Targeting proliferating cell nuclear antigen (PCNA) for cancer therapy

Caroline K. Søgaard[a] **and Marit Otterlei**[a,b,*]
[a]Department of Clinical and Molecular Medicine, Faculty of Medicine and Health Sciences, NTNU Norwegian University of Science and Technology, Trondheim, Norway
[b]APIM Therapeutics A/S, Trondheim, Norway
*Corresponding author. e-mail address: marit.otterlei@ntnu.no

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Abstract

Proliferating cell nuclear antigen (PCNA) is an essential scaffold protein in many cellular processes. It is best known for its role as a DNA sliding clamp and processivity factor during DNA replication, which has been extensively reviewed by others. However, the importance of PCNA extends beyond its DNA-associated functions in DNA replication, chromatin remodelling, DNA repair and DNA damage tolerance (DDT), as new non-canonical roles of PCNA in the cytosol have recently been identified. These include roles in the regulation of immune evasion, apoptosis, metabolism, and cellular signalling.

The diverse roles of PCNA are largely mediated by its myriad protein interactions, and its centrality to cellular processes makes PCNA a valid therapeutic anticancer target. PCNA is expressed in all cells and plays an essential role in normal cellular homeostasis; therefore, the main challenge in targeting PCNA is to selectively kill cancer cells while avoiding unacceptable toxicity to healthy cells. This chapter focuses on the **stress-related roles of PCNA,** and how targeting these PCNA roles can be exploited in cancer therapy.

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1. Structure and function of PCNA

PCNA is a ring-shaped homotrimer with each PCNA monomer consisting of two globular domains connected by a flexible interdomain connecting loop (IDCL). The monomers are linked in a head-to-tail manner to form a circle that has a positivily charged inside which are able to encircle and slide along DNA (Gulbis et al., 1996) (Fig. 1). However, this trimeric ring structure is not dependent on DNA as this also is the structure of cytosolic PCNA (Belyakova et al., 2017; González-Magaña & Blanco, 2020). PCNA has no enzymatic activity, but act as a scaffold protein exerting its functions by anchoring interacting proteins in a highly coordinated fashion at the site of action (reviewed in Choe & Moldovan, 2017; Slade, 2018). Binding to PCNA may modulate the activity of interacting proteins. Conserved PCNA-interacting motifs regulates the various protein – PCNA interactions.

The PCNA-interacting peptide-box (PIP-box) was the first acknowledged PCNA-interacting motif. The PIP-box is defined by the consensus sequence Q-x-x-ψ-x-x-ϑ-ϑ (canonical PIP-box: ψ = hydrophobic, ϑ = aromatic, x = any residue), where at least one of the aromatic amino acids

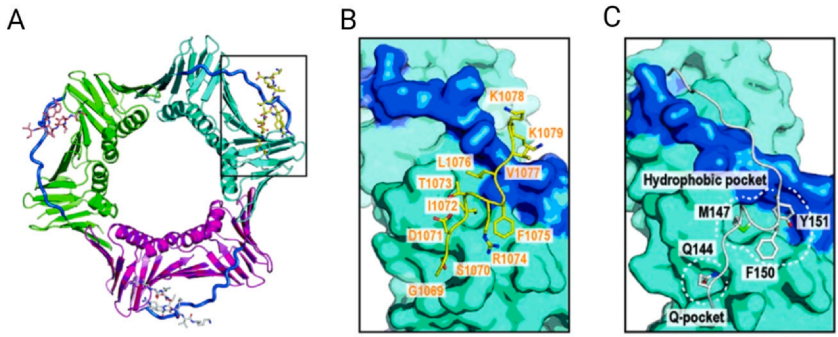

Fig. 1 Structure of PCNA bound to PIP-box and APIM peptides. (A) Three PCNA monomers (shown in green, cyan and magenta) are linked head-to-tail to form a ring structure. The hydrophobic pocket below the interdomain connecting loop (IDCL) (shown in blue) on each monomer is the major binding site for many PCNA-interacting proteins. An APIM-peptide bound to PCNA in this region is shown as a stick on each monomer (yellow, pink, grey). (B) Close-up of the binding of an APIM-peptide (yellow stick presentation) to PCNA, with the IDCL highlighted in blue. (C) Close-up of the binding of a PIP-box peptide (grey stick presentation) to PCNA, with the IDCL highlighted in blue. *From Hara, K., et al. (2018). Structure of proliferating cell nuclear antigen (PCNA) bound to an APIM peptide reveals the universality of PCNA interaction. Acta Crystallographica Section F:Structural Biology Communications 74(Pt 4), 214–221, reproduced with permission from the International Union of Crystallography.*

are vital for PCNA interaction (Warbrick, 1998). A second highly conserved sequence facilitating binding to PCNA was later discovered in human AlkB homolog 2 (hABH2), and hence termed AlkB homolog 2 PCNA-interacting motif (APIM). APIM is defined by the consensus sequence [K/R]-[F/Y/W]-[L/I/V/A]-[L/I/V/A]-[K/R], and also in this motif, the aromatic amino acid is vital for binding to PCNA (Gilljam et al., 2009). Co-crystal structures of PCNA bound to PIP-box peptides have revealed that the IDCL region of PCNA and the hydrophobic pocket underneath are important for this interaction (Bruning & Shamoo, 2004; Gulbis et al., 1996). Later, it was discovered that also APIM-peptides bind in the same region (Bacquin et al., 2013; Ciccia et al., 2012; Hara et al., 2018; Muller et al., 2013; Sebesta et al., 2017), and that the aromatic amino acids in both motifs are docked in the hydrophobic pocket (Fig. 1). Putative APIM and PIP-box motifs are found in more than 600 proteins (>300 APIM-containing proteins, >300 PIP-box-containing proteins) (Gilljam et al., 2009; Olaisen et al., 2015), but it remains to be determined how many of these are functional PCNA-interacting motifs. Nevertheless, the many known interactions that occur in the region around the hydrophobic pocket of PCNA have made this region particularly interesting as a target for cancer therapy (reviewed in Wang, 2014; Altieri & Kelman, 2018).

Although the PIP-box and APIM are the main motifs conferring PCNA binding, it should be noted that several other proteins not containing APIM or PIP-box are still able to bind to PCNA. For example, the translesion synthesis (TLS) scaffold protein REV-1 binds to PCNA via its BRCA1 C-terminus (BRCT) domain (Guo et al., 2006), and the natural killer (NK) cell natural cytotoxicity receptor NKp44 binds to PCNA via a sequence without of APIM and PIP-box similarity (Shemesh et al., 2018). This supports that PCNA-interacting proteins may bind outside the hydrophobic pocket/IDCL region (reviewed in Naryzhny, 2008; Maga & Hubscher, 2003).

2. Regulation of PCNA interactions

2.1 Interacting motifs and affinity differences

The plethora of PCNA interactions must be tightly regulated for PCNA to perform its multiple functions correctly. Although the PIP-box and APIM have overlapping binding sites on PCNA, their amino acid sequence and

interaction points are different, resulting in different binding affinities (Hara et al., 2018; Sebesta et al., 2017). More than 700 fold differences in affinity for PCNA have been measured in vitro between different PIP-box variants; the canonical p21 PIP-box has the highest known affinity (Bruning & Shamoo, 2004), whereas non-canonical PIP-boxes; i.e., PIP-box sequences without a glutamine (Q) in the first position, generally have lower affinities (González-Magaña & Blanco, 2020). Different affinities for PCNA also apply to APIM-variants (Ræder et al., 2018), and interaction studies with recombinant PCNA in vitro suggest that APIM generally have a lower affinity than canonical PIP-boxes (Olaisen et al., 2015; Prestel et al., 2019). Not only the motifs, but also the overall charge of the flanking regions can strongly influence the binding affinity for PCNA (Prestel et al., 2019). For example, the region adjacent to the hydrophobic pocket on PCNA is negatively charged and the positively charged flanking regions to the PIP-box in p21 also interact with PCNA and are likely to increase the affinity of p21 for PCNA (Kroker & Bruning, 2015). Similarly, a variant of hABH2 in which glutamine is substituted for lysine (Q to K) three amino acids downstream of APIM has increased affinity for PCNA in vitro (Fu et al., 2015).

While proteins containing canonical (high affinity) PIP-boxes appear to be essential for replication and bind to PCNA by default, the non-canonical PIP-box and APIM-containing proteins appear to be more important in the response to cellular stress. For example, APIM is verified as a functional motif in several stress-related proteins such as the DNA repair and DDT proteins hABH2, RAD51B, Xeroderma pigmentosum group A (XPA), F-box DNA helicase 1 (FBH1), zinc finger RANBP2-type containing 3 (ZRANB3), helicase like transcription factor (HLTF), SNF2 histone linker PHD RING helicase (SHPRH), protein reversionless 3-like (REV3L) (the catalytic subunit of polymerase (POL) ζ) and general transcription factor (TFII-I) (Bacquin et al., 2013; Ciccia et al., 2012; Fattah et al., 2014; Gilljam et al., 2009, 2012; Ræder et al., 2018; Seelinger & Otterlei, 2020), and non-canonical PIP-boxes are found in the TLS polymerases POL η, POL ι, and POL κ (González-Magaña & Blanco, 2020). Studies have demonstrated that overexpression of canonical PIP-box-peptides inhibited colony formation (Mattock, Lane, & Warbrick, 2001; Warbrick, 2006), while overexpression of APIM-peptides in the same cell lines only reduced growth when combined with external stress. This suggests that APIM-peptides block a subset of PCNA – protein interactions that are more important in stressed cells, while canonical

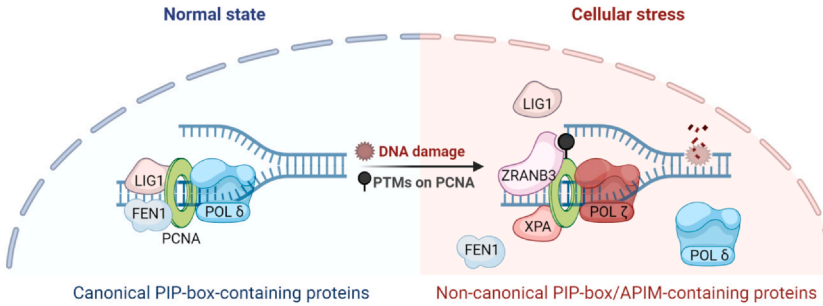

Fig. 2 Affinity switch from canonical PIP-box to non-canonical PIP-box/APIM-containing proteins during stress. In the normal cellular state, high affinity PIP-box-containing proteins (blue) bind predominantly to PCNA and participate in essential "housekeeping" processes such as DNA replication. DNA damage, or other cellular stress can induce post-translational modifications (PTMs) on PCNA, causing an affinity switch from canonical PIP-box-containing proteins to non-canonical PIP-box/APIM-containing proteins (red), which are involved in stress-related pathways such as DNA repair and DNA damage tolerance.

PIP-box-peptides block PCNA – protein interactions also involved in replication (Gilljam et al., 2009). Furthermore, APIM-peptides were shown to pull down PCNA variants with a more acidic isoelectric point than total PCNA, suggesting that APIM has a higher affinity for PCNA with post-translational modifications (PTMs) (Gilljam et al., 2009). These findings have led to the affinity switch model (Gederaas et al., 2014a) (Fig. 2), which proposes that stress-induced PCNA modifications lead to an affinity switch from high-affinity PIP-box-containing proteins to non-canonical PIP-box/APIM-containing proteins.

PTMs on PCNA. PTMs identified on PCNA include, among others, ubiquitination, ISGylation, NEDDylation, SUMOylation, acetylation and phosphorylation (Fig. 3).

PTMs on PCNA are not known to directly affect the underlying structure of PCNA, but rather to change the repertoire of interaction partner possibilities by causing steric hindrance or creating additional binding modules (reviewed in Slade, 2018; Mailand, Gibbs-Seymour, & Bekker-Jensen, 2013; Ulrich, 2009). An example of a scenario in which PTMs on PCNA are introduced is stalled replication forks caused by DNA lesions/aberrations. Stalled replication forks can trigger ubiquitination of PCNA at K164, which is important for the initiation of DDT pathways (Leung et al., 2018; reviewed in Mailand et al., 2013; Kanao & Masutani, 2017; Niimi et al., 2008; Zhang et al., 2021). Monoubiquitination of

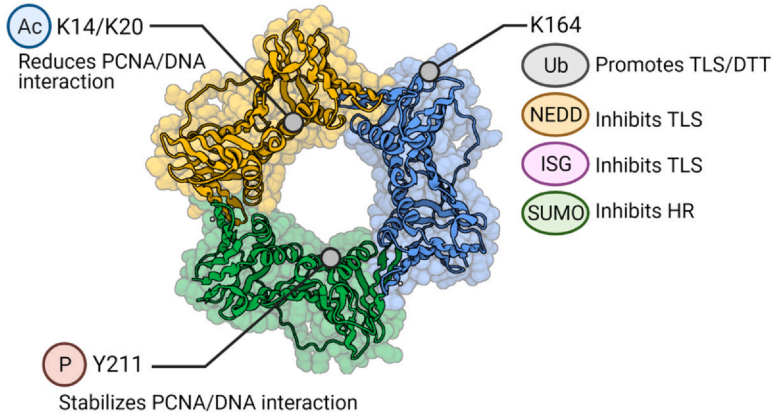

Fig. 3 PCNA modifications. PCNA exerts its functions through numerous protein interactions that can be regulated by various post-translational modifications (PTMs), including ubiquitination (Ub), NEDDylation (NEDD), ISGylation (ISG), SUMOylation (SUMO), acetylation (Ac) and phosphorylation (P).

PCNA at K164 is thought to be associated with error-prone TLS, whereas polyubiquitination favours error-free bypass via template switch (TS); however, this is not categorical (reviewed in Kanao & Masutani, 2017). Several TLS polymerases contain ubiquitin-binding motifs in addition to their non-canonical PIP-boxes which increase their affinity for ubiquitinated PCNA (Bienko et al., 2005). Ubiquitination of PCNA can thus stimulate a switch from replicative polymerases to TLS polymerases (Kannouche, Wing, & Lehmann, 2004). However, other modifications also play a role in selecting which DDT pathway to use to deal with stalled replication forks. For example, ZRANB3 contains both APIM and PIP-box PCNA-interacting motifs in addition to an NPL4 zinc finger (NZF) protein domain that mediates binding to polyubiquitin chains. Together, the PIP-box, APIM and NZF facilitate high affinity binding of ZRANB3 to polyubiquitinated PCNA at stalled replication forks to promote fork reversal, TS and replication restart (Ciccia et al., 2012).

TLS polymerases have lower fidelity than normal DNA polymerases, and their activity must therefore be tightly regulated to avoid increased mutation rates (reviewed in Mailand et al., 2013; Goodman, 2002; Kanao & Masutani, 2017; Zhang et al., 2021). Several of the mechanisms to avoid excessive TLS and/or other forms of DDT involve regulation of PCNA PTMs. The ubiquitin-specific protease 1 (USP1) is active in the absence of DNA damage and deubiquitinates PCNA to repress the initiation of

inappropriate DDT (Huang et al., 2006). In addition, ISGylation of monoubiquitinated PCNA at K164/K168 by interferon-stimulated gene 15 (ISG15) triggers deubiquitination of PCNA and the release of TLS polymerases (Park et al., 2014). NEDDylation of PCNA, also at K164, by NEDD8 in a response to oxidative stress and UV irradiation antagonizes PCNA ubiquitination and the recruitment of POL η and thus TLS (Guan, Yu, & Zheng, 2018).

SUMOylation of PCNA by the small ubiquitin-like modifier 1 (SUMO1) also occurs predominantly at K164. The PCNA-associated recombination inhibitor (PARI) contains a SUMO-interacting motif (SIM) in addition to its PIP-box, and SIM enhances the affinity of PARI for SUMOylated PCNA. The PCNA-PARI interaction interferes with the nucleofilament formation which is essential for the initial steps of homologous recombination (HR), thereby preventing inappropriate HR and helping to maintain chromatin stability (Moldovan et al., 2012).

Acetylation of lysines (K) on PCNA can neutralise the positively charged core of the ring, thereby reducing the ability of PCNA to slide along DNA. Acetylation of K20 on PCNA detected after methyl methanesulfonate (MMS)-induced DNA damage, has been shown to promote HR and suppress DDT, probably by reducing the replication rate (Billon et al., 2017). Acetylation of K14 on PCNA after UV-induced DNA damage has been suggested to mark PCNA for degradation following nucleotide excision repair (NER) (Cazzalini et al., 2014).

Phosphorylation of PCNA at Y211 by nuclear epidermal growth factor receptor (EGFR) or by the Ron receptor tyrosine kinase and c-Abl, has been reported to stabilise chromatin-bound PCNA and facilitate DNA replication and repair (Wang et al., 2006; Zhao et al., 2014).

Other modifications on PCNA have also been proposed, including methylation, S-nitrosylation, mono- and poly-ADP-ribosylation and methyl esterification (also called caPCNA) (reviewed in Choe & Moldovan, 2017; Mailand et al., 2013; Slade, 2018), but their roles are not well understood. Nevertheless, PTMs on PCNA are mainly observed in response to cellular stress and these can induce a change in protein binding partners (reviewed in Zhu et al., 2014; Lehmann et al., 2007). Cancer cells must adapt to higher levels of stress than normal cells as a consequence of dysfunctional pathways such as reduced cellular checkpoint control, DNA repair failure, increased levels of DNA aberrations, oxygen deprivation, nutrient limitation, increased/altered signalling events and increased proliferation rates (reviewed in Fouad & Aanei, 2017). The stressed nature of

cancer cells therefore implies higher levels of modified PCNA and targeting primarily processes mediated by modified PCNA as an anti-cancer strategy is therefore likely to increase the selectivity for cancer cells. This could be particularly effective in combination with stress-inducing treatments.

2.2 Vital PCNA – protein interactions during replication stress

DNA lesions that halt DNA replication, such as abasic sites (AP sites), modified/abnormal DNA bases, DNA-DNA (inter/intrastrand crosslinks), transcription-replication conflicts, and DNA-protein crosslinks, can cause replication fork collapse, and DNA strand breaks. It is therefore essential for cell survival that these lesions are repaired or circumvented. PCNA is central to most of these repair and/or DDT pathways; that is, PCNA has recently been proposed to play a critical role in the transfer of active RNA-Pol II complexes to nascent DNA during transcription-replication conflicts via interaction with RNA-Pol II (Fenstermaker et al., 2023). Interestingly, the RPB1 subunit of RNA Pol II contains an APIM motif (Gilljam et al., 2009; Olaisen et al., 2018). PCNA naturally plays a role in DNA synthesis following lesion excision, but PCNA also interacts with several DNA repair and DTT proteins and stimulates their activity in earlier steps of the DNA repair and/or DDT pathways (reviewed in Choe & Moldovan, 2017; Moldovan et al., 2007). Many chemotherapeutic drugs induce genotoxic lesions; however, DNA repair and DDT pathways can reduce the therapeutic effect by repairing/tolerating these lesions. Targeting the role of PCNA in these pathways may therefore be beneficial in cancer therapy (Fig. 4).

2.2.1 Targeting DNA repair

Inhibiting DNA repair pathways to increase the sensitivity to chemotherapy may be particularly effective in DNA repair-defective cancer cells which rely on redundant DNA repair pathways to survive excessive DNA damage (reviewed in Helleday et al., 2008; Chang et al., 2021; Desai, Yan, & Gerson, 2018; Gavande et al., 2016; Nickoloff et al., 2017; Puigvert, Sanjiv, Helleday, & Targeting, 2016). Targeting this type of vulnerability has been successfully achieved by inhibiting poly (ADP-ribose) polymerase 1 (PARP1). PARP1 recognises and marks DNA single-strand breaks (SSBs), leading to the recruitment of DNA repair proteins and enhanced repair of these lesions (First PARP inhibitor ok'd for breast cancer, 2018; Bornstein & Jimeno, 2016). If left unrepaired, SSBs will

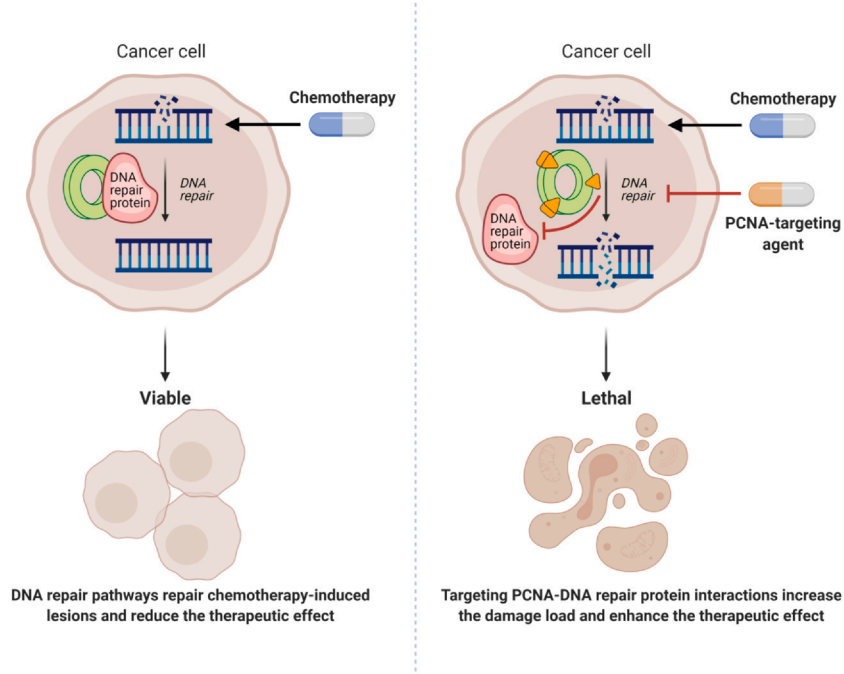

Fig. 4 Targeting PCNA – DNA repair protein interactions. Chemotherapy-induced lesions can be repaired by DNA repair pathways, reducing the therapeutic effect (left panel). Targeting PCNA - protein interactions could impair PCNAs role in several DNA repair pathways and thus increase the DNA damage load and enhance the efficacy of chemotherapeutics (right panel).

progress to double-strand breaks (DSBs) when they encounter the replication fork. PARP inhibitors are therefore particularly effective in rapidly growing cancers with DSB repair deficiencies (e.g., BRCA1/2) (Fong et al., 2009; Kaufman et al., 2015). It should be noted that although PARP1 is important in DNA repair, it is also involved in normal replication and epigenetics. The underlying mechanism for the effects achieved with PARP inhibitors is therefore likely to be more complex than just impaired SSB repair (reviewed in Ray Chaudhuri & Nussenzweig, 2017). For example, PARP1 has a role in controlling replication rate, and PARP1 inhibition has been shown to increase the replication rate and thereby compromising genome integrity (Maya-Mendoza et al., 2018). PARP1 is also proposed to be one of PCNA's many interaction partners (Frouin et al., 2003; Isabelle et al., 2010), and both PARP1 and PARP2 contain APIM (Olaisen et al., 2018). Many of the stress-related functions of

PARP1 may therefore also be impaired by inhibiting PARP´s ability to interact with PCNA.

Several other studies support the potential of targeting the role of PCNA in DNA repair to increase the DNA damage load and enhance the effect of chemotherapeutics i.e.; increased levels of 1-methyladenine (1meA) were found in MMS-treated cells overexpressing an APIM-peptide that blocks APIM-containing proteins from interacting with PCNA (Gilljam et al., 2009), increased levels of 6–4 photoproducts and cyclobutene-pyrimidine dimers (CPDs) were found after UV irradiations in cells expressing XPA with an APIM mutation compared to cells expressing wild-type XPA (Gilljam et al., 2012), and increased levels of DNA breaks were observed in bladder cancer cells treated with cisplatin in combination with a APIM-containing cell-penetrating peptide (ATX-101) compared to either single agent alone (Søgaard et al., 2018a).

2.2.2 Targeting DDT

Targeting modified PCNA during replicative stress could inhibit several DDT proteins from performing their function, e.g., PCNA ubiquitination largely controls the initiation of the DDT pathways (Hoege et al., 2002). The RAD6 - RAD18 complex is largely responsible for the mono-ubiquitination of PCNA, whereas the two ubiquitin ligases HLTF and SHPRH promote the polyubiquitination of PCNA. Monoubiquitination is thought to favour TLS whereas polyubiquitination is thought to favour TS (Hoege et al., 2002; Kannouche et al., 2004; Motegi et al., 2006, 2008; Unk et al., 2006, 2008; Watanabe et al., 2004). However, HLTF and SHPRH also play a role in TLS by activating POL η and POL κ (Lin et al., 2011). Both HLTF and SHPRH interact with PCNA through their APIM sequences, and their APIM-PCNA interactions are important for their dual roles in DDT (Seelinger & Otterlei, 2020; Seelinger, Søgaard, & Otterlei, 2020).

The TLS polymerases POL η, POL κ and POL ι are "inserters", i.e., they are responsible for the insertion of bases opposite the lesion, and POL ζ is the main "extender" TLS polymerase, i.e., it extends the DNA synthesis from the inserted and often mismatched base (reviewed in Livneh, Ziv, & Shachar, 2010). POL η, POL κ and POL ι interact with PCNA via their non-canonical PIP-box and monoubiquitin binding domains (Bienko et al., 2005; Haracska et al., 2001, 2005; Masuda et al., 2015; Ogi, Kannouche, & Lehmann, 2005), while POL ζ interacts with PCNA via an APIM in the catalytic subunit REV3L (Ræder et al., 2018).

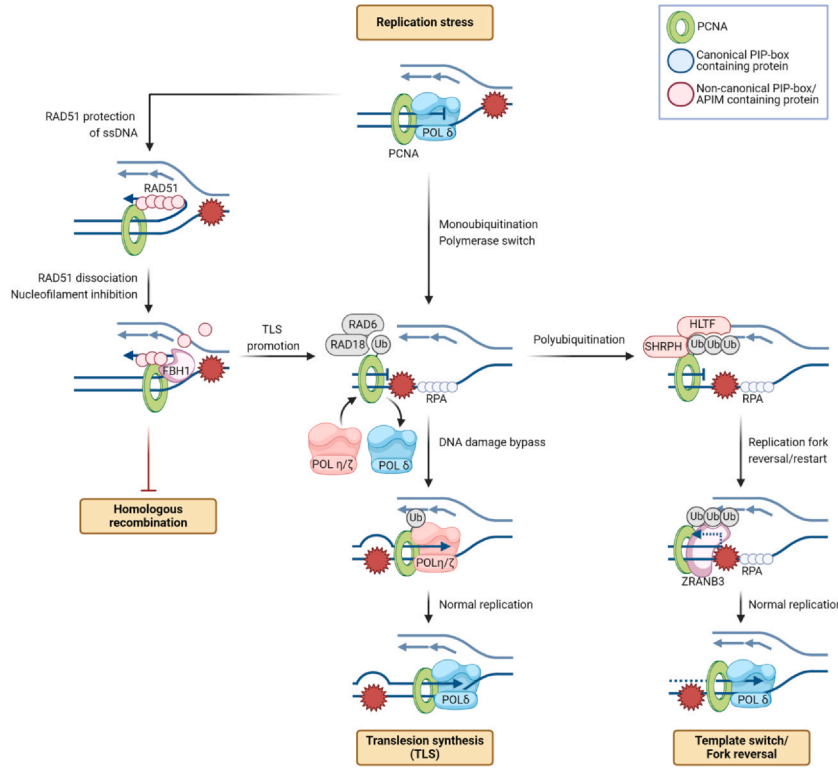

Fig. 5 Examples of PCNA modifications and interaction partners during DNA damage tolerance (DDT). Replication stress, such as DNA damage, and prolonged stalling of the replication fork activates DDT pathways through ubiquitination of PCNA. The RAD6 – RAD18 complex is recruited to accumulated RPA – ssDNA at stalled replication forks. This complex monoubiquitinates PCNA, which stimulates TLS, whereas HLTF/SHPRH is involved in the polyubiquitination of PCNA, which promotes TS (reviewed in Kanao & Masutani, 2017). Monoubiquitination of PCNA leads to a switch from replicative to TLS polymerases, such as POL η and POL ζ, which can bypass the lesion. Both PCNA and REV1 are scaffolding proteins in TLS. POL ζ consists of the subunits REV3L, REV7, p50 and p66, and can bind to PCNA directly via the APIM in REV3L or the PIP-box in p66, or indirectly via REV7 binding to REV1. FBH1 also plays a role in DDT via PCNA. FBH1 is involved in both fork regression and in its interaction with PCNA stimulates its anti-recombinase activity causing dissociation of RAD51 from ssDNA and thus impairing nucleofilament formation and invasion, which are important steps in HR. By inhibiting HR, FBH1 rather promotes TLS. Only a selection of PCNA functions in DDT are shown.

Whether mono- or polyubiquitin on PCNA increases POL ζ affinity is unknown, but a site-specific cleavage N-terminally in REV3L stabilise its remaining catalytic domain by preventing ubiquitin-dependent proteasomal degradation (Wang et al., 2020). Thus, both the strength of the

PCNA-polymerase interactions and the stability of the polymerases are important for efficient TLS (Fig. 5).

ZRANB3 is an important DDT protein that supports replication fork reversal via interacting with polyubiquitinated PCNA via its APIM and PIP-box motifs (Ciccia et al., 2012; Sebesta et al., 2017; Vujanovic et al., 2017) (Fig. 5). Another protein important in DDT during replication stress is FBH1. It has both helicase and anti-recombination activity and is involved in ubiquitination (Chu et al., 2015). Like ZRANB3, it is important for replication fork reversal and FBH1 also contains an APIM and a PIP-box, both of which are required for its interaction with PCNA (Bacquin et al., 2013; Fugger et al., 2015). Binding to PCNA stimulates the anti-recombinase activity of FBH1, resulting in the displacement of RAD51, and thereby inhibiting the formation of the RAD51 nucleoprotein filament required for HR initiation (Bacquin et al., 2013) (Fig. 5).

DDT may contribute to chemoresistance by allowing chemotherapy-induced DNA lesions to be bypassed by employing the intrinsic mutagenic TLS polymerases, as this will generate multiple mutations. In support of this, the TLS polymerase POL ζ has been reported to contribute to cisplatin resistance, and knockdown of its catalytic subunit REV3L has been shown to reduce the development of cisplatin resistance (Wu et al., 2004). Suppression of POL ζ/REV3 has also been shown to be beneficial in the treatment of already cisplatin-resistant lung tumours (Doles et al., 2010), likely due to impaired DDT of cisplatin-induced lesions. Targeting DDT in cancer therapy may therefore be valuable both by increasing the efficacy of the therapy by inhibiting DNA damage evasion and by reducing the development of acquired resistance, as TLS polymerases are intrinsically mutagenic (Xie et al., 2010; reviewed in Yamanaka et al., 2017; Korzhnev & Hadden, 2016). Targeting PCNA-protein interactions important for DDT or ubiquitination of PCNA are both proposed therapeutic strategies (reviewed in Korzhnev & Hadden, 2016). Functional non-canonical PIP-boxes or APIMs have been demonstrated in several important DDT proteins, some of which are mentioned above, e.g., POL η, POL κ, POL ι, REV3L, TFII-I, FBH1, HLTF, RAD51B, SHPRH and ZRANB3 (Bacquin et al., 2013; Ciccia et al., 2012; Fattah et al., 2014; Gilljam et al., 2009; Masuda et al., 2015; Ræder et al., 2018; Seelinger & Otterlei, 2020; Seelinger et al., 2020). Blocking the ability of these proteins to bind to modified PCNA can potentially reduce DDT.

2.3 Cytosolic roles of PCNA

Several cell types, including primary monocytes, multiple myeloma (MM) cells, neutrophils, bladder cancer cells and neuroblastoma cells have been shown to contain substantial amounts of cytosolic PCNA (Gederaas et al., 2014b; Gravina et al., 2022; Muller et al., 2013; Witko-Sarsat et al., 2010; Yin et al., 2015), and in recent years, a variety of functional roles for cytosolic PCNA have emerged. These includes processes regulating metabolism, cell signalling, apoptosis and immune responses.

PCNA in cellular signalling. The phosphoinositide-3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) and mitogen-activated protein kinase (MAPK) pathways signalling pathways are important signalling pathways downstream of receptor tyrosine kinases (RTKs), and these pathways are associated with a wide range of biological processes including regulation of proliferation, apoptosis, and metabolism. In addition, these signalling pathways interconnect and regulate many other signalling pathways. The PI3K/Akt/mTOR and MAPK pathways regulate cell growth and survival and are often activated in cancer. Several proteins involved in cellular signalling contain PCNA-interacting motifs and are therefore putative PCNA-interacting proteins (Olaisen et al., 2015, 2018). For example, APIM sequences are found in (i) three PI3Ks (PI3Kα, PI3Kγ, PIK3C2B), microtubule-associated serine/threonine kinase 3 (MAST3) and polo-like kinase 3 (PLK3) in the PI3K/Akt/mTOR pathway, and in (ii) SOS Ras/Rac guanine nucleotide exchange factor 1/2 (SOS1/2), serine/threonine protein kinase MST4, neurofibromin 1 (NF1), thousand and one amino acid protein kinase 2 (TAOK2) and mitogen-activated protein kinase-activated protein kinase 2 (MK2/MAPKAP2) in the MAPK pathways (Fig. 6).

The functionality of APIM in most signalling proteins have not yet been experimentally demonstrated; however, targeting PCNA with a cell-penetrating APIM-containing peptide (APIM-peptide) reduces the activation of PI3K/Akt/mTOR pathways in various cells (Gravina et al., 2022; Olaisen et al., 2015; Røst et al., 2023; Søgaard et al., 2018a), and inhibition of, or knock-out of, p38/Hog1 in combination with APIM-peptide treatment has been shown to cause cisplatin hypersensitivity in both mammalian and yeast cells, suggesting a functionally conserved property of PCNA (Olaisen et al., 2018). Further supporting a functional role of PCNA-signalling protein interactions is the detection of several proteins of the MAPK and PI3K/Akt/mTOR pathways in PCNA pull-downs,

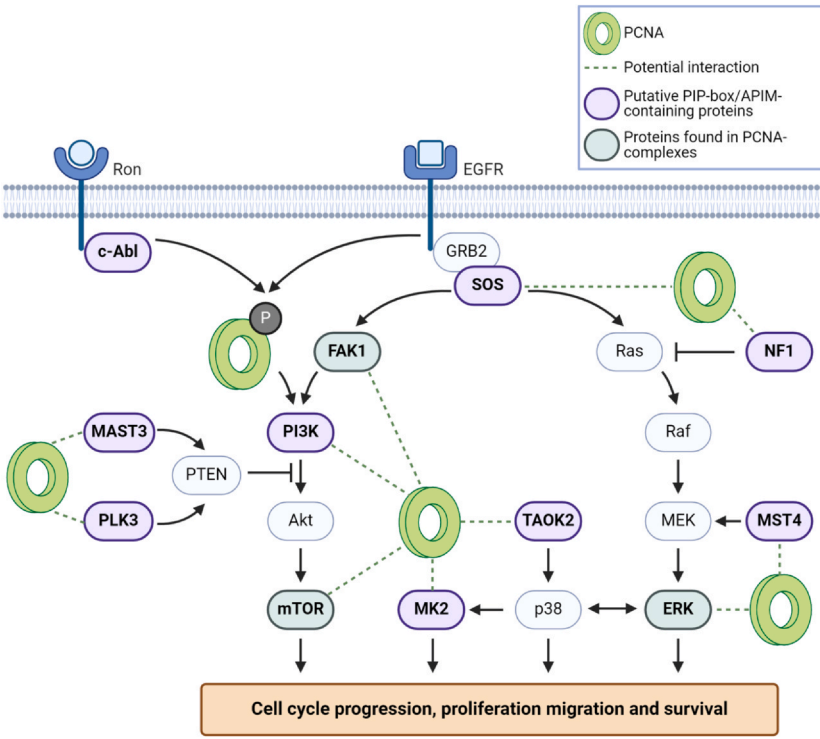

Fig. 6 Potential roles of PCNA in cellular signalling. Receptor tyrosine kinases activate downstream PI3K/Akt/mTOR and MAPK signalling pathways. These signalling pathways support cellular proliferation, progression and survival (reviewed in Iqbal & Iqbal, 2014; Wee & Wang, 2017). Many proteins involved in these pathways contain a putative PCNA-interacting motif (proteins in purple), or are found in complex with PCNA (proteins in green) and may therefor be potential PCNA-interacting partners (dashed lines).The Ron and EGFR receptors are also involved in the phosphorylation of PCNA, which has been shown to be involved in the activation of the Akt signalling pathway (Peng et al., 2019).

including MST4, NF1, MAPK1/ERK2, focal adhesion kinase 1 (FAK1) and mTOR (Olaisen et al., 2015) (Fig. 6).

Because proteins involved in the PI3K/Akt/mTOR and MAPK pathways are de-regulated in many cancers, there has been considerable effort to improve cancer therapy by targeting signalling components in these pathways. However, crosstalk between multiple signalling pathways can compensate for the inhibition of single targets and resistance to such therapies is therefore a major challenge (reviewed in Yap, Omlin, & de Bono, 2013). Targeting the roles of PCNA' in cellular signalling during

cellular stress may therefore be an interesting approach in cancer therapy, as this approach de-regulates multiple pathways simultaneously and this may reduce the risk of resistance by cellular reprogramming (Shaffer et al., 2017). In support of this approach, increased efficacy of an epidermal growth factor receptor (EGFR)/vascular endothelial growth factor (VEGFR) inhibitor in combination with an APIM-peptide was shown in a syngeneic orthopedic breast cancer model in mice (Søgaard et al., 2019). Targeting the PIP-box and APIM binding site on PCNA also reduced the ability of cells to generate resistance via mutations (Raeder et al., 2018).

Y211-phosphorylated PCNA has been shown to promote cancer progression and cell migration through epithelial-mesenchymal transition (EMT) and activation of the ataxia telangiectasia mutated (ATM)/Akt/Snail signalling pathway (Peng et al., 2019). This further supports the involvement of PCNA in signalling and suggests that inhibition of PCNA phosphorylation may have anti-cancer potential. EGFR and the Ron receptor tyrosine kinase (via c-Abl) can interact with and phosphorylate PCNA (Y211), thereby promoting cell growth (Shaul, 2000; Zhao et al., 2012). The c-Abl-PCNA interaction and the levels of phosphorylated PCNA both increase after ionising radiation in breast cancer cells, suggesting that the phosphorylation of PCNA and its association with c-Abl is an important response to DNA damage/stress. A peptide called the Y211F peptide interferes with the c-Abl/EGFR-PCNA interaction by competition, and thus PCNA phosphorylation, and has been shown to have anti-cancer activity (Zhao et al., 2012).

2.3.1 PCNA in the regulation of metabolism

Cancer cells often exhibit a metabolic phenotype known as the Warburg effect (Vander Heiden, Cantley, & Thompson, 2009). This effect is characterised by a high rate of glycolysis, even in the presence of oxygen, followed by an increased lactic acid production. This reprogramming/switching of energy metabolism is thought to support the increased demand for energy and biosynthetic precursors, allowing rapid proliferation of cancer cells (reviewed in Vander Heiden et al., 2009). Interestingly, increased glycolysis in chemotherapy-resistant leukaemia cells and acute myeloid leukaemia (AML) blast cells correlates with increased levels of cytosolic PCNA, suggesting a role for PCNA in regulating metabolism (Ohayon et al., 2016). In support of a direct role for PCNA in the regulation of cellular metabolism, seven of the glycolytic enzymes that catalyse steps 4–10 in the glycolysis pathway are

reported to be in complex with PCNA in pull-down experiments. These are aldolase (ALDA), triosephosphate isomerase (TPI), glyceraldehyd-3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (PGK1), phosphoglycerate mutase (PGM), enolase (ENO1) and pyruvate kinase (PKM2) (Naryzhny & Lee, 2010; Ohayon et al., 2016). Of these, ENO1 contains APIM (Olaisen et al., 2018), and mutation of APIM greatly reduced the binding of ENO1 to PCNA and resulted in reduced levels of ENO1 protein (Røst et al., 2023). Other phenotypic consequences of the APIM mutation were reduced proliferation and glucose consumption rates, increased levels of glycolytic intermediates above the step catalysed by ENO1, and reduced levels below. This reduction in glycolysis also resulted in reduced activation of the Akt signalling pathway and increased activation of enzymes in the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (Røst et al., 2023). Thus, disrupting the ability of ENO1 to interact with its scaffold PCNA directly affects cellular metabolism and indirectly affects cellular signalling (Fig. 7). Recently, a critical role for PCNA in the regulation of glycolysis in neutrophils has been reported, and the interactions between PCNA and several glycolytic enzymes, including ENO1, have been reported (Aymonnier et al., 2023). PCNA has also been shown to stimulate the enzymatic activity of ALDA and GAPDH (Naryzhny & Lee, 2010). Further PCNA directly interacts with nicotinamide phosphoribosyltransferase (NAMPT), which catalyses the first step of nicotinamide adenine dinucleotide (NAD+) biosynthesis, an essential cofactor in glycolysis (Ohayon et al., 2016). The PCNA-NAMPT interaction was not inhibited by p21-peptides, suggesting that NAMPT does not bind in the hydrophobic pocket as APIM and PIP-box-containing proteins do.

Targeting PCNA with an APIM-containing peptide drug (ATX-101) resulted in a metabolic shift characterised by both a reduction in glycolytic intermediates, likely mediated in part by reduced ENO1 and GAPDH levels, and reduced nucleoside phosphate pools (Røst et al., 2023). Interestingly, 6-phosphogluconate dehydrogenase (6PGD), which regulates the third step in the pentose phosphate pathway (PPP) and contains a putative PIP-box (Olaisen et al., 2018), was both enzymatically inhibited and subsequently degraded by ATX-101 treatment (Røst et al., 2023) (Fig. 7). In addition, 6PGD has recently been detected in PCNA pull-downs from neutrophils (Aymonnier et al., 2023). These findings support the role of PCNA in the regulation of primary metabolism.

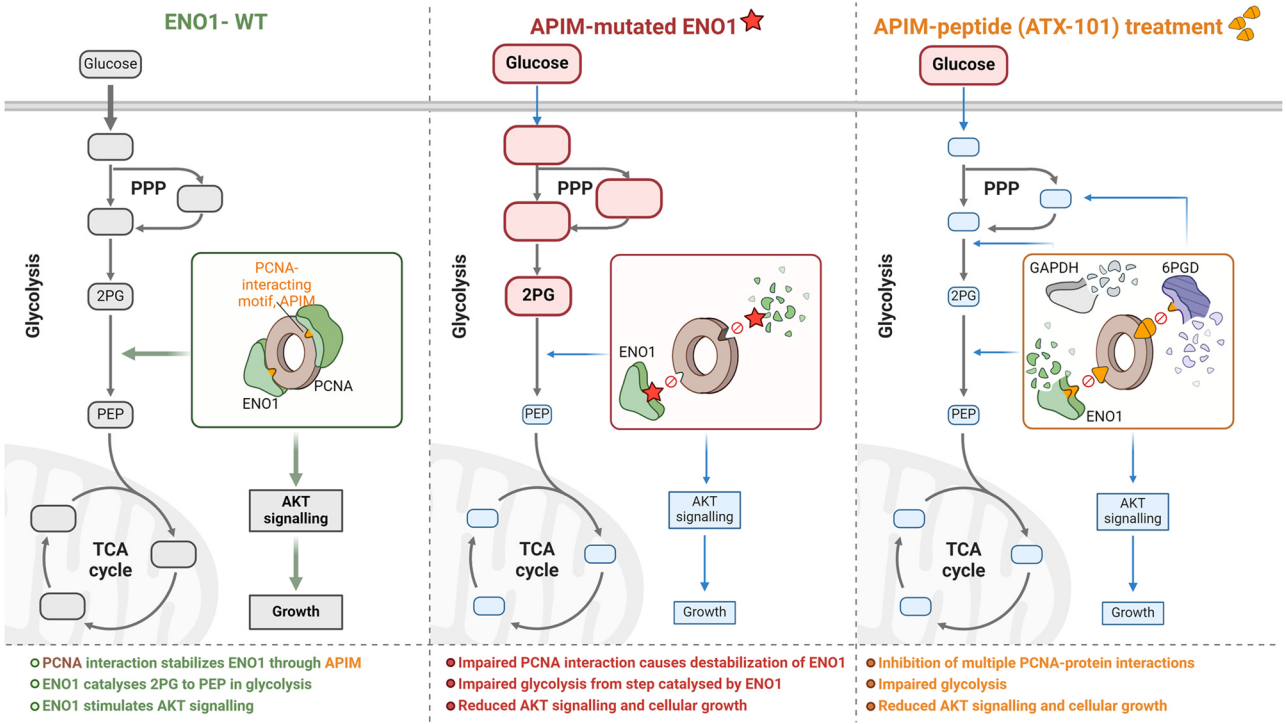

Fig. 7 PCNA stabilises ENO1 and regulates glycolysis. ENO1 catalyses glycolysis and stimulates Akt signalling. Interaction with PCNA stabilises ENO1 (left panel). Mutation of APIM in ENO1 leads to destabilisation of ENO1 and impaired glycolysis and Akt signalling (middle panel). Targeting PCNA with an APIM-containing peptide disrupts several PCNA-protein interactions, resulting in impaired glycolysis and reduced Akt signalling (right panel) (Røst et al., 2023).

2.3.2 PCNA in the regulation of apoptosis

Neutrophils play an important role in the early stages of infection and non-cancer inflammation. The resolution of these responses depends on neutrophils undergoing apoptosis (Galli, Borregaard, & Wynn, 2011), and defects in neutrophil clearance can lead to chronic inflammation (Landskron et al., 2014). During neutrophil differentiation, PCNA is transported to the cytosol where it plays a direct role in the regulation of apoptosis through interactions with procaspases 3, 8, 9 and 10 (Witko-Sarsat et al., 2010). Recently, it has been shown that the parasite *Toxoplasma gondii* exploits this anti-apoptotic function of PCNA to prolong its lifespan in infected neutrophils. *T. gondii* infection resulted in increased PCNA expression, increased PCNA-procaspase interactions, and delayed neutrophil apoptosis. Interestingly, this delay in apoptosis could be reversed by targeting PCNA with an APIM-peptide (Lima et al., 2021). PCNA has also been shown to interact with and regulate the p47phox subunit of NADPH oxidase, which is responsible for the production of reactive oxygen species (ROS) in neutrophils. ROS production is important for defence against pathogens, but excessive levels can cause tissue damage. Targeting PCNA with a small molecule (T2AA, see below) that interacts with the hydrophobic pocket of PCNA, same binding site as PIP-box and APIM, impaired the activation of NADPH oxidase and resulted in reduced ROS production and had an anti-inflammatory effect (Ohayon et al., 2019). This effect is likely to be caused not only by inhibition of the p47phox-PCNA interaction, but rather by the sum of the effects that targeting PCNA has on cellular signalling, primary metabolism, and apoptosis. Nevertheless, targeting PCNA may be a potential strategy for the treatment of neutrophil-dominated inflammatory diseases (Chiara et al., 2012).

The anti-apoptotic function of PCNA is not specific to neutrophils, as it has been shown that PCNA binds procaspase-9 in neuronal cells, and that targeting PCNA with an APIM-peptide in MM and glioblastoma cells reduces the amount of procaspase-3 binding to PCNA and/or induces activation of caspase 3, 7, 8 and 9 (Gravina et al., 2022; Muller et al., 2013; Yin et al., 2015). This suggests a direct role for PCNA in the regulation of apoptosis in many cell types, including cancer cells.

2.3.3 PCNA in the regulation of the tumour microenvironment (TME)

Evasion of the immune system is one of the hallmarks of cancer and can be mediated by the development of an immunosuppressive TME that supports

cancer growth and survival (reviewed in Muenst et al., 2016). The metabolic state known as the Warburg effect is observed in many cancer cells as well as in activated immune cells and therefore metabolism is important for the immunoreactivity of the TME.

Monocytes and neutrophils are important cells in our innate immune system and are also part of the TME. They produce and secrete cytokines and chemokines as a response to damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) when these bind to toll-like receptors (TLRs) (reviewed in Galli et al., 2011). DAMPs are derived from the host cells, including tumour cells, dead cells or products released by dying cells or stressed cell. Both monocytes and neutrophils contain significant amounts of cytosolic PCNA (Müller et al., 2013; Witko-Sarsat et al., 2010). Targeting PCNA in TLR-stimulated monocytes with an APIM-containing peptide has been shown to affect PI3K/Akt and MAPK signalling, resulting in reduced secretion of cytokines and chemokines, e.g., IL-4 and IL-10 (Olaisen et al., 2015). Cytokines and chemokines are important for the recruitment and activation of immune cells. For instance, IL-4 and IL-10 are anti-inflammatory cytokines important for the differentiation of monocytes into M2 macrophages. These macrophages promote tumourigenesis and have immunosuppressive roles (reviewed in Wu & Dai, 2017; Kwaśniak et al., 2019). Therefore, altered cytokine secretion is likely to affect the tumour ecosystem (Fig. 8). PCNA has recently been shown to be important for the regulation of the glycolysis in both monocytes and neutrophils (Aymonnier et al., 2023; Røst et al., 2023). In addition to cytokines, regulation of caspases, in particular caspase-8, has recently been shown to be important for immunologically hot (inflamed) or cold TME (Gong et al., 2023). As PCNA has been shown to be important for the regulation of apoptosis via interaction with, among others, procaspase 8 (Witko-Sarsat et al., 2010), it is likely that PCNA has a direct impact on immunogenicity in the TME. A direct link between the role of PCNA in metabolism and regulation of the immune response may be mediated through interaction with 6PGD. Targeting PCNA has recently been shown to impair 6PGD activity (Røst et al., 2023), and inhibition of 6PGD has been reported to reprogramme the metabolism of CD8 + T cells and increase their tumouricidal activity (Daneshmandi et al., 2021). In addition, pyruvate dehydrogenase kinase (PDK1) has recently been implicated in the regulation of glycolysis in T cells (Menk et al., 2018). PDK1 is rapidly activated via interaction with the T cell receptor subunits of the Src-related protein tyrosine kinase Lck and

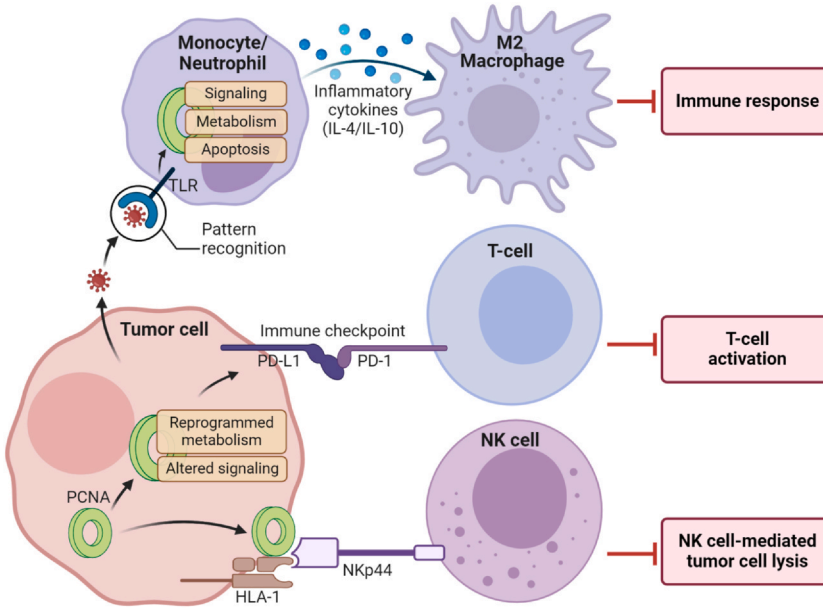

Fig. 8 PCNA regulation of the immune response involves altered signalling and reprogrammed metabolism in multiple immune cells. Cytokines are secreted by neutrophiles/monocytes/macrophages in response to toll-like receptor (TLR) stimulation. Blocking PCNA-protein interactions has been shown to reduce the secretion of IL-4 and IL-10, suggesting that PCNA affects cytokine secretion (Olaisen et al., 2015). In addition, all immune cells have PCNA and the regulation of pathways involving PCNA will affect their role and activity in the tumour microenvironment (TME). Tumour cells also express PD-L1, which can bind PD-1 on the surface of T cells, and immune checkpoint interaction inhibits T cell activation and thus impairs the immune response in the TME (reviewed in Chen et al., 2016). PD-L1 expression is stimulated by oncogenic MAPK and PI3K/Akt signalling. PCNA may therefore be involved in immune signalling through its role in cellular signalling. PCNA is recruited to the surface of tumour cells and, together with HLA-1, forms a ligand for the natural cytotoxicity receptor NKp44 on natural killer (NK) cells. This interaction inhibits NK cell-mediated lysis of the tumour cells and may be an immune evasion mechanism (Horton et al., 2013).

zeta-chain-associated protein kinase-70 (ZAP70). Interestingly, PDK1 contains a putative PIP-box and ZAP70 contains a putative APIM (Gilljam et al., 2009; Olaisen et al., 2018). Thus, PCNA may be an important scaffold in a process that regulates metabolism during immune responses.

Pairs of inhibitory ligands and receptors, such as programmed death ligand 1 (PD-L1) and programmed death 1 (PD-1), also known as immune checkpoints, are part of the mechanism for avoiding inappropriate and excessive immune responses (reviewed in Chen et al., 2016). These

receptors are found on both immune cells and on cancer cells, i.e., cancer cells often express inhibitory receptors such as PD-L1 on their surface that prevent the T cells from exerting their cytotoxic activity. This is one of the mechanisms cancer cells use to evade the immune system (Fig. 8). Immune checkpoint inhibitors can boost the immune response and these drugs have ushered in new era of cancer therapy. The expression levels of immune checkpoint receptor pairs are tightly regulated, e.g., PD-L1 expression is upregulated in response to inflammatory cytokines and oncogenic signalling such as activated MAPK and PI3K/Akt signalling pathways (Du et al., 2020; Liang et al., 2020). Therefore, targeting the pathways that stimulate PD-L1 expression is considered promising for improving the response to PD-L1 inhibitors in cancer therapy (reviewed in Chen et al., 2016). PCNA is therefore likely to influence expression of immune checkpoint receptors and the immune response in the TME via its role as a regulator of cellular signalling (Fig. 8).

Finally, NK cells serve as a primary defence against infected cells and cancer cells, and are important immune cells in the TME (Lodoen & Lanier, 2006; reviewed in Muenst et al., 2016). The NKp44 is expressed on activated NK cells, and the binding of ligands to this receptor is important for NK-mediated lysis of cancer cells (Moretta et al., 2001; Vitale et al., 1998; reviewed in Parodi et al., 2019). PCNA can be recruited to the cell surface of cancer cells and, together with human leukocyte antigen class 1 (HLA-1), forms an inhibitory ligand for NKp44 (Horton, Mathew, & Mathew, 2013; Rosental et al., 2011). This interaction inhibits NK cell activation and lysis of the interacting cancer cell and may therefore be an additional mechanism by which cancer cells evade the immune system (Rosental et al., 2011, 2012; Fig. 8). Targeting this blockade of NK cells is an interesting strategy to improve cancer therapy (reviewed in Yang et al., 2020).

A summary of the various stress responses reported to be regulated by PCNA is shown in Fig. 9.

2.4 Drugs targeting PCNA

Targeting the roles of PCNA in DNA replication is likely to result in undesirable toxicity in patients because this is an essential process in healthy cells as well, e.g., blocking replication will affect rapidly replicating normal cells such as haematological cells and epithelial cells of the gastrointestinal tract. It is also worth considering that many drugs that effectively inhibit the rapid proliferation of cancer cells in preclinical studies often fail in

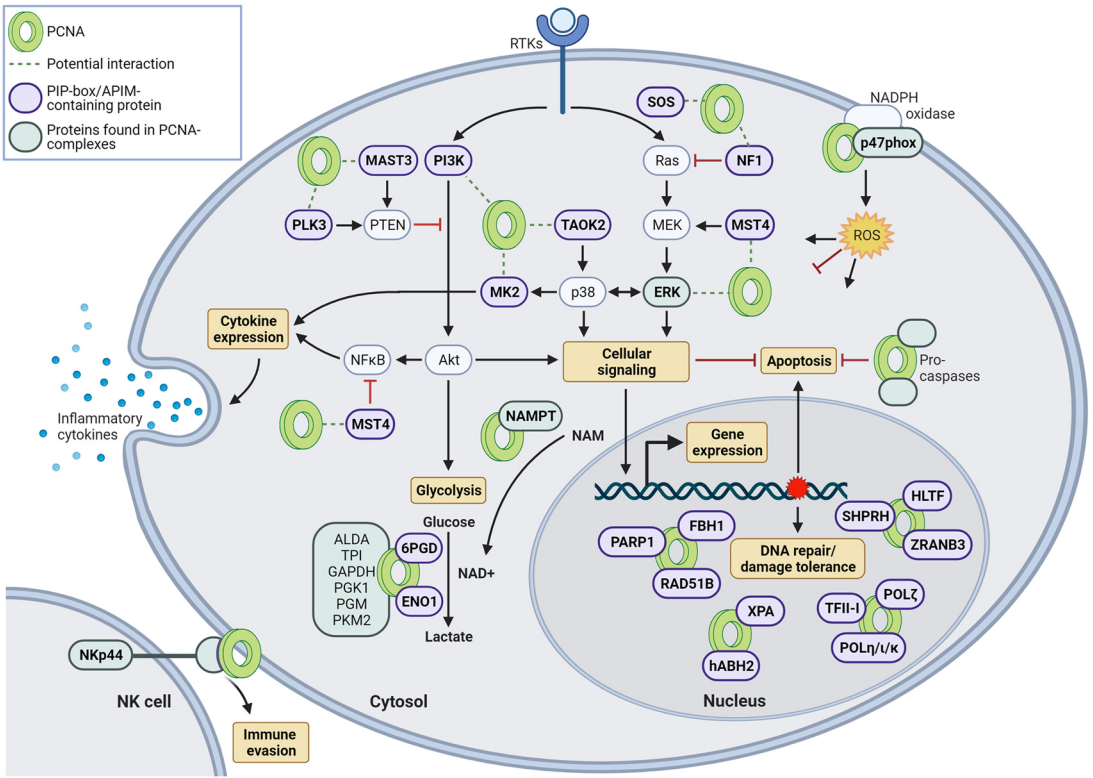

Fig. 9 See figure legend on opposite page.

clinical trials. This is probably because most cancer cells in patients have much longer doubling times than those in most cell lines used in preclinical animal cancer models (reviewed in Chan, Koh, & Li, 2012). The aim should therefore be to target the stress-related functions rather than rapid proliferation.

The only known disease-causing mutation in PCNA is serine (S) 228 mutated to isoleucine (I), S228I. This mutation does not affect the overall PCNA expression levels, and importantly, does not disrupt DNA replication (Baple et al., 2014); however, these patients have a severe repair deficiency phenotype (Baple et al., 2014; Green, Baple, & Crosby, 2014). Amino acid 228 is in proximity to the protein binding site on PCNA and the S228I mutation therefore causes conformational changes in the IDCL region. This alters the binding affinity for several PCNA interaction proteins but has the greatest effect on proteins with low affinity PCNA interacting motifs (Duffy, Hilbert, & Kelch, 2016). The knowledge gained from this disease supports the possibility of targeting PCNA without inhibiting all PCNA-protein interactions, and this should be exploited in the design of anti-cancer drugs targeting PCNA to avoid excessive toxicity and increase cancer specificity.

Four main strategies for targeting PCNA have been developed and/or are currently in progress: (i) targeting PCNA stability and/or loading, (ii) targeting the hydrophobic pocket, (iii) targeting the PCNA-NKp44 interaction, and (iv) competing with PCNA (reviewed in Wang, 2014; Altieri & Kelman, 2018; Cardano, Tribioli, & Prosperi, 2020) (Fig. 10).

Fig. 9 Scaffolding functions of PCNA important in cellular stress responses. Nuclear roles: Several APIM-containing proteins involved in DNA repair and DNA damage tolerance have been shown to depend on their APIM-PCNA interactions for optimal function (Bacquin et al., 2013; Ciccia et al., 2012; Fattah et al., 2014; Gilljam et al., 2009, 2012; Raeder et al., 2018; Seelinger & Otterlei, 2020). **Cytosolic roles**: Several cytosolic proteins involved in cellular signalling and metabolism, including glycolytic enzymes, contain APIM or PIP-box motifs (proteins in purple) or are found in complex with PCNA (proteins in green) (Naryzhny & Lee, 2010; Ohayon et al., 2019; Olaisen et al., 2015, 2018; Witko-Sarsat et al., 2010). Studies have shown that impairment of PCNA scaffolding function affects multiple signalling pathways including the PI3K/AKT and MAPK pathways and cytokine production following toll-like receptor stimulation (Olaisen et al., 2015, 2018; Søgaard et al., 2019), regulation of apoptosis (Müller et al., 2013; Witko-Sarsat et al., 2010; Yin et al., 2015), NK cell cytotoxicity (Rosental et al., 2011; Shemesh et al., 2018), and cellular defence against ROS (Ohayon et al., 2019).

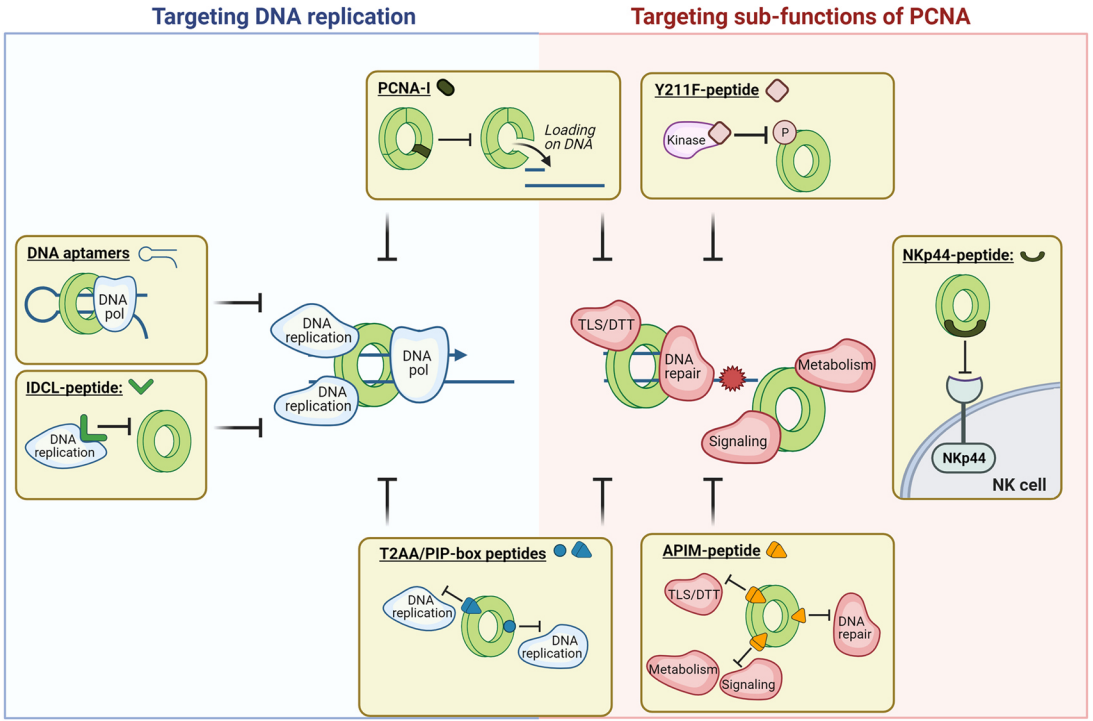

Fig. 10 See figure legend on opposite page.

2.5 PCNA inhibitors

A class of compounds called PCNA inhibitors (PCNA-I) bind to the interface between the monomers of PCNA. This binding stabilises the homotrimer and prevents it from opening and loading onto DNA, thereby impairing DNA replication and causing replication stress and cell death. These inhibitors have been shown to reduce the proliferation of several cancer cells at lower concentrations than non-cancerous cells, although the mechanism is unclear (Dillehay et al., 2015; Tan et al., 2012). Another strategy to interfere with PCNA-chromatin interaction is the use of DNA aptamers, which are short DNA sequences with high specificity and affinity for their target. An anti-PCNA aptamer has been shown to compete with primer-template DNA for binding of the PCNA-DNA polymerase δ/ε complex, and thereby interfering with DNA replication (Kowalska et al., 2018).

Another class of inhibitors target PCNA by directly interfering with PCNA-protein interactions. The small molecule inhibitor T2AA, a derivative of the thyroid hormone T3 that lacks hormonal activity, binds to the hydrophobic pocket of PCNA. T2AA inhibits the PCNA-p21 PIP-box interaction, which is the strongest known, thus, inhibiting most protein interactions that occur at this site (Punchihewa et al., 2012), at least when

Fig. 10 Strategies for targeting PCNA. Several strategies to target PCNA have been and are being investigated to target PCNA. Many strategies aim to inhibit the role of PCNA in DNA replication. PCNA-I binds to the interface between monomers and stabilises the PCNA ring, preventing it from opening and loading onto DNA (Dillehay et al., 2015). DNA aptamers compete with primer-template DNA for the binding of the PCNA-DNA polymerase complex (Kowalska et al., 2018). IDCL-peptides are based on sequences from the IDCL region and compete with PCNA for its protein interaction partners, such as DNA replication proteins, rather than targeting PCNA directly (Lingeman, Hickey, & Malkas, 2014). Both the small molecule T2AA and the p21-based PIP-box peptides bind with high affinity to the hydrophobic pocket of PCNA and prevent even high affinity proteins from binding to PCNA and thereby inhibiting DNA replication (Punchihewa et al., 2012; Warbrick et al., 1995). Other strategies aim to target only a subset of PCNA functions. The Y211F-peptide competes with PCNA for the kinases responsible for PCNA phosphorylation, thereby inhibiting this modification of PCNA. This only affects the sub-function of PCNAs that is dependent on phosphorylation (Wang et al., 2006). The APIM-peptide is a cell-penetrating peptide containing APIM, which binds with lower affinity to the same protein binding site on PCNA as the PIP-box. Therefore, APIM-peptides mainly block a subset of PCNA-interacting proteins (Muller et al., 2013). The NKp44-peptide is based on the part of the NKp44 receptor that binds PCNA. It targets PCNA and blocks NKp44-PCNA binding and thereby PCNA-induced inhibition of NK cell-mediated tumour cell lysis (Shemesh et al., 2018).

PCNA is unmodified. As a result, T2AA effectively disrupts PCNA functions in DNA replication, TLS and DNA repair (Actis et al., 2013; Inoue et al., 2014; Punchihewa et al., 2012). A low affinity variant of T2AA, AOH1160, targets a slightly different part of the IDCL/hydrophobic pocket of PCNA (Gu et al., 2023). It does not prevent the canonical PIP-box-peptides from binding to PCNA, but AOH1160 has been shown to interfere with DNA replication and to inhibit DNA repair. Nevertheless, AOH1160 induces apoptosis in cancer cells and reduces cancer growth in xenograft models without causing significant toxicity to non-cancerous cells. AOH1160 also enhances the efficacy of cisplatin therapy, supporting that inhibition of selective PCNA-protein interactions may be beneficial in combination with genotoxic stress (Gu et al., 2018). A variant of AOH1160, AOH1996, can be administered orally, has shown promising activity in preclinical models and is currently in Phase I. Interestingly, the main mode of action of AOH1996 is suggested to be an increased interaction between RNA Pol II complexes and PCNA during transcription-replication conflicts; however, the molecular mechanisms for this are not shown (Gu et al., 2023).

2.6 PCNA-targeting or competing peptides

In general, the use of peptides as drugs is considered challenging in terms of delivery as they often have short half-lives in the blood. However, the advantage of using peptides is that peptides are larger than small molecules and are therefore often more specific. In addition, natural peptide motifs are likely to respond to the normal regulation of affinity mediated by PTMs. Several peptides targeting PCNA-protein interactions are currently under development.

1. **The NKp44-peptide.** A cell-penetrating peptide linked to a peptide from the PCNA binding region of NKp44 (R11-I-KKKRK-W-EAS-ALVCIRLVTSSKPRTVA) was shown to interact with PCNA and partially block PCNA from acting as a ligand for NKp44. Inhibition of the NKp44-PCNA interaction increased NK cell activation and tumour cell lysis (Shemesh et al., 2018). The similarly composed APIM-containing peptide (ATX-101, Ac-MDRWLVK-W-KKKRK-I-R11 (Muller et al., 2013)) was unable to block the NKp44-peptide from interacting with PCNA, suggesting that these peptides have different affinities for PCNA or different binding sites on PCNA. The NKp44-peptide reduced tumour growth in animal models of breast cancer and melanoma, and the anti-cancer effect was mediated by the NKp44-

derived part of the peptide, as a truncated form of the same peptide containing mainly the cell penetrating part had no effect. However, the mode of action of the NKp44-peptide is not solely due to the blocking the PCNA-NKp44 interaction, as the peptide kills cancer cell lines also in the absence of NK cells and reduces tumour growth in mice that do not express the NKp44 receptor (Shemesh et al., 2018). This suggests that this interaction site on PCNA has a biological function beyond binding to NKp44. The NKp44-peptide binds normal/unmodified PCNA; however, in vivo studies in mice showed low toxicity (Shemesh et al., 2018). An alternative strategy to target the NKp44-PCNA interaction is the use of an antibody, called 14-25-9, which blocks the interaction site. In support of its functionality, IFN-γ release by NK cells in vitro was increased in the presence of 14-25-9 when co-cultured with cancer cells. *In vivo,* 14-25-9 increased NK cell activity and reduced tumour growth in a patient-derived head and neck squamous cell carcinoma xenograft model (Kundu et al., 2019).

2. **The Y211F-peptide.** The Y211F-peptide contains a cell-penetrating peptide (HIV-TAT) linked to the 12 amino acids flanking tyrosine 211 on PCNA, but with tyrosine 211 being replaced by phenylalanine. The Y211F-peptide interferes with PCNA phosphorylation by binding to the relevant phosphorylating kinases and thereby inhibiting PCNA phosphorylation by competition. This is likely to affect only the subset of PCNA functions that are dependent on 211 tyrosine phosphorylation. This phosphorylation is known to increase the stability of chromatin bound PCNA, but it is not essential for DNA replication. The levels of Y211 phosphorylated PCNA correlate better with poor survival in prostate and breast cancer than total PCNA levels, suggesting that this PCNA modification is important for cancer progression (Ortega et al., 2015; Wang et al., 2006; Yu et al., 2013; Zhao et al., 2011). Y211 phosphorylation of PCNA has been shown to impair the initiation of mismatch repair (MMR), probably by reducing the interaction between PCNA and MMR proteins, and possibly by introducing more misincorporations during DNA synthesis through the recruitment of error-prone TLS polymerases (Ortega et al., 2015). Reduced MMR, increased TLS and thus increased mutation frequency may drive the development of malignancy and drug resistance. In addition, phosphorylated PCNA has been reported to promote cancer progression through the activation of the Akt signalling pathway, cell migration and other EMT-associated features (Peng et al., 2019). Given the association

between cancer cells and phosphorylated PCNA, the Y211F-peptide are likely to primarily target cancer cells (Zhao et al., 2011). Suppressed viability and increased apoptosis were observed in breast cancer cells treated with the Y211F-peptide, but not in mammary epithelial cells derived from normal breast tissue, supporting a selectivity for cancer cells (Yu et al., 2013). Importantly, the Y211F-peptide increased the radiosensitivity in breast cancer cell lines and reduced the growth of aggressive triple-negative breast cancer cells and hormone-insensitive prostate cancer cells in xenograft models (Yu et al., 2013; Zhao et al., 2011).

3. **PIP-box-peptides.** The strong interaction between PCNA and p21 motivated the first strategy to inhibit PCNA functions with peptides. Peptides with the PIP-box of p21 and the cell-penetrating peptide of HIV-TAT, were shown to be delivery to the nucleus and to induce apoptosis in cancer cells by interfering with PCNA-protein interactions (Baker, Howl, & Nicholl, 2007). More recently, this strategy has been further developed by peptidomimetics that covalently modify the p21-based PIP-box to confine the peptide to the same 3_{10}-helical structure as it has in p21 when bound to PCNA. This strategy increased the biostability and specificity of the peptide (Wegener et al., 2018; reviewed in Horsfall, Abell, & Bruning, 2020). However, peptides derived from the PIP-box of p21 also bind PCNA with high affinity and block DNA replication also in normal cells (Warbrick et al., 1995).
4. **IDCL-peptide.** Instead of designing peptides based on the binding sequences in PCNA-interacting proteins, an alternative strategy is based on peptide sequences from the protein binding site and IDCL region of PCNA. The caPeptide is a cell-penetrating peptide containing eight amino acids homologous to a part of the IDCL region that is accessible and important for protein binding. This peptide, R9-cc-caPeptide, reduced the levels of FEN-1 and LIG1 bound to PCNA, interfered with DNA replication and DNA repair and reduced cancer cell growth. The cytotoxicity induced by the R9-cc-caPeptide was reported to be higher for cancer cells than for non-cancerous cells. Furthermore, the R9-cc-caPeptide has been shown to enhance the efficacy of cisplatin in several cancer cell lines and in xenograft cancer models (Gu et al., 2014; Lingeman et al., 2014; Smith et al., 2015).
5. **The APIM-peptide, ATX-101.** The PIP-box-based cell-penetrating peptides bind with high affinity to the hydrophobic cavity of PCNA and block the interaction site for replicative proteins (Warbrick et al., 1995;

Warbrick, 2006). An alternative strategy is to target PCNA-protein interactions that occur mainly during stress, i.e., proteins that contain low affinity binding motifs under normal conditions, for example by using APIM-containing peptides (Gilljam et al., 2009). An APIM-containing peptide, ATX-101, is currently under development for cancer therapy (Lemech et al., 2023; Muller et al., 2013). ATX-101 inhibits protein-PCNA interactions but has a 3-fold lower affinity for recombinant, unmodified PCNA in vivo than canonical PIP-box-peptides. It is rapidly imported into cells and increases the efficacy of multiple chemotherapeutics (Muller et al., 2013; Olaisen et al., 2015; Prestel et al., 2019). ATX-101 rapidly induces apoptosis in all phases of the cell cycle in cancer cell lines without affecting cell cycle progression (Muller et al., 2013; Søgaard et al., 2018a, 2018b), and enhances the anti-cancer efficacy of MMC in vivo also in an endogenously induced slow-growing bladder cancer model (Gederaas et al., 2014b). The data supports that ATX-101 does not inhibit replication, but affects multiple stress defence pathways and processes. These include: (i) reduced DNA repair (Gilljam et al., 2012; Søgaard et al., 2018a), (ii) reduced TLS/mutagenesis (Ræder et al., 2018; Seelinger & Otterlei, 2020), (iii) increased apoptosis (Gravina et al., 2022; Muller et al., 2013; Søgaard et al., 2018a), (iv) altered signalling and immunity (Olaisen et al., 2015, 2018; Søgaard et al., 2018a, 2019), and altered primary metabolism (Røst et al., 2023). This multi-targeting strategy is likely to explain the broad anti-cancer activity observed across multiple indications and in combination with various chemotherapeutics and kinase inhibitors detected in preclinical animal cancer models (MM, bladder, breast, prostate, glioblastoma) (Gederaas et al., 2014b; Søgaard et al., 2018a, 2018b, 2019). Intravenously administrated ATX-101 is rapidly distributed to all tissues including the brain (Søgaard et al., 2018a), and has also recently been shown to reduce the growth of intracranial glioblastoma both as a single agent and in combination with radiotherapy in mice (Gravina et al., 2022).

The growth and survival of non-cancerous cell lines, primary monocytes and lymphocytes are largely unaffected by ATX-101 at concentrations that kill multiple cancer cell lines (Olaisen et al., 2015). However, primary metabolism and cellular signalling, and consequently cytokine and chemokine production following TLR ligand stimulation were strongly reduced by ATX-101 in monocytes (Muller et al., 2013; Røst et al., 2023). Thus, altered immune signalling induced by ATX-101 is likely to affect the TME.

ATX-101 was the first-in-class PCNA-targeting drug at clinical stage. Phase I data showed that the drug was well tolerated with no myelosuppression or any other grade 3 or 4 toxicities (Lemech et al., 2023). This supports that ATX-101 mainly targets PCNA functions that are important during cellular stress and that toxicity in normal cells is low. Interestingly, a disease stabilisation was observed in 70% of the efficacy population, which included heavily pre-treated, late stage solid tumour patients (all-comers). The prolonged stable disease observed in these patients (median 4.2 months) may be explained, at least in part, by the multiple effects of targeting stress-related functions of PCNA, including effects on primary metabolism and cellular signalling in both cancer cells and activated immune cells in the TME. ATX-101 is currently undergoing two Phase Ib/II studies on ovary cancer (NCT04814875) and sarcoma (NCT05116683).

3. Conclusion/summary

The canonical roles of PCNA in DNA replication and repair are well known and represent interesting targets for cancer therapy. However, the recently discovered roles of PCNA in cellular signalling, metabolism and apoptosis may be equally important in a cancer therapy setting. As a scaffold protein, PCNA interacts with multiple proteins in a highly coordinated manner depending on growth and cell cycle status, stress levels and PTMs on itself and interacting proteins. Recent data support the possibility of targeting sub-populations and/or non-canonical roles of PCNA, opening a new era for PCNA as a cancer drug target. To date, only one drug has completed Phase I clinical trials with favourable toxicity, but with advances in the understanding of the multiple roles of PCNA, several drugs targeting PCNA are expected to be developed in the coming years.

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