which directs it into the membrane of the endoplasmic reticulum (Saito *et al.*, 2008). A nuclear targeting signal was predicted in a sequence of many PPMs, as well (Petri *et al.*, 2007; Chuman *et al.*, 2009; Zhou *et al.*, 2013; Osawa *et al.*, 2024). Out of them, PPM1D and PPM1G were characterised to have an important role in the DDR signalling and will be further described in this work with emphasis on PPM1G.

3.1.1. PPM1D

The *PPM1D* gene, located on the longer arm of human chromosome 17 (Dyer *et al.*, 2025), encodes a 605 amino acids long enzyme (Fiscella *et al.*, 1997). Except for the full-length protein, there is also a shorter alternative splicing variant 430 amino acids long, synthesised from messenger RNA (mRNA) with a 111 bp insertion containing a stop codon. It includes a conserved catalytic domain (1–420 residues) and an additional 10 residues specific to the PPM1D430 variant (Chuman *et al.*, 2009). The protein phosphatase magnesium-dependent 1D (PPM1D), originally named wild-type p53-induced phosphatase 1 (Wip1), consists of the conserved catalytic core (see

Fig. 3A/C/D) with two PPM1D-specific segments, B-loop and P-loop (in **Fig. 3A/D**), and a distinctive C-terminal extension. The B-loop, composed mostly of basic amino acids, and the proline-rich P-loop are located inside the sequence of the catalytic domain but stretch out of it in a tertiary structure (Chuman *et al.*, 2008).

Kumar *et al.* have recently carried out a study in which they have accomplished to produce high-quality crystals and, by X-ray diffraction, created an experimental three-dimensional (3D) model of the PPM1D's catalytic domain, which can be seen in **Fig. 3A** (Kumar *et al.*, 2024). The resolution of the structure was 1.8 Å. All amino acid residues were in the preferred or allowed regions of the Ramachandran diagram with no outliers, indicating a good quality of the model. In order to obtain the crystallisable protein, several residues had to be substituted in the catalytic domain and two regions were deleted to allow sufficient concentration and prevent aggregation. The two deleted regions were part of a flexible P-loop and an unstructured C-terminal segment. SUMOstar fusion tag was also added to the N-terminal of PPM1D to increase protein solubility (Kumar *et al.*, 2024).

The organisation of the observed catalytic core corresponded to the conserved cores of other PPM phosphatases with resolved 3D structures (see structural alignment with PPM1A in **Fig. 3C**). It is composed of a β-sandwich from two five-stranded antiparallel β-sheets with two and three antiparallel α-helices flanking the β-sandwich from each side. Three metal-binding sites, M1, M2 and M3, were recognised in the structure of the catalytic domain of PPM1D. For coordination of metal to the M1 site, carboxylate oxygens of three aspartic acids (Asp105, Asp314 and Asp366) and three water molecules are required. M2 metal ion is bound through the backbone carbonyl of Gly106, carboxylate oxygen of Asp105 and four molecules of water. Glu22, Asp23, Asp366 and Asn367 support the binding of metal to the M2 site by interacting with the water molecules (Kumar *et al.*, 2024). The structure was not crystallised with the third metal bound. Nonetheless, the M3 site is known to bind with a lower affinity and carboxylate oxygens of two aspartic acid residues (Asp192 and Asp314) are needed for a metal chelation (Tanoue *et al.*, 2013).

Inside the catalytic domain, a 76 residues long flap subdomain can be found. It stretches from Pro219 to Asp295 and consists of two short α-helices and three β-strands (see **Fig. 3A/D**). Part of the flap subdomain is the PPM1D-specific B-loop (Kumar *et al.*, 2024). B-loop contains one of PPM1D's nuclear localisation signals (NLSs) (Chuman *et al.*, 2008)

and its basic amino acid residues were suggested to play a crucial role in the recognition of substrates (Hayashi *et al.*, 2011). The other NLS is situated outside of the catalytic domain in the C-terminal domain, which is not included in 3D model and alignments in

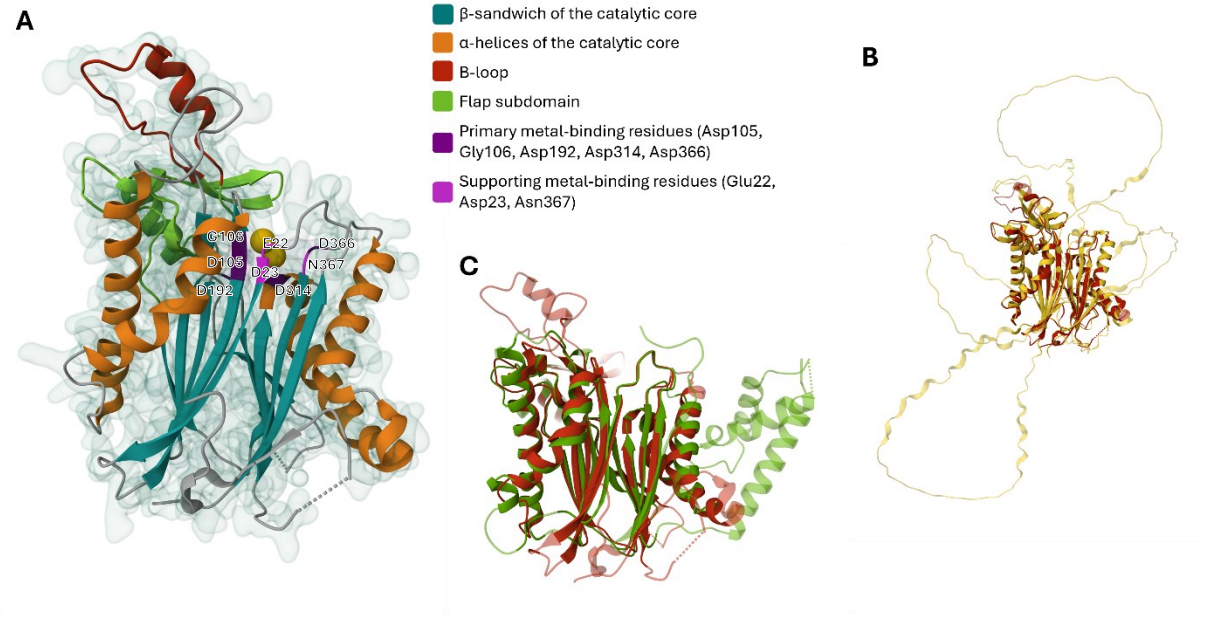


Figure 3. Structure and alignments of PPM1D with PPM1A. A) The model of crystalised PPM1D created by Kumar *et al.* is shown as a cartoon and a Gaussian volume. β-sandwich and α-helices of the catalytic core are coloured cyan and orange, respectively. The flap subdomain is shown in green and the B-loop in red. Two magnesium ions are displayed as yellow spheres. Primary and supporting metal-binding residues are labelled and coloured in purple and magenta, respectively. B) Structural alignment of the new 3D crystal model and the AlphaFold model (Jumper *et al.*, 2021; Varadi *et al.*, 2024) of PPM1D showing the prediction quality of the catalytic domain. The crystal model is shown in red and AlphaFold in yellow. C) Structural alignment of the crystal model of PPM1D and PPM1A (RCSB PDB Entry ID: 1A6Q; Das *et al.*, 1996). PPM1D is shown in red and PPM1A in green. D) Pairwise sequence alignment of PPM1D and PPM1A. In grey are highlighted conserved amino acids between PPM1D and PPM1A, in red are primary conserved metal-binding residues and in magenta are the supporting ones. In blue is the flap subdomain disrupted by the B-loop in yellow. In green is the P-loop, and Lys336 with Cys346 forming the NOS bridge are coloured orange. Inspired by Tanoue *et al.*, 2013 and Kamada et al., 2020, the first few amino acids and the C-terminals were excluded as they are not highly conserved. The structural alignment was done using the TM-align method from the RCSB PDB Pairwise Structure Alignment tool (Bittrich *et al.*, 2024) and the pairwise sequence alignment by EMBOSS Needle using BLOSUM62 from EMBL-EBI (Madeira *et al.*, 2024).

Fig. 3 (Chuman *et al.*, 2009). A covalent crosslink through a nitrogen-oxygen-sulphur, the NOS bridge, has been identified between Lys336 and Cys346 in the PPM1D structure. It forms under oxidative conditions and appears to rigidify the conformation, therefore, it may work to modulate and preserve catalytic activity (Kumar *et al.*, 2024). The active site of PPM1D has a higher electronegativity because of the number of acidic residues, like aspartic and glutamic acid. The positively charged metal ions and side chains of arginine and lysine residues work as neutralising agents, creating positive electrostatic potential, which is important for substrate binding (Yamaguchi *et al.*, 2007).

PPM1D preferentially recognises and dephosphorylates two sequence motifs, pS/pTQ and pTXY, with a higher affinity for diphosphorylated pTXpY peptides (Yamaguchi *et al.*, 2005, 2007). Acidic residues surrounding these consensus sequences enhance the catalytic activity of PPM1D through better binding because of the electrostatic interaction of negatively and positively charged residues in both proteins (Yamaguchi *et al.*, 2007; Chuman *et al.*, 2008). pTXpY dephosphorylation site can be found in the p38 mitogen-activated protein kinases (MAPKs). It is part of stress signalling in response to cytokines, UV or IR and influences p53 activation via phosphorylation at Ser33 and Ser46 (Bulavin *et al.*, 1999). S/TQ motifs are phosphorylated by ATM/ATR/DNA-PK kinases from the PIKK family, which play a critical role in initiating the DDR, as mentioned earlier.

PPM1D is one of the major phosphatases in the DDR and works to downregulate the signalling. Through the dephosphorylation of its substrates, it abrogates the cell cycle checkpoints and restores the homeostatic state. The basal expression levels of PPM1D are low since the transcription is induced in response to genotoxic stress in a p53-dependent manner (Fiscella *et al.*, 1997). The *PPM1D* gene possesses a conserved p53 response element in the promoter in the 5' untranslated region (UTR) (Rossi *et al.*, 2008). Until this date, many proven and putative substrates have been discovered (Gräf *et al.*, 2022). Among them, the ones with the highest relevance for attenuating the DDR are ATM, p53, MDM2, Chk1, Chk2, γH2AX and KAP1, as shown in **Fig. 4**.

p53 is directly dephosphorylated at Ser15 by PPM1D (Lu et al., 2005), which enables interaction with MDM2, leading to ubiquitination and degradation of p53 (Haupt *et al.*, 1997). Moreover, MDM2 is a substrate of PPM1D, as well. PPM1D stabilises MDM2 via dephosphorylation of Ser395 and prevents its autoubiquitination through enhanced

interaction of ubiquitin-specific-processing protease 7 (USP7) with MDM2 (X. Lu *et al.*, 2007). PPM1D can suppress the p53-dependent pathway in many ways. Either directly through promoting the negative feedback loop of p53-MDM2, as said, or indirectly through inhibition of other upstream DDR effector proteins, which stabilise p53 (see **Fig. 2** and **Fig. 4**). These actions of PPM1D mediate the oscillation of p53 levels during the response to the genotoxic stress (Batchelor *et al.*, 2008).

When ATM is recruited to the sites of DSBs by the MRN complex, it dissociates from its dimer, which holds it in an inactive state, and undergoes autophosphorylation at Ser1981 (Bakkenist and Kastan, 2003). Active ATM then initiates the DDR cascade events. PPM1D targets this phosphoserine and dephosphorylates it, thereby inactivating the master upstream kinase of the DDR and its subsequent effects (Shreeram *et al.*, 2006). As already mentioned, Chk1 and Chk2 get phosphorylated by ATR and ATM kinases, respectively.

Activated checkpoint kinases induce cell cycle checkpoints and further phosphorylate p53 at multiple sites (Shieh *et al.*, 2000; Ou *et al.*, 2005). Chk1 is specifically dephosphorylated at Ser345 by PPM1D, which reduces its catalytic activity and inhibits kinase function (Lu et al., 2005). PPM1D targets Chk2 at Thr68, which delays its oligomerisation and T-loop autophosphorylation (Oliva-Trastoy *et al.*, 2007). However, pThr387 located in the activating T-loop cannot be dephosphorylated by PPM1D, which indicates that already fully activated Chk2 might not be highly affected by PPM1D (Ahn and Prives, 2002; Oliva-Trastoy *et al.*, 2007).

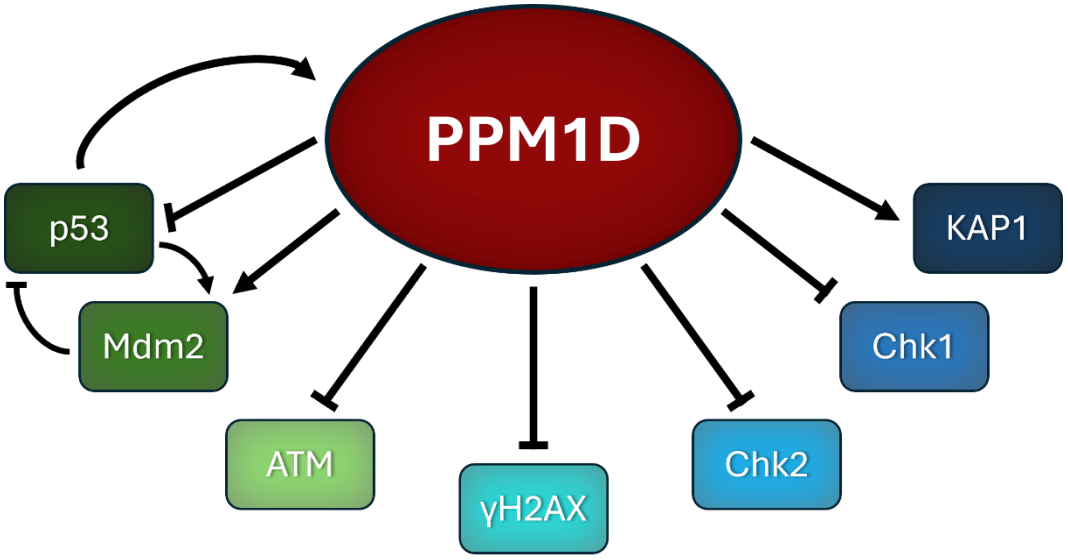


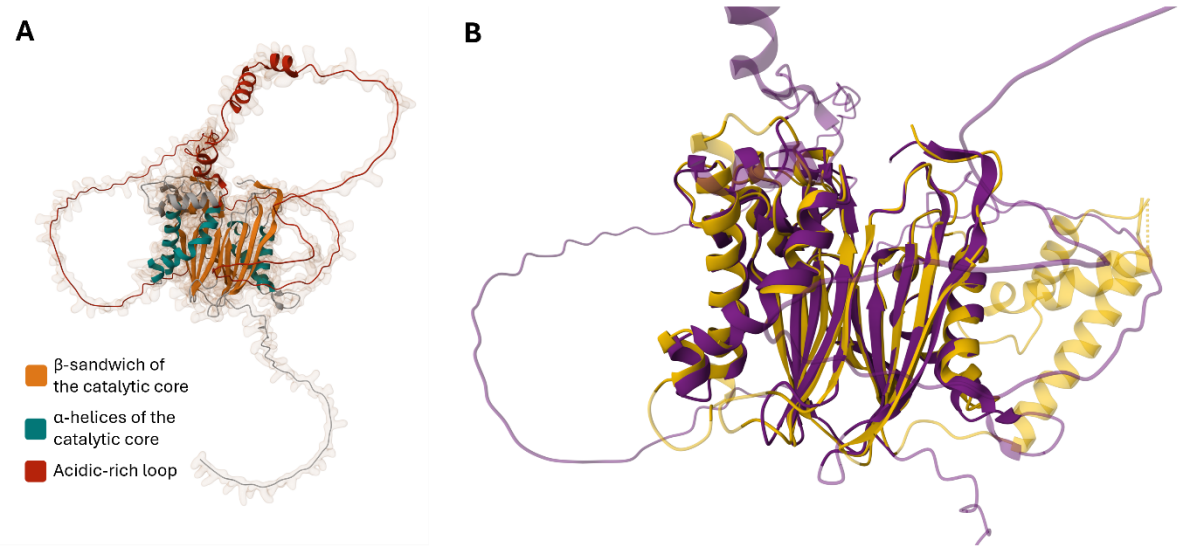
Figure 4. Substrates dephosphorylated by PPM1D with relevance in the DDR. Detailed information and functions of proteins are available in the text.

PPM1D has been shown to play a critical role in controlling cell cycle arrest in the G2 phase. Although PPM1D does dephosphorylate several checkpoint-inducing proteins, its main role is maintaining recovery competence. This is done through destabilising p53 (Lindqvist *et al.*, 2009), since p53, through the action of p21, is essential for sustaining G2 arrest (Bunz *et al.*, 1998). Besides that, PPM1D also determines G2 checkpoint length. It counteracts ATM-dependent phosphorylation of KRAB-associated protein 1 (KAP1) at Ser824, and dephosphorylated KAP1 promotes release from the cell cycle arrest, through repression of p21 expression (Li *et al.*, 2007; Jaiswal *et al.*, 2017). Additionally, PPM1D was shown to be tightly bound to chromatin and localised to DNA damage foci. There, it dephosphorylates γH2AX, reverting it back to its original state before DNA lesions appeared and thus attenuates the DSB repair and the checkpoint signalling (Cha *et al.*, 2010; Macůrek *et al.*, 2010; Moon *et al.*, 2010).

3.1.2. PPM1G

Protein phosphatase magnesium-dependent 1G (PPM1G) is the second member of the PPM family with a reported role in the DDR. The human *PPM1G* gene is located on the shorter arm of chromosome 2 (Dyer *et al.*, 2025). The protein translated from this gene is 546 amino acids long (Travis and Welsh, 1997). PPM1G contains the conserved catalytic core typical for the phosphatases of the PPM family (see structural alignment in **Fig. 5B**). It shares approximately 34 % sequence identity with PPM1A (their alignment is shown in **Fig. 5C**) (Travis and Welsh, 1997), but have all conserved metal-binding residues, namely Glu40, Asp41, Asp60, Gly61, Asp441 and Asp496 (Murray et al., 1999), even the Asp348, corresponding to Asp146 or Asp192, implicated in the binding of the third metal in PPM1A or PPM1D, respectively (see **Fig. 5C**).

The two unique regions can be found in PPM1G (see **Fig. 5A/C**). One of them is a lysine-rich sequence located at the C-terminal (Gudipaty *et al.*, 2015). It also contains the predicted NLS sequence (Petri *et al.*, 2007), which corresponds with the fact that PPM1G was found to be predominantly localised inside the nucleus (Murray et al., 1999; Kumar et al., 2019). The second one is a long unique acidic-rich loop that interrupts the conserved catalytic domain and cannot be found in any of the other PPM phosphatases. It is approximately a 200 amino acids long region with a high concentration of aspartic



C

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PPM1A     1  MGAFLDKPKMEKHNAQGQGN---GLRYGLSSMQGWRVEMEDAHTAVIGLPSGLESWSFFAVYDGHAGSQVAKYCC   72
PPM1G     1  MGAYLSQPNTVKCSGDGVGAPRLPLPYGFSAMQGWRVSMEDAHNCIPELDS---ETAMFSVYDGHGGEEVALYCA   72
PPM1A    73  EHLLDHITNNQDFKGSAGAPSVENVKNGIRTGFLEIDEHM---RVMSE-----------------------------  117
PPM1G    73  KYLPDIIKDQKAYKEG-------KLQKALEDAFLAIDAKLTTEEVIKELAQIAGRPTEDEDEKEKVADEDDVDNE  140
PPM1A   117  -------------------------------------------------------------------------------  117
PPM1G   141  EAALLHEEATMTIEELLTRYGQNCHKGPPHSKSGGGTGEEPGSQGLNGEAGPEDSTRETPSQENGPTAKAYTGFS  215
PPM1A   117  -------------------------------------------------------------------------------  117
PPM1G   216  SNSERGTEAGQVGEPGIPTGEAGPSCSSASDKLPRVAKSKFFEDSEDESDEAEEEEEDSEECSEEEDGYSSEEAE  290
PPM1A   118  ------------------------------KKHGADRSGSTAVGVLISPQHTYFINCGDSRGLLCRNRKVHFFTQ  162
PPM1G   291  NEEDEDDTEEAEEDDEEEEEEEMMVPGMEGKEEPGSD-SGTTAVVALIRGKQLIVANAGDSRCVVSEAGKALDMSY  364
PPM1A   163  DHKPSNPLEKERIQNAGGSV-MIQRVNGSLAVSRALGDFDYKCVHGKGPTEQLVSPEPEVHDIERSEEDDQFIIL  236
PPM1G   365  DHKPEDEVELARIKNAGGKVTMDGRVNGGLNLSRAIGDHFYKRNKNLPPEEQMISALPDIKVLTLT-DDHEFMVI  438
PPM1A   237  ACDGIWDVMGNEELCDFVRSRLEVTDD------LEKVCNEVVDTCLYKGSR------DNMSVILICFP--NAPKV  297
PPM1G   439  ACDGIWNVMSSQEVVDFIQSKISQRDENGELRLLSSIVEELLDQCLAPDTSGDGTGCDNMTCIIICFKPRNTAEL  513
PPM1A   298  SPEAVKKEAELDKYLECRVEEIIKKQGEGVPDLVHVMRTLASENIPSLPPGGELASKRN   356(382)
PPM1G   514  QPESGKR----------KLEEVLSTEG-------------AEENGNS---DKKKKAKRD   546
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Figure 5. Structure and alignments of PPM1G with PPM1A. A) Predicted 3D structure of PPM1G by the AlphaFold method (Jumper *et al.*, 2021; Varadi *et al.*, 2024) shown as a cartoon and a Gaussian volume. β-sandwich and α-helices of the catalytic core are coloured orange and cyan, respectively. The acidic-rich loop is shown in red. B) Structural alignment of predicted structure of PPM1G and PPM1A (RCSB PDB Entry ID: 1A6Q; Das *et al.*, 1996). PPM1G is shown in purple and PPM1A in yellow. C) Pairwise sequence alignment of PPM1G and PPM1A. In grey are highlighted conserved amino acids between PPM1G and PPM1A, in red are conserved metal-binding residues and in magenta is aspartate used for binding of the third metal, in yellow is the acidic-rich loop, and in green is the lysine-rich loop. In alignment production, the unique acidic-rich loop was excluded and added afterwards with adjustments inspired by Murray et al., 1999. The C-terminal of PPM1A was excluded as it shared no sequence identity with PPM1G's C-terminal. The structural alignment was done using the TM-align method from the RCSB PDB Pairwise Structure Alignment tool (Bittrich *et al.*, 2024) and the pairwise sequence alignment by EMBOSS Needle using BLOSUM62 from EMBL-EBI (Madeira *et al.*, 2024).

and glutamic acid residues. That causes slower mobility during SDS electrophoresis and results in a higher observed molecular weight than the predicted one from the amino acid sequence (Travis and Welsh, 1997). The crystal structure of PPM1G has not yet been determined, but an AlphaFold 3D model has been created (displayed in **Fig. 5A**). This

model shows the conserved PPM catalytic core with high confidence. An unstructured C-terminal end and acidic-rich loop cannot be predicted with good accuracy because of their flexibility.

PPM1G is ubiquitously expressed in all human tissues. Higher levels were detected within the skeletal muscle, heart, testis (Travis and Welsh, 1997) and during embryonic development, PPM1G was enriched in neural tissues (Foster *et al.*, 2013). It was observed that PPM1G-dependent signalling is essential for the proper development of the central nervous system, since when both *PPM1G* alleles were knocked out, it resulted almost exclusively in lethality in mouse embryos with severe defects connected to neural tube development. Loss of PPM1G led to enhanced apoptosis and stress signalling through activated p38 MAPKs (Foster *et al.*, 2013).

4. The role of PPM1G in the DNA damage response

4.1. Interaction with the histone dimer H2A-H2B

The first study that connected PPM1G with the DDR was concerning chromatin remodelling, particularly the deposition of histone dimer H2A-H2B. Nucleosomes are basic structural units that ensure higher organisation of genetic material through the winding of DNA in eukaryotes. They form an octamer consisting of one H3-H4 heterotetramer and two H2A-H2B heterodimers (Luger *et al.*, 1997). After exposure to the DNA-damaging agents, the rare histone H2A variant, H2AX, undergoes phosphorylation, which can facilitate the binding of the DDR effectors to the damage sites, as said in the second chapter. In order to effectively attenuate the signalling, these histones have to either be dephosphorylated or exchanged for their non-phosphorylated form.

When observing under which conditions the histone dimer H2A-H2B can be incorporated into the nucleosome, a soluble fraction ensuring exchange activity was isolated from the cellular extract. PPM1G was copurified along with two types of known histone H2A-H2B chaperones, nucleosome assembly protein 1-like 1 (NAP1L1) and 4 (NAP1L4) (Rodriguez et al., 1997; Okuwaki et al., 2010). Research of a potentially novel function of PPM1G was then conducted. It was found out that PPM1G can indeed facilitate the incorporation of H2A into the chromatin by itself (shown in **Fig. 6**), even when the system had been depleted of ATP. The cumulative effect of PPM1G with NAP1L1 or NAP1L4 chaperons was observed only after all three purified proteins were added together (Kimura *et al.*, 2006).

Tests were done with mutated PPM1G lacking the acidic-rich loop to assess its importance in chaperone activity. The co-immunoprecipitation assay confirmed that PPM1G directly interacts with H2A-H2B dimers by its acidic-rich loop. Since histones are highly basic proteins, PPM1G could bind them non-specifically through electrostatic interactions, however, PPM1G was found to associate specifically with the H2A-H2B heterodimer and not with histones H1, H3 or H4. Its function is further supported by the fact that the mutated phosphatase without the acidic-rich region did not contribute to H2A exchange, and via supercoiling assay, it was established that PPM1G can carry out, even though only weakly, nucleosome assembly de novo (Kimura *et al.*, 2006).

Because PPM1G does bind histones H2A-H2B, it was expected that they could be its substrates, too. It turned out that while PPM1G can dephosphorylate its associated histones, the histone incorporation and dephosphorylation are not strictly connected because even the catalytically dead mutant of PPM1G was able to enhance histone exchange. Histones that remained part of the nucleosomes were resistant to PPM1G activity, which indicates that PPM1G can dephosphorylate only nucleosome-free H2A-H2B (shown in **Fig. 6**). Some of the phosphorylation sites that PPM1G targeted were pSer139 of H2AX (γH2AX) and pSer14 of H2B (Kimura *et al.*, 2006), which are closely connected to the DDR (Fernandez-Capetillo et al., 2004).

Several phosphatases have been designated to be able to dephosphorylate γH2AX. The main phosphatases for γH2AX are considered to be predominantly PPM1D, described in the previous chapter, and also PP2A (Chowdhury *et al.*, 2005; Li *et al.*, 2015). Nonetheless, cells with knockout PPM1G, compared to the wild-type cells, still showed higher sensitivity to IR with caffeine present in the growing medium (Kimura *et al.*, 2006), since caffeine works as an inhibitor for the PIKK-dependent signalling network in the DDR (Sarkaria *et al.*, 1999). When calyculin A, an inhibitor of PP2A phosphatases, was added and cells were subjected to IR, PPM1G-deficient cells showed higher levels of γH2AX and H2B phosphorylated at Ser14. These findings point to the fact that PPM1G is another phosphatase that can act on γH2AX and phosphorylated H2B. It probably serves as one of the secondary or backup phosphatases and may be involved in the recovery phase of the DDR (Kimura *et al.*, 2006).

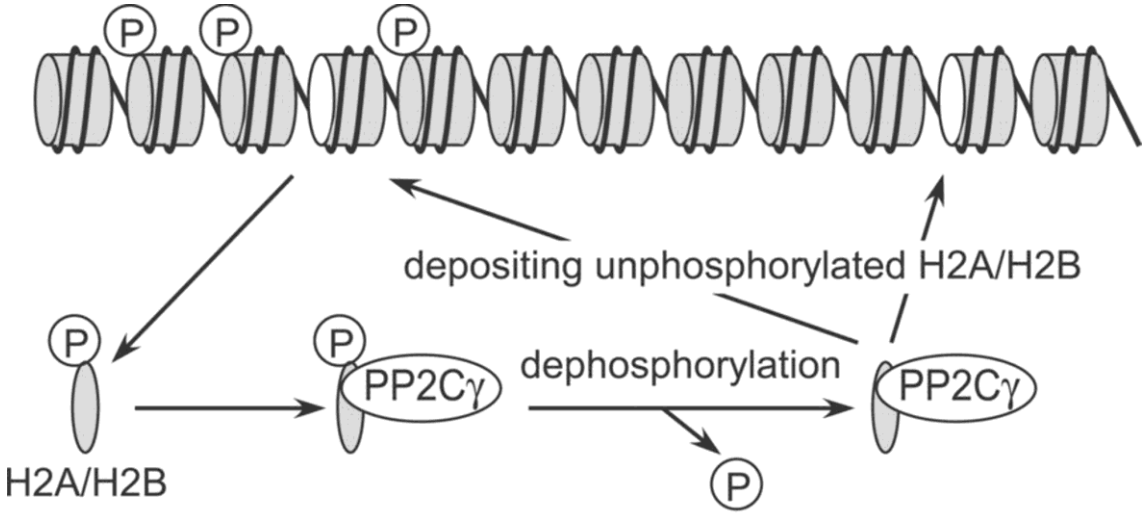

Figure 6. Suggested role of PPM1G in dephosphorylation and deposition of H2A-H2B histone dimer. Adapted from Kimura *et al.*, 2006, edited.

4.2. PPM1G is an ATM target

Several phosphorylation sites on either serine or threonine of PPM1G were identified in large-scale phosphoproteomic screens, and furthermore, PPM1G was found among the proteins that undergo phosphorylation initiated by DNA-damaging agents (Matsuoka *et al.*, 2007; Dephoure *et al.*, 2008; Beli *et al.*, 2012). Two PIKK SQ consensus sites, Ser183 and Ser201, were identified in the sequence of PPM1G. Both are located inside the acidic-rich loop (Beli *et al.*, 2012). Through either knockdown or inhibition of ATM, it was discovered that phosphorylation of PPM1G is ATM-dependent (Khoronenkova *et al.*, 2012; Gudipaty *et al.*, 2015). Phosphorylation of PPM1G was proposed to play a role in the regulation of p53 response (Khoronenkova *et al.*, 2012) and in transcription mediated by nuclear factor κB (NF-κB) (Gudipaty *et al.*, 2015) in response to the DDR (see below).

4.3. Stabilization of p53 through dephosphorylation of USP7S

USP7 can regulate cellular levels of p53 through the actions of its downstream effector E3 ubiquitin-protein ligases MDM2 and HUWE1 (HECT, UBA and WWE domain-containing E3 ubiquitin-protein ligase 1) (Li *et al.*, 2004; Khoronenkova and Dianov, 2013). It is common for E3 ubiquitin ligases to auto-ubiquitinate themselves, which then leads to their proteasomal degradation (Fang *et al.*, 2000; Chen *et al.*, 2005). USP7 cleaves off ubiquitin from MDM2 and HUWE1, thus restoring their stability and controlling their cellular levels. MDM2 and HUWE1 (also known as Mcl-1 ubiquitin ligase E3 [Mule] or ARF-binding protein 1 [ARF-BP1]) downregulate p53 during the normal cellular state and in the final stage of the DDR, through its ubiquitination and degradation (Haupt *et al.*, 1997; Chen *et al.*, 2005). On the other hand, to effectively initiate the DDR, p53 must accumulate inside the nucleus to activate transcription of its dependent genes. It was proposed that this process is mediated through the signalling axis of ATM-PPM1G-USP7S, shown in **Fig. 7** (Khoronenkova *et al.*, 2012; Khoronenkova and Dianov, 2013).

For a long time, it was not known how USP7 could be regulated until a biochemical study was conducted. Besides structural requirements and other PTMs, it revealed that a specific USP7 isoform is phosphorylated at Ser18 (referred to as USP7S) (Fernández-Montalván *et al.*, 2007). To identify the protein kinase responsible for Ser18 phosphorylation, the cellular extract was fractionised, and an *in vitro* kinase assay was

conducted. Eventually, casein kinase 2 (CK2) with all its subunits was found via mass spectrometry in the final fraction that still demonstrated USP7S phosphorylation activity. Additionally, it was confirmed that USP7S is co-purified with CK2 and contains a high-score consensus sequence for CK2, which includes Ser18 (Khoronenkova *et al.*, 2012).

It was theorised that phosphorylation of USP7S might be responsible for the regulation of its function and stability. When CK2α or CK2α' subunits were knocked down, it, as expected, led to decreased phosphorylation of Ser18 on USP7S, which was coupled with lower levels of the USP7S isoform and MDM2. To confirm that the phosphorylation was a key player in the regulation of USP7S's stability and not some additional functions of CK2, serine was substituted for alanine in USP7S (referred to as USP7S18A). USP7S18A was then shown to be less stable and degraded faster than the wild-type form. More interestingly, even with the overexpression of USP7S18A, to even up the original levels of the wild-type USP7S isoform, efficient stabilisation of MDM2 was not achieved. This indicates that the phosphorylation of Ser18 is essential for USP7S stability as well as for its enzymatic activity (Khoronenkova *et al.*, 2012).

Since it was established that phosphorylation at Ser18 is essential for USP7S function, research for the regulatory mechanism of this phosphorylation was conducted. Through mass spectrometry, PPM1G was identified to be the phosphatase responsible for the dephosphorylation of pUSP7S. The dephosphorylation activity of PPM1G on pUSP7S was confirmed by both *in vitro* dephosphorylation assay and *in vivo* overexpression, which led to decreased levels of pUSP7S. So, whether Ser18 at USP7S is or is not phosphorylated depends on which one of the CK2 or PPM1G enzymatic activities will prevail (Khoronenkova *et al.*, 2012). In contrast to PPM1D, whose expression is activated by p53 after DNA damage (Rossi *et al.*, 2008), PPM1G is commonly present in unstressed cells. That means its activity must be switched on upon detection of the DNA damage, otherwise, USP7S would be dephosphorylated even during the normal cell cycle. PPM1G was found to be a substrate of ATM, as described before, and phosphorylation by ATM is essential for its ability to dephosphorylate pUSP7S (Khoronenkova *et al.*, 2012).

This signalling pathway enlightens how the p53 protein levels are regulated during DDR and shows a novel axis through which p53 can be stabilised (pictured in **Fig. 7**). When the ATM is activated at the site of the DSBs, it then phosphorylates PPM1G. Activated PPM1G

interacts with the USP7S isoform, which is phosphorylated at Ser18 in the normal state. Dephosphorylation of USP7S leads to its destabilisation and increased proteasomal degradation. A decrease in the cellular levels of pUSP7S causes a subsequent decrease in levels of MDM2 and HUWE1 ubiquitinases, as their self-ubiquitination and degradation cannot be prevented. This further means they cannot ubiquitinate p53 (Khoronenkova *et al.*, 2012; Khoronenkova and Dianov, 2013). So, p53 is upregulated and can initiate transcription of its dependent genes (reviewed in Fischer, 2017), leading to activation of DNA repair, cell cycle checkpoints or apoptosis. One of these genes is also PPM1D, which starts a negative feedback loop to eventually downregulate p53 at the end of the DDR, as already mentioned in its separate chapter. ATM-PPM1G-USP7S pathway reveals that PPM1G could possess an opposite role from PPM1D in the initiation of the DDR.

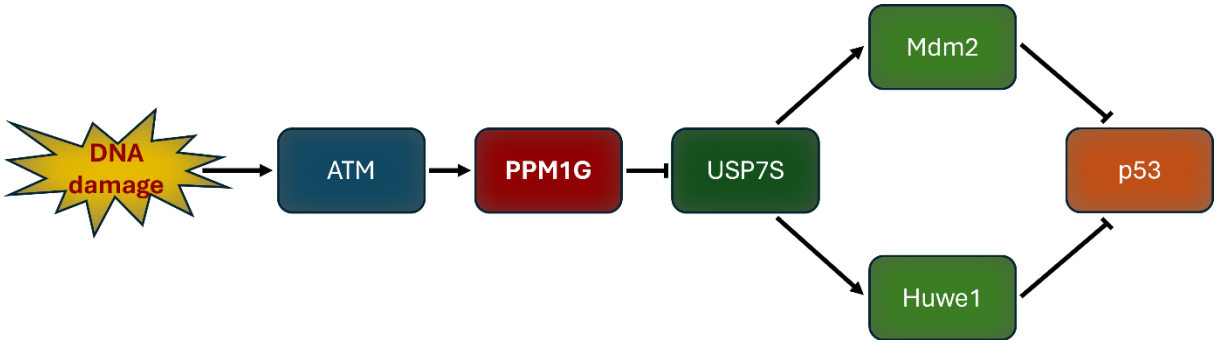

Figure 7. Novel suggested signalling axis: ATM-PPM1G-USP7S. The proposed mechanism is described in detail in the text. Scheme was created according to papers by Khoronenkova et al., 2012 and Khoronenkova and Dianov, 2013.

Although this model looks elegant, the effect on p53 stability was not experimentally verified *in vivo*, and the results are inconclusive. The only observed correlation between PPM1G and p53 was connected to the PPM1G knockdown, as p53 remained at a basal level after induction of DNA damage by IR, compared to raised levels in the wild-type. That would suggest a positive effect on p53. However, the observed behaviour could have been caused by small interfering RNA (siRNA) off-targets, since they did not test for them and used only one siRNA. Moreover, the authors did not conduct a rescue experiment to determine whether a siRNA-resistant PPM1G would restore p53 stability. Apart from these, the opposite relations between PPM1G and p53 and the overall effect of PPM1G on the DDR have been suggested. According to the effect on γH2AX described in the previous chapter and co-dependencies displayed on the depmap portal (DepMap, 2024) PPM1G appears to have an opposite role in the DDR, in inhibition rather than promotion.

Nevertheless, talking about PPM1G-dependent dephosphorylation of γH2AX, it has to be mentioned that PPM1G is most likely only redundant during this process, and the connection to ATM or the importance of phosphorylation of PPM1G in this mechanism was not tested. Since then, no other study about its direct interaction with H2A-H2B dimer and dephosphorylation of γH2AX has been conducted to possibly bring more detail or support this role. It was only confirmed that PPM1G is briefly recruited to the sites of DNA damage marked by γH2AX as early as 5 minutes after irradiation (Beli *et al.*, 2012).

On the other hand, PPM1G function in stabilisation and accumulation of p53 has recently been indirectly supported in an investigation of hepatocellular carcinoma (HCC) with gain-of-function (GOF) mutant p53 (Hu *et al.*, 2024). It was discovered that both mRNA and protein levels of PPM1G were significantly elevated in HCC cells when compared to healthy liver tissue or adjacent non-tumour tissues. Cells overexpressing PPM1G showed a faster proliferation rate, which promotes tumour development, but only a slight change in cell migration was observed. Increased protein expression of GOF p53 was associated with PPM1G overexpression in HCC, and knockdown of PPM1G resulted in a decrease in p53 expression. However, the cellular mechanisms behind this correlation or their interaction were not studied. Higher levels of PPM1G and, therefore, p53 were connected to worse prognosis and clinical outcomes. These findings point to an opportunity for utilising the PPM1G as a molecular target in the treatment of HCC (Hu *et al.*, 2024).

4.4. 7SK RNP binding and transcriptional pause release in the DDR

Protein expression can be controlled in many stages, however, regulation of transcription is the most important one. PPM1G has been mentioned to play a role in the precursor mRNA (pre-mRNA) splicing and initiation of translation (Murray et al., 1999; Allemand et al., 2007; Liu et al., 2013; Chen et al., 2021; Wang et al., 2024). These roles will be briefly presented later in a separate chapter. Another function concerning the protein expression, PPM1G holds, is connected to the promotion of transcription elongation. PPM1G was found to ensure a release of the positive transcription elongation factor b (P-TEFb) from inhibitory 7SK small nuclear ribonucleoprotein particle (snRNP), which leads to transcriptional pause release (McNamara *et al.*, 2013).

Transcriptional elongation is regulated by two types of elongation factors, negative and positive ones. Negative elongation factors bind to the RNA polymerase II (Pol II) soon after initiation, when a short RNA molecule is synthesised, and cause transcriptional pausing (Rougvie and Lis, 1988). The two most notable ones, which cooperate together, are 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole sensitivity-inducing factor (DSIF) and the negative elongation factor (NELF) (Wu *et al.*, 2003). In order to release halted Pol II, positive elongation factors have to be set in action. The one that is affected by PPM1G is P-TEFb, which is a heterodimer of cyclin-dependent kinase 9 (CDK9) and cyclin T1, T2 or K subunit (Peng *et al.*, 1998; Fu *et al.*, 1999). Free P-TEFb can phosphorylate the C-terminal domain of Pol II, DSIF and NELF, abrogating their negative effect, thereby promoting effective transcriptional elongation (Marshall *et al.*, 1996; Fujinaga *et al.*, 2004; Yamada *et al.*, 2006).

During the normal state, P-TEFb is reversibly associated with the 7SK snRNP complex, which impairs its catalytic activity and is only released upon stimulation, so it can promote transcriptional pause release (Yang *et al.*, 2001). 7SK snRNP consists of 7SK RNA, which is approximately 331 nucleotides long non-coding RNA, protein hexamethylene bisacetamide-inducible proteins 1 and/or 2 (HEXIM1/2), La-related protein 7 (LARP7) and 7SK snRNA methylphosphate capping enzyme (MePCE) (Muniz et al., 2013; Fujinaga et al., 2014). The assembly into an inhibiting complex is conditioned by phosphorylation at Thr186 in the T loop of the CDK9 subunit, which supports its direct interaction with 7SK RNA and kinase inhibitor HEXIM1/2 (Li *et al.*, 2005).

Since the function of P-TEFb is hampered by its phosphorylation at Thr186, some activating phosphatase has been searched for. PPM1G was observed to interact with 7SK snRNP, and because of that, its dephosphorylation activity on P-TEFb was tested in comparison to catalytically inactive PPM1G with mutated Asp496 into alanine (McNamara *et al.*, 2013). PPM1G did indeed dephosphorylate P-TEFb at Thr186. Furthermore, dephosphorylation caused the release of P-TEFb from 7SK snRNP (depicted in **Fig. 8**). The role in promoting transcriptional elongation is also strengthened by the fact that PPM1G was found to colocalise in the promoter-proximal region, as well as Pol II and the 7SK snRNP complex with bound P-TEFb. This close setting enables rapid induction of transcription (McNamara *et al.*, 2013).

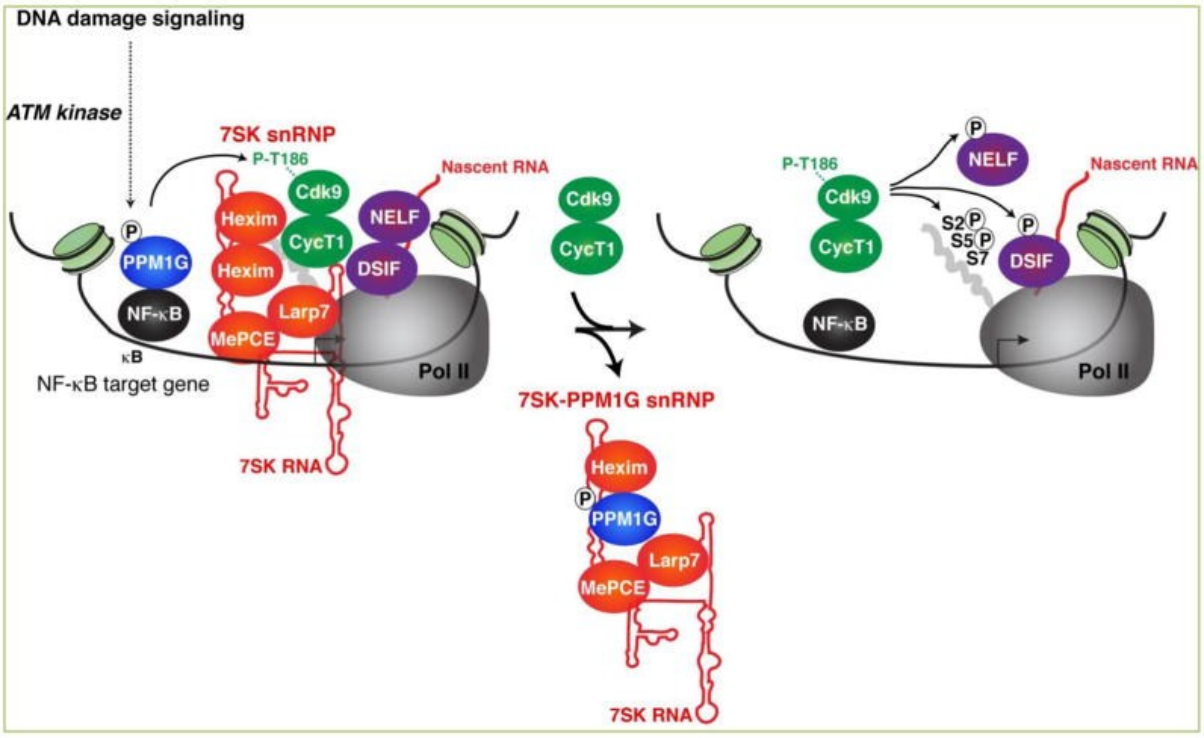

Figure 8. Mechanism of how PPM1G contributes to transcriptional pause release and gene expression. The scheme illustrates how PPM1G dephosphorylates and thereby releases P-TEFb when recruited by NF-κB to the promoter and phosphorylated by ATM. At the same time, PPM1G creates a complex with the rest of the 7SK snRNP and inhibits repeated interaction with P-TEFb. Adapted from review Gudipaty and D'Orso, 2016.

The localisation of PPM1G to the promoter region is currently understood to be facilitated by two transcriptional factors. The first one identified was the Tat protein from the human immunodeficiency virus 1 (HIV-1), which binds to the acidic-rich loop of PPM1G (McNamara *et al.*, 2013). The second factor is NF-κB, which is involved in inflammatory pathways (McNamara *et al.*, 2013). However, NF-κB is also activated during the DDR to mediate transcriptional activation. When DNA damage was induced by etoposide treatment, the presence of PPM1G at the promoter-proximal region was greatly enhanced compared to the low basal levels without stimulus (Gudipaty *et al.*, 2015).

PPM1G and NF-κB cooperate as coactivators since a significant downregulation of NF-κB-dependent genes encoding interleukin 8 and NF-kappa-B inhibitor alpha has been observed following the knockdown of PPM1G. Besides, PPM1G-depleted cells were more sensitive to apoptosis induced by proinflammatory cytokines, which indicates that PPM1G, along with P-TEF, is crucial for the expression of pro-survival genes (McNamara *et al.*, 2013). Tuning of NF-κB-dependent transcription upon stimulation is further controlled by the tumour suppressor $p14^{ARF}$ through direct binding of PPM1G (Hyder *et al.*,

2020). $p14^{ARF}$ inactivates PPM1G coactivator function, thereby inhibiting transcriptional pause release and promoting apoptosis. Moreover, $p14^{ARF}$ is stabilised by interaction with PPM1G and promotes assembly of the ternary complex $p14^{ARF}$-PPM1G-NF-κB at a promoter site, with PPM1G serving as a scaffolding protein (Hyder *et al.*, 2020).

Except for the dephosphorylation of P-TEFb and coactivation of the NF-κB-dependent genes, PPM1G was additionally detected to bind to 7SK RNA and HEXIM1 and form a complex with them. The phosphorylation of CDK9 in P-TEFb has two opposing effects, as it causes inhibition through recruitment to 7SK snRNP but also has a critical role for its ability to hyperphosphorylate the C-terminal domain of Pol II. Thereby, after release from the inhibiting complex, the T loop is once again phosphorylated, which could promote anew sequestration by HEXIM1. This futile cycle is prevented by action PPM1G, disassembling the original one and creating a new 7SK-PPM1G snRNP complex as shown in **Fig. 8** (Gudipaty *et al.*, 2015).

PPM1G interacts with stem I of 7SK RNA via the lysine-rich region at the C-terminal. This leads to the ineffective binding of HEXIM1 to 7SK RNA as they interact through stem I, as well. Because of that, HEXIM1 cannot tether and inactivate P-TEFb even if its T loop is phosphorylated. Moreover, PPM1G directly binds HEXIM1 through the domain that normally mediates its interaction with P-TEFb and sterically blocks it (Gudipaty *et al.*, 2015). During the DDR, PPM1G gets phosphorylated at Ser183 by ATM. This phosphorylation was found to be a crucial regulatory mechanism for the promotion of transcriptional elongation, as it is involved in PPM1G's interaction with the 7SK snRNP complex and in the formation of the HEXIM1-PPM1G-7SK RNA complex (Gudipaty *et al.*, 2015).

Through this delicate network of interactions with multiple transcriptional factors, PPM1G has been confirmed to play a critical role in the regulation of transcriptional elongation of at least a subset of P-TEFb-dependent genes whose expression is mediated by NF-κB in response to DNA damage. The second role in promoting transcriptional elongation is then during HIV-1 infection, as it enhances expression of its P-TEFb-dependent genes through the same mechanism. Nonetheless, it remains unknown whether PPM1G could have broader effects as a general transcriptional coactivator.

5. Other roles of PPM1G in the nucleus and cytoplasm

Except for the roles in the DDR, PPM1G has also been studied in connection with other cellular processes. It has been discovered that PPM1G is an essential player in the regulation of protein expression concerning transcription, discussed in the previous chapter, translation and pre-mRNA splicing. PPM1G is a negative controller of cap-dependent translation via two possible mechanisms. The first one is through 4E binding protein 1 (4E-BP1), which in its hypophosphorylated state binds the eukaryotic translation initiation factor 4E (eIF4E) of the cap-complex and inhibits the initiation of translation (Gingras *et al.*, 2001). PPM1G can potentially dephosphorylate 4E-BP1 on all four phosphorylation sites, which includes Thr37, Thr46, Ser65 and Thr70, and by that enabling the interaction between eIF-4E and 4E-BP1 (Liu *et al.*, 2013).

A recent study stated that PPM1G does not dephosphorylate 4E-BP1, but rather the cap-free eIF4E at Ser209 to reduce its binding to the cap-complex. Furthermore, a potential 4E-binding motif was found inside the primary sequence of PPM1G, and through its point mutation, PPM1G's capacity to bind and dephosphorylate eIF4E was significantly reduced, indicating its importance in this interaction (Wang *et al.*, 2024). In the earlier study, the link between eIF4E and PPM1G was not investigated, but the importance of phosphorylated Ser209 in translation is also still debated. Because of that, additional research will be required to specify how PPM1G regulates eIF4E and cap-dependent translation.

Over the years, several functions of PPM1G regarding the pre-mRNA splicing have been proposed. The first discovered role of PPM1G was in the formation of the A-complex, a spliceosome precursor (Murray et al., 1999). It was proven that the acidic-rich region is essential for the assembly of the A-complex (Allemand *et al.*, 2007). Among the proteins that were found to interact with PPM1G directly is Y-box binding protein 1 (YB-1), which regulates alternative splicing of the surface glycoprotein CD44 (Allemand *et al.*, 2007).

Moreover, PPM1G dephosphorylates serine/arginine-rich splicing factor 3 (SRSF3), which afterwards influences alternative splicing of genes linked to the cell cycle and regulation of transcription (Chen *et al.*, 2021). Recently, PPM1G has also been found to correlate with exon 6 skipping in the splicing of transducing β-like protein 1X (TBL1X) pre-mRNA and

increased stability of this transcript variant (Hu *et al.*, 2025). However, further research will be necessary to determine the role of PPM1G in this regulatory mechanism. Both of these functions were discovered in connection with the HCC, as PPM1G expression is elevated in the HCC cells. Upregulation of PPM1G was linked to worse prognosis, like in the case with the GOF p53 (Hu *et al.*, 2024), and was identified as a potential biomarker for HCC metastasis (Chen *et al.*, 2021; Hu *et al.*, 2025).

Although phosphatases of the PPM family were said to function as monomers, PPM1G has lately been suggested to form a holoenzyme with regulatory subunit B56δ (Kumar *et al.*, 2019). B56δ is originally one of the possible regulatory subunits of protein phosphatase 2A (PP2A) from the PPP family (McCright *et al.*, 1996), however, PP2A was not found in this new PPM1G complex. Surprisingly, B56δ directly binds PPM1G and causes cytoplasmatic localisation of this enzymatic complex through PPM1G sequestration, even though PPM1G is predominantly a nuclear phosphatase. Inside the cytoplasm, a new substrate for PPM1G was determined as an α-catenin. PPM1G binds α-catenin through the acidic-rich loop and dephosphorylates it at Ser641 (Kumar *et al.*, 2019).

A different study found that dephosphorylation sites are at Ser655 and Thr658, and did not observe any effects on Ser641 (Li *et al.*, 2025), so further research would be needed to determine the actual dephosphorylation sites of PPM1G on α-catenin. It was discovered that the dephosphorylation of α-catenin is critical for its localisation to the cytoplasmic membrane and assembly of adherens junctions. PPM1G-B56δ is, therefore, a key holoenzyme for controlling cell adhesion and migration. In this context, PPM1G can act as a tumour suppressor as it directs α-catenin to the membrane and inhibits cell migration (Kumar *et al.*, 2019).

6. Conclusion

Formerly, the main focus was on the roles of protein kinases in cellular signalling, with phosphatases playing only the second fiddle to them. They were regarded as simply counteracting enzymes important in switching off the pathways. However, the true nature of the functions of protein phosphatases in the DDR and other signalling networks is far more complex. Through dephosphorylation of response effectors, they can influence gene expression, mediate protein degradation and even regulate duration and dynamics of cellular response.

Many nuclear Ser/Thr phosphatases from both PPP and PPM have been shown to play a part in the DDR. From the metal-dependent family, the one with well-established roles is PPM1D. It was discovered as a p53-dependent gene. In the DDR, PPM1D initiates the p53 negative feedback loop through destabilisation of p53 by MDM2 interaction and inhibition of upstream kinases that phosphorylate and activate p53. On account of that, it causes pulses in p53 levels, and the p53-dependent transcription gets dampened. This leads to downregulation of p21 protein levels, enables exit from cell cycle arrest in G2 phase and determines checkpoint length.

The second phosphatase of the PPM family, which displayed promising connections to the DDR, was PPM1G. The primary aim of this thesis was to summarise and elucidate its role in the processes related to the DDR. However, despite the original assumption, the roles of PPM1G in the DDR, regarding stabilisation of p53 and histone chaperone or γH2AX dephosphorylation, are blurry and not well-supported. Furthermore, actions of PPM1G extend even outside the DDR.

PPM1G's first discovered role was as a pre-mRNA splicing factor, which was then followed by a couple of articles assessing its role in general and alternative splicing. In some of these studies, PPM1G was also connected to the HCC. Overexpression of PPM1G was related to the tumour development, metastasis and a worse prognosis. This points to the fact that PPM1G could be used as a biomarker and potentially therapeutic molecular target for the HCC. Nevertheless, more detailed clinical as well as basic research must be conducted to clarify its cellular functions and effects in the HCC.

Since PPM1G was repeatedly confirmed as an ATM target after induction by DNA damage, its role very likely lies somewhere in the DDR. Even though it might only be one of its functions, it can be of high significance and worth investigating as it can elucidate its therapeutic potential in cancer. A wide phosphoproteomic screen after exposure to genotoxic agents in PPM1G knockout cells could help to estimate its potential substrates and their subsequent targets in the DDR, which would then possibly point to further research.

Because of the proposed function of PPM1G as a histone chaperone and colocalization with and dephosphorylation of γH2AX, a detailed investigation should be given to the mechanisms through which PPM1G is deposited onto chromatin. Whether it is dependent only on interaction with histones through its acidic-rich loop, or whether it is facilitated by some adaptor proteins? If it can be found primarily in the heterochromatin or transcriptionally active euchromatin, since it was shown that PPM1G promote transcriptional pause release and localises to promoters of NF-κB-dependent and P-TEFb-dependent genes in response to the DDR.

Its effect on p53 stability and activity should be cleared up, as well. PPM1G appears to dephosphorylate the USP7 isoform with Ser18 at its N-terminal (USP7S), which leads to the downregulation of MDM2 and HUWE1. Besides, from the findings of the study by Khoronenkova *et al.*, 2012, USP7S is not the predominantly represented isoform of USP7 inside the cell, so the majority of USP7 is not affected by PPM1G, as it lacks this phosphoserine. Moreover, USP7 not only deubiquitinates MDM2 and HUWE1 but also p53, so its degradation should negatively impact p53 levels, as well. Therefore, *in vivo* monitoring of p53, its expression targets and checkpoint activation in PPM1G knockout cells compared to the wild-type control group, could put its role in the regulation of p53 stability straight and once again elucidate its therapeutic potential in cancer treatment.

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