Novel mechanisms of DNA damage and replication stress signaling: Functional roles of the WDR82/PNUTS-PP1 phosphatase complex

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"It is (…) remarkable that after a seemingly miraculous feat of morphogenesis a complex metazoan should be unable to perform the much simpler task of merely maintaining what is already formed"

From "Pleiotropy, Natural selection and the evolution of senescence"
George C. Williams 1957

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Oslo, April 2020

Lise E. Sandquist

LIST OF PAPERS

List of papers included in the thesis, referred to as Papers I-III in the text:

Paper I

Regulation of ATR activity via the RNA polymerase II associated factors CDC73 and PNUTS-PP1

Landsverk HB, Sandquist LE, Sridhara SC, Rødland GE, Sabino JC, de Almeida SF, Grallert B, Trinkle-Mulcahy L, Syljuåsen RG.

Nucleic Acid Research. 2019 Feb 28;47(4):1797-1813.

Paper II

WDR82/PNUTS-PP1 prevents transcription-replication conflicts by limiting RNA polymerase II residence time

Landsverk HB*, Sandquist LE*, Bay LTE*,Steurer B, Campsteijn, C, Grallert B, Landsverk, OJB, Marteijn JA, Petermann E, Trinkle-Mulcahy L, Syljuåsen RG.

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Paper III

WDR82 protects cancer cells against inhibitors of Wee1 kinase

Sandquist LE, Landsverk HB, Syljuåsen RG.

Manuscript

1 Introduction

Cancer represents a serious health problem. A recent study showed that cancer has bypassed cardiovascular disease as the main cause of death in higher income countries [1]. Cancer is a disease of the genome and age is the greatest risk factor for cancer. With an increasing population of elderly, cancer is becoming an ever more important health burden worldwide [2]. Interestingly, unrepaired or misrepaired DNA damage is a major contributor to aging as well as cancer and the two processes of ageing and carcinogenesis are tightly interconnected.

However, not all tissues develop cancers at an equal rate. The lifetime risk of cancer development for a specific tissue type strongly correlates with the total number of cell divisions for the stem cells that maintain the tissue homeostasis [3]. In fact, no other environmental or inherited factors are known to show such a strong correlation across tumors [3]. This finding implies that the process of DNA replication and cell division represents a major risk for cancer development, and factors that cause increased cell proliferation or problems with the replication process will add to the risk. Sustained proliferation and replication stress are hallmarks of cancer [4], and stalled replication forks have been shown to induce genomic rearrangements associated with cancer [5, 6]. Understanding the underlying mechanisms of DNA damage and replication stress signaling, and how the cells cope with it, will thus help understanding the etiology of cancer.

1.1 The DNA damage response

The DNA is the blueprint for everything that makes up an organism, and thus, maintenance of the DNA is essential for an organism to be healthy and survive. Organisms have evolved specialized surveillance systems to preserve the integrity of the DNA; these systems are collectively called the DNA damage response (DDR) [7]. The DNA damage response is a network of signaling pathways that respond to DNA damage and replication stress, and cause cellular changes that will eventually lead to protection of the genome. The term “DNA damage” refers to a number of different lesions, both physical and chemical, that compromises the structure of DNA [8] and DNA damage can arise from both internal and external sources.

An internal source of DNA damage may for instance be reactive oxygen species (ROS) generated during normal cell metabolism. ROS can oxidize the bases that make up the DNA, causing base conversions and mismatches [9]. These mismatches may cause local structural distortion of the DNA [10]. In addition, ROS can also cause single strand DNA breaks (SSBs) [7]. Another internal source of DNA damage is the synthesis of DNA during S-phase (also termed DNA replication). The process of DNA replication is inherently dangerous since the chromatin has to be opened up and the single strands of DNA exposed. Single-stranded DNA (ssDNA) is more vulnerable to chemical and enzymatic degradation than double stranded DNA, and needs to be protected by ssDNA binding proteins [11]. Replication forks may stall at obstacles, and if they are not sufficiently protected, they may collapse and cause double strand breaks. In addition, replication causes torsional stress in the DNA helix that will have to be resolved by topoisomerases. Topoisomerases cause coordinated DNA breakage to relieve the torsional stress, and any errors in this process may generate unwanted DNA breaks [7]. Furthermore, if the DNA polymerase erroneously incorporate the wrong base into the newly formed DNA strand during the replication, there will be a mismatch in base pairing.

External sources of DNA damage can be physical or chemical. A major physical source of DNA damage is UV light from the sun. UV light causes the formation of photodimers that can distort the DNA helix and block both replication and transcription [12]. It is estimated that

exposure to UV light can cause up to 100,000 DNA lesions per cell per day [13]. Moreover, if we undergo medical treatment such as radiotherapy or medical examination by X-ray imaging, we will be exposed to ionizing radiation (IR). IR can cause many different types of DNA damage, but the most harmful is double stranded DNA breaks (DSBs) [7]. We are also continuously exposed to low amounts of IR due to cosmic background radiation or naturally occurring radioactive materials in the environment (e.g. radon). Moreover, nuclear accidents and nuclear weapon testing can cause exposure to IR. An example of a chemical source of DNA damage, and a major contributor to cancer prevalence, is cigarette smoke. A cigarette contains >4500 chemicals of which at least 60 have been proven carcinogenic. These chemicals cause a large variety of alterations to DNA [14].

Many different repair mechanisms have evolved to handle DNA damage. Mismatch repair (MMR), base excision repair (BER) and nucleotide excision repair (NER) tackle smaller errors such as single base alterations or short stretches of DNA containing helix distorting lesions (<30nt for NER) [15]. Transcription coupled repair (TCR) is a sub pathway of NER that acts on DNA alterations that stall the RNA polymerase [16]. More extensive damage, such as interstrand and intrastrand crosslinks or SSBs, can be handled by the interstrand crosslink repair pathway (also called the Fanconi repair pathway), NER or the single strand break repair pathway (SSBR), respectively. The two main pathways for DSB repair are homologous recombination (HR), which requires a sister chromatid present, and non-homologous end joining (NHEJ) [7, 17].

The ultimate goal of the DNA damage response is to protect the DNA in order for the organism to stay healthy. DNA damage signaling leads to a number of different effects including DNA repair, protection of replication forks, cell cycle arrest, apoptosis etc. For the sake of this thesis, I will not go into detail about repair mechanisms, but rather focus on how the cell can detect damage and recruit the apical kinases that set off the DNA damage response signaling cascades. I will mainly focus on the response to DNA double strand breaks (DSBs), the most deadly type of DNA damage, and on the response to replication stress. In a later chapter, I will look more specifically into how replication stress activates the ATR signaling pathway, and how transcription can be a source of replication stress.

1.1.1 The apical kinases of the DNA damage response

Central to the DNA damage response are the three related kinases Ataxia telangiectasia mutated (ATM), ATM and RAD3-related (ATR) and DNA dependent protein kinase (DNA-PK). Their kinase domains are similar to that of the phosphoinositide 3-kinase (PI3K) and they thus comprise the family of PI3K-related kinases (PIKKs) [18]. Despite the similarity in the kinase domain of the PIKKs and the PI3K, the PIKKs do not phosphorylate lipids but rather serine or threonine residues followed by a glutamine (so-called ST/Q sites) [19]. ATM, ATR and DNA-PK also share other common domains apart from their kinase domain: They have HEAT-repeats at the N-terminus, followed by FAT domains, the kinase domain and a FATC domain at the C-terminus. Another similar feature is that they all require accessory proteins for recruitment to DNA damage sites: DNA-PK is recruited by Ku80 [20, 21], ATM by NBS1 [22], and ATR by ATRIP [23].

Both ATM and DNA-PK respond to DNA double strand breaks. However, while ATM activation leads to phosphorylation of a number of different proteins involved in DNA repair and checkpoint activation, DNA-PK has a lesser role in signaling and mainly responds to DSBs to facilitate NHEJ [18]. ATR mainly responds to ssDNA formed as intermediates in DNA repair, or during replication stress [24]. Collectively, ATM and ATR can phosphorylate more than 700 proteins [25], most of which have a role in DDR. Unlike ATR, neither ATM nor DNA-PK are essential for cell survival, highlighting the importance of ATR in protecting the genome [26]. More details into how ATR functions is given in a later chapter (1.2.3).

1.1.2 Sensing DNA damage

One can think of sensing DNA damage in the context of recruiting proteins involved in processing of the lesion, or in the context of activating a large scale DNA damage signaling response which causes cell cycle arrest and other drastic effects on the cell (**Figure 1**). The proteins that “sense” the DNA damage do not necessarily cause a full-blown activation of

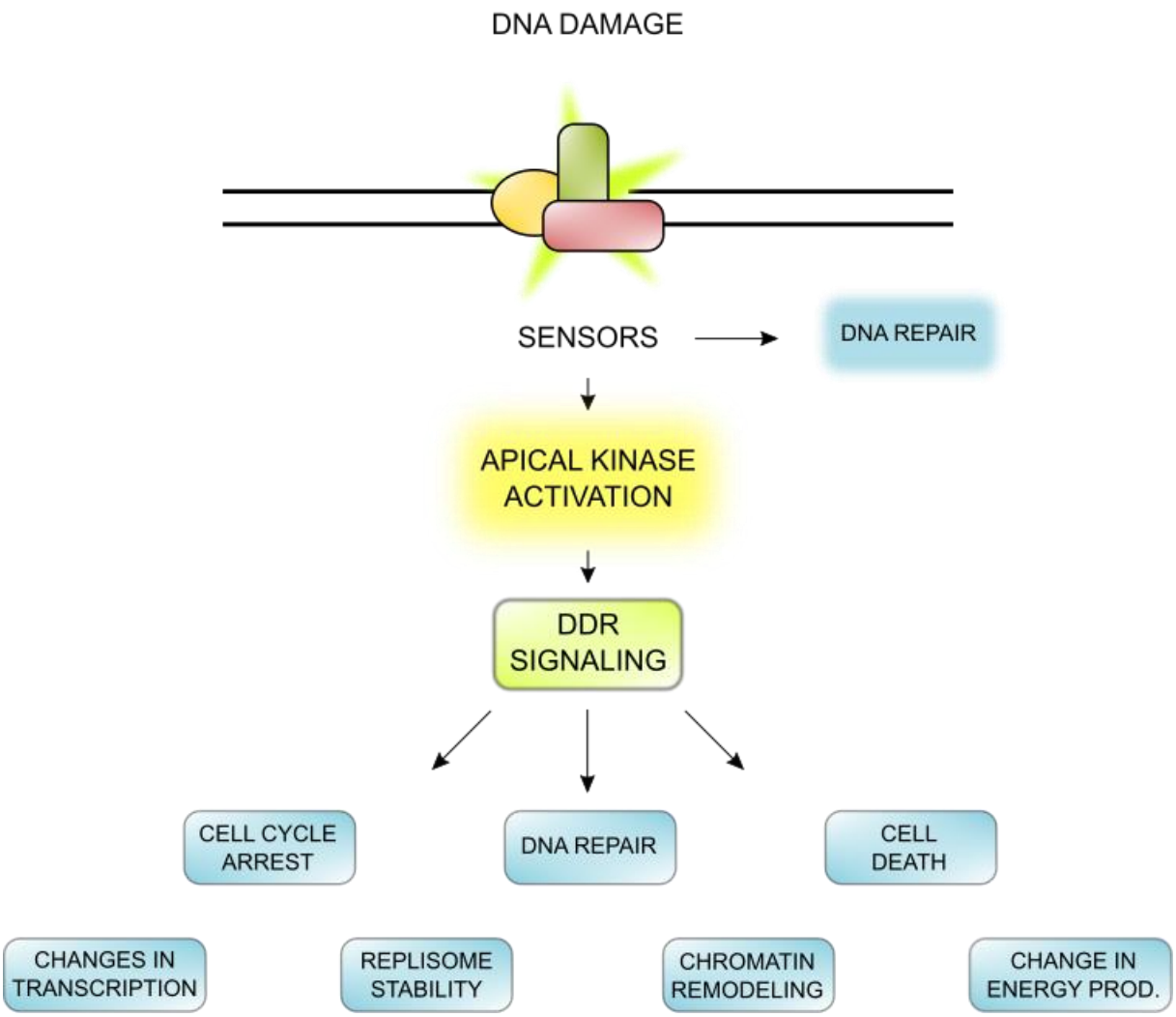

Figure 1: The DNA damage response

DNA damage is detected by sensor proteins that can stimulate processing of the DNA lesion and recruit factors such as the apical kinases that sets off signaling cascades leading to many different outcomes for the cell.

the DNA damage signaling response on their own, but they may promote recruitment of factors that do. Two structures of DNA that are important for full activation of the DDR are DSBs and ssDNA [8]. As mentioned in the previous section, ATM and DNA-PK respond to double strand breaks and ATR responds to ssDNA [18].

Double strand breaks can form after exposure to IR or chemicals, but can also form secondarily for instance when replication forks encounter ssDNA breaks, or when stalled replication forks collapse [7, 27]. Two factors that are rapidly recruited to DSBs, and thus can be seen as DNA double strand break sensors, are PARP1 and the Ku complex (consisting of Ku70/80) [28]. The Ku complex is highly abundant and is recruited to DSBs within seconds of DNA break formation [29]. Ku binds DNA ends with a high affinity and recruits the catalytic subunit of DNA-PK (DNA-PKcs) to form the kinase active DNA-PK holoenzyme [21], and thereby stimulates DSB repair via NHEJ [18]. PARP1 is one of the most abundant proteins in a cell, and it too is rapidly recruited to DNA damage [29]. PARP1 is one of 17 members of the Poly (ADP-ribose) polymerases (PARPs), enzymes that attach negatively charged poly (ADP-ribose) (PAR) chains to proteins in a process called PARylation. PARP1 can detect both SSBs and DSBs through different binding modes, and binding of PARP1 to DNA induces a conformational change that activates its PARylation activity [30]. PARylation can stimulate recruitment of factors involved in the DDR, including the MRN complex and ATM [31]. Recruitment may involve direct interaction between PAR and the PAR domain of ATM [32], and/or via the recruitment of the MRN factor NBS1 [33]. ATM interacts with a conserved motif in the C-terminus of NBS1, a motif that is also found in both ATRIP and Ku80, and has been shown to be important for ATR and DNA-PK recruitment, respectively [22].

ATM binding to chromatin stimulates its kinase activity; however, the exact mechanism for how ATM is activated at DSBs remains unclear, but as mentioned above, it probably involves the MRN complex [18]. Activated ATM stimulates DNA resection by CtIP and MRE11 to facilitate DSB repair via HR [34]. The long stretches of ssDNA formed upon resection are bound by the ssDNA binding protein RPA, a protein important for canonical ATR activation [24] (see section 1.2). ATR responds to ssDNA covered by RPA, but should RPA be considered a DNA damage sensor? ATR recruitment to RPA-ssDNA is insufficient to activate ATR [35], and full activation of the ATR branch of the DDR can only be achieved when multiple factors are tethered on ssDNA [8, 24]. Thus, defining a single DNA damage sensor in the context of canonical ATR signaling is difficult.

Notably, sensing DNA damage may also occur in a process called “recognition by proxy” [36]. For example, RNAPII can act as a damage sensor in this way, which means that RNAPII

does not directly recognize the DNA lesion, but its stalling stimulates recruitment of repair factors, such as factors involved in transcription coupled nucleotide excision repair (TC-NER) [37]. It has been proposed that RNAPII may in fact be the ultimate DNA damage sensor since it can stall at many different types of lesions, it continually scans the genome in the process of pervasive transcription, and it is incredibly stable on chromatin. This makes it 100-10,000 fold more specific than any other DNA damage sensing protein [38].

1.2 Replication stress and the replication stress response

1.2.1 DNA replication

DNA replication is the coordinated process of duplicating the DNA to provide identical copies for the two daughter cells. DNA replication is tightly regulated to ensure the whole genome is copied, and that it is only copied once [39]. In eukaryotes, replication is initiated from a vast number of replication origins located at intervals to cover the whole genome [40]. To prevent shortage of factors needed to replicate, origins are activated, or fired, throughout S-phase and can thus be divided into early and late replicating origins [39, 41]. Although origins are called early and late replicating origins, the process of origin activation is likely a continuum, as shown in yeast [42]. The process of replication starts with a licensing step which involves loading of the "pre-replication complex" (pre-RC) at the origins. Licensing is restricted to telophase [43] or G1 phase due to low CDK activity [44]; this is to prevent re-licensing of already replicated sequences in S-phase. The pre-RC consists of the "origin recognition complex" (ORC), Cdc6, Cdt1 and the DNA replication helicase MCM2-7 [45]. The DNA helicase is inactive until cells enter S-phase and CDK activity rises [44]. Firing of an origin is controlled by phosphorylation of the pre-RC by the kinases CDK2/Cyclin E and Dbf4-Dependent kinase (DDK), which promote binding of CDC45 and GINS to MCM2-7 to form the pre-initiation complex (pre-IC) [39]. The interaction between CDC45, GINS and the MCM2-7 activates the helicase [46], leading to melting of the DNA double strand helix and formation of the replication bubble. PCNA tethers the DNA polymerases to DNA, forming two replication forks at each end of the bubble [39]. DNA replication is performed by the DNA polymerases α, δ and ε [47]. Each replication fork is moving bi-directionally away from the origin, and the replication fork is disassembled if it encounters another active replication fork [39]. Many more origins are licensed than fired, and in a normal unperturbed S-phase, most origins are not fired at all, but are rather passively replicated. The decision to fire an origin or not is dependent on the replication timing program, which is dependent on several factors including epigenetic marks, 3D genome architecture and transcriptional activity [48]. The dormant origins are origins that can fire later in the replication program if they are not already replicated. Thus, if a replication fork meets a barrier and stalls, these licensed

dormant origins can be activated to ensure that replication of that section of DNA is completed [42].

1.2.2 What is replication stress?

Replication stress can be defined as anything that will stall or slow down replication [27]. Replication can stall when it encounters natural pausing elements or hard to replicate genomic regions. For example, parts of the genome contain replication fork barriers that actively prevent replication at certain locations, such as in the ribosomal DNA (rDNA) [49]. Furthermore, some DNA sequences are prone to forming secondary structures, e.g. G4-quadruplexes and hairpin loops that will block replication [50]. Highly repetitive DNA sequences, such as satellite DNA and the regions around telomeres and centromeres also frequently lead to replication errors. Further, replication can stall when it encounters DNA lesions. As mentioned before, both internal and external sources can cause the formation of DNA lesions. For example, modifications in the bases that make up the DNA can cause the DNA to distort and block replication, the action of topoisomerases can cause single strand nicks in the DNA, and DNA damaging agents can cause DNA breaks that will interfere with replication [7].

Replication is a process that requires many different factors, including the proteins involved in the replication machinery itself, but also histones and free nucleotides for incorporation into the newly formed chromatin. Many of these factors are limiting, and as mentioned previously, this is one of the reasons why replication has to be tightly regulated. If replication timing is disrupted, such as if firing too many origins at once, for instance by inhibiting the Wee1 kinase (see chapter 1.2.4.1), nucleotide pools may become depleted and replication will stall [51]. Drugs that interfere with nucleotide production, such as hydroxyurea [52], will cause replication stalling/stress on a global level [53]. In addition, other drugs that inhibit replication by different mechanisms, e.g. aphidicholin which directly inhibits DNA polymerase alpha [54], or camptothecin which stalls replication by creating DNA protein adducts [55], will inherently cause replication stress.

Moreover, as the processes of transcription and replication both occur on the same DNA template, interference or conflicts between the two processes is a major source of replication stress. This topic will be covered in detail in a later chapter (1.2.2).

1.2.3 ATR activation in response to replication stress

In order to protect itself from the dangers of replication stress, the cell has evolved a replication stress response to ensure that the genome is duplicated in a timely and secure fashion. ATR is the master regulator of the replication stress response, thereby being essential for cell viability in the absence of DNA damaging agents [26, 56, 57]. Because of this, mutations in the ATR gene are uncommon; however, mutations that reduce the function of ATR occur in the rare Seckel syndrome [58]. Seckel syndrome patients share clinical features with many other syndromes caused by mutations in DNA damage response proteins, many of which are congenital, suggesting that the failure to protect against endogenous DNA damage is the underlying cause of these syndromes [59]. As mentioned earlier, replication stress is a major source of endogenous DNA damage. In addition to its role in the replication stress response, ATR has other roles in the cell, e.g. in meiotic silencing [60] and telomere maintenance [61].

The canonical ATR pathway (**Figure 2**) is activated in response to the presence of ssDNA coated with RPA. During replication stress, if the polymerases stall at lesions that do not block the helicase, the helicase will continue to unwind the DNA, generating ssDNA [62]. Single stranded DNA may form e.g. as an intermediate during NER or after resection of DSBs [63]. RPA coats most of the ssDNA in the cell, also during normal cell metabolism [11], and the presence of ssDNA-RPA is not sufficient to activate ATR [35, 62, 64, 65]. However, ssDNA-RPA can recruit ATR via its obligate partner ATRIP [23], and may act as a platform to tether other ATR activating proteins. In addition to ssDNA, activation of ATR has, at least in some cases, been shown to require the presence of a ssDNA-dsDNA junction with a free 5'-end (5' junction) [35]. In the presence of RPA [66], this structure is loaded with the RAD9-RAD1-HUS1 (9-1-1) checkpoint clamp complex with the help of the RAD17-RFC2-5 clamp loader, and can stimulate TOPBP1 recruitment [67, 68]. TOPBP1 recruitment also partially

depends on the MRN complex and RHINO, although the exact details remain unclear [24]. TOPBP1 can interact with ATR-ATRIP and enhance ATR kinase activity via its ATR activation domain (AAD) [69]. Recently, another AAD containing protein, ETAA1, was found in eukaryotes, and a role in ATR activation was described [70-72]. In contrast to TOPBP1, ETAA1 does not seem to require a 5' junction and loading of the 9-1-1 complex, as it can bind directly to RPA and stimulate ATR activity [70, 71, 73]. TOPBP1 and ETAA1 apparently act in parallel to stimulate ATR kinase activity toward different substrates: TOPBP1 is required for CHK1 phosphorylation while ETAA1 is required for RPA phosphorylation [70, 71]. Some reports suggest TOPBP1 functions mainly during replication stress while ETAA1 functions during unperturbed S-phase [74, 75].

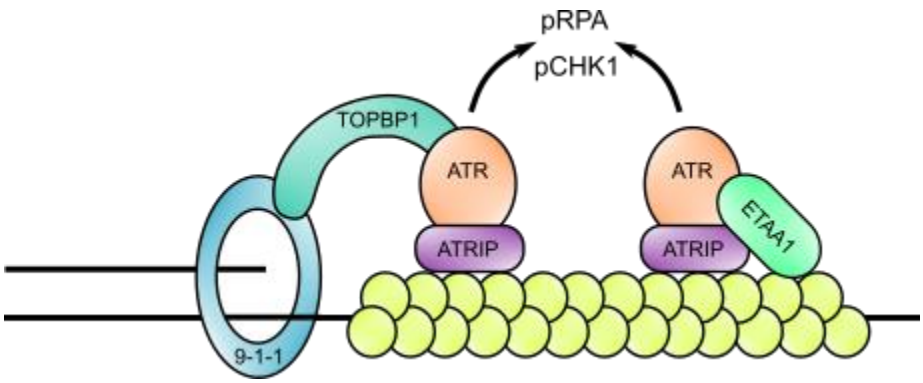

Figure 2: The canonical ATR pathway The canonical ATR pathway: ATR is recruited to ssDNA coated with RPA via ATRIP, however ATR binding to RPA is not sufficient to activate ATR. The 9-1-1 complex can be loaded at ssDNA-dsDNA junctions and stimulate TOPBP1 recruitment. TOPBP1 can stimulate ATR activity. Alternatively, ETAA1 can bind to RPA and stimulate ATR activity. Activation of ATR by TOPBP1 and ETAA1 leads to phosphorylation of the downstream ATR targets RPA and CHK1. Adapted from [70].

In cases where the replisome stalls ahead of the helicase, such as when it encounters a DNA crosslink or upon collisions with the transcription machinery, the helicase cannot keep unwinding the DNA to produce ssDNA. Exactly how ATR is activated in these circumstances is not clear [24]. The stalled replication fork can be remodeled into a so-called chicken foot structure; In this case the replisome reverses, the parental strands reanneal and the newly formed DNA anneals, generating a structure resembling a four way Holliday junction [76].

Reversed forks form upon treatment with compounds that cause replication stress, including topoisomerase inhibitors, interstrand crosslinking agents, base damaging agents and more, and are dependent on RAD51 [77] and enzymes such as SMARCAL, ZRANB3 and HTLF [78]. In order to activate ATR at a reversed fork, according to the model of canonical ATR activation, endonucleases would have to generate ssDNA and a 5' junction. One such endonuclease is DNA2; It is involved in resection during HR [34], and has been reported to resect reversed forks as well. One study found that DNA2, together with the WRN helicase, resected reversed forks, and that depletion of DNA2 prevented ATR signaling after HU as shown by decreased phosphorylation of ATR substrates CHK1 and RPA [79]. However, in yeast, Dna2 has been shown to have a direct role in Mec1 (yeast homolog of ATR) activation that is independent of its endonuclease activity, but dependent on an N-terminal domain also found in other Mec1 activating proteins [80]. Thus, whether DNA2 is important for generating an ATR activating structure at regressed forks, or whether DNA2 has a role in ATR activation in human cells, is still unclear. A direct role for DNA2 in mammalian ATR activation has not been described.

1.2.4 Downstream effects of ATR activation

Once ATR is activated at a stalled replication fork, it sets off a network of signaling cascades that affect fork -stabilization, -repair, and -restart, origin firing and cell cycle progression [24]. ATR can phosphorylate a large number of proteins [25]. However, one protein that has been extensively studied, and is central to many of the downstream effects of ATR activation, is Checkpoint Kinase 1 (CHK1). One important effect of CHK1 activation is regulation of Cyclin Dependent Kinase (CDK) activity [81]. CDKs control cell cycle progression, replication initiation and activation of nucleases, processes important for maintaining genome stability [81]. Upon replication fork stalling, CHK1 is brought into the vicinity of ATR with the aid of CLASPIN [82], and ATR can phosphorylate CHK1 on serine 317 and serine 345, which causes its activation [83, 84]. Phosphorylation of the serine 345 residue has been shown to be especially important since replacing it with alanine completely disrupts the biological function of CHK1 in response to genotoxic stress [85]. Once activated, CHK1 can

autophosphorylate on serine 296, which promotes CHK1 release from chromatin and allows it to reach its targets throughout the nucleoplasm [86].

1.2.4.1 The control of CDK activity by CHK1 and Wee1

CHK1 is, together with Wee1, central in the regulation of CDK activity [81]. This is because CDK activity is negatively regulated by inhibitory phosphorylation of tyrosine 15 [87]. Wee1 kinase is responsible for adding the inhibitory phosphorylation to CDK [87], while CHK1 regulates the activity of the CDC25 phosphatases that removes it [88]. CHK1 does so by phosphorylating CDC25A, which promotes its degradation by the ubiquitin-proteasome pathway [89, 90]. With CDC25A lacking, the inhibitory phosphorylations on CDK1 and CDK2

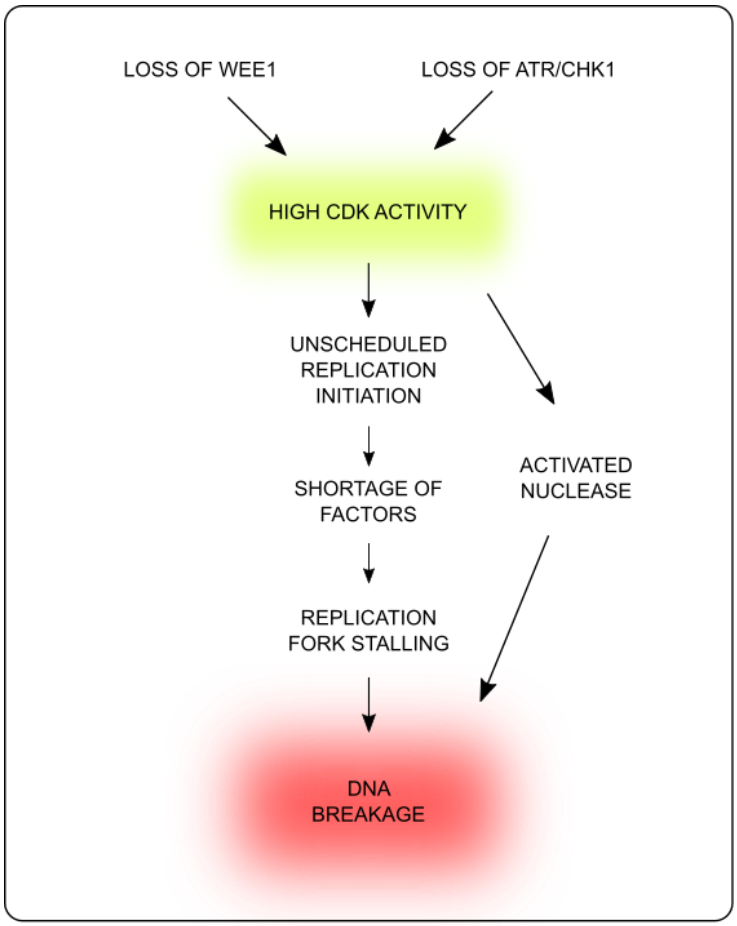

Figure 3: High CDK activity lead to DNA breakage. High CDK activity, such as after inhibition of Wee1 or ATR/CHK1, leads to unscheduled replication initiation which cause shortage of factors involved in replication contributing to replication fork stalling and DNA breakage. High CDK activity may also activate nucleases (such as Mus81) which will aberrantly digest the DNA to cause DNA breakage.

will remain [88]. The detrimental effects of disrupting CHK1 or Wee1 function illustrate the importance of proper regulation of CDK activity: depletion of CHK1 by siRNA or treating cells with CHK1 inhibitors cause CDK dependent destabilization of the genome [91], and the DNA damage observed after CHK1 inhibition is dependent on CDC25A [92]. Similar to CHK1, Wee1 depletion or inhibition also causes CDK dependent DNA damage in S-phase [51, 92]. The exact mechanisms how DNA damage is induced are not fully understood, but may involve CDK-dependent unscheduled replication initiation and subsequent nucleotide shortage, replication stalling and CDK-dependent activation of endonuclease Mus81 [51, 81] (**Figure 3**).

1.2.4.2 Checkpoint activation

One important function of the replication stress response is the activation of cell cycle checkpoints, which are regulated by CDK activity. CDK1 and CDK2 are required for cell cycle progression via their interaction with Cyclins, and in S-phase CDK2 activity is important for loading of CDC45 at origins, and thus activation of the DNA helicase (See section 1.2.1). CHK1 activation upon replication stalling causes downregulation of CDK2 activity, and thus prevents origin firing in a global manner. This is referred to as the S-phase checkpoint. Global downregulation of origin firing may actually stimulate firing of dormant origins in the local vicinity of the stalled fork by an unknown mechanism [42]. This may allow the replication program to "catch up", and prevent unscheduled replication timing [93]. The S-phase checkpoint is also activated in response to DSBs and typically delays cell cycle progression, though does not permanently arrest it.

In order to prevent entry into mitosis with persistent replication problems, the replication stress response will activate the G2 checkpoint. Coupling the replication stress response with cell cycle arrest prevents the cell from entering mitosis with under-replicated areas, and thereby preventing genomic instability. The G2 checkpoint is also activated by DSBs, thus preventing cells with unrepaired DNA breaks from entering mitosis. The G2 checkpoint can be activated by ATR-CHK1, but also ATM-p53-p21 [94]. Wee1 dependent phosphorylation of CDKs is crucial for checkpoint activation, and inhibiting Wee1 kinase suppresses the G2 checkpoint [95]. Inhibition of ATR or CHK1 also abrogates the G2 checkpoint.

1.3 Transcription as a threat to genome stability

1.3.1 The transcription cycle

Transcription is the process of reading the DNA code to produce RNA. RNA is used in the production of proteins, acting as messenger RNA, transfer RNA and as part of the ribosome. RNA also has other important functions, e.g. in gene expression via transcriptional and post-transcriptional regulation, and in epigenetic regulation [96]. Transcription is performed by the RNA polymerases, and RNA polymerase II (RNAPII) transcribes most of the genome in eukaryotic cells. RNAPII is a large multi-subunit protein complex where RPB1 is the largest and catalytic component. RPB1 contains an unstructured C-terminal domain (CTD) which is conserved in fungi, plants and animals [97]. The RNAPII CTD is made up of 26 (in yeast) to 52 (in mammals) $Y_1S_2P_3T_4S_5P_6S_7$ heptad repeats, and is essential for life. One important feature of the CTD is that it can be post-translationally modified, mainly by phosphorylation [97-99], and the most studied modifications are on serine 2 and serine 5. The pattern of different modifications can act as a "CTD code" [100], which can dictate progress in the transcription cycle and the binding of proteins involved in RNA processing [99].

The transcription cycle can be divided into several steps, including initiation, elongation and termination (***Figure 4***). Initiation of transcription starts with assembly of the general transcription factors at the promoter to form the pre-initiation complex (PIC). A minimal set of factors that are required for transcription initiation *in vitro* are the general transcription factors (GTFs) TFIIA, TFIIB, TFIIF, TFIIE and TFIIH [101]. TFIIH contains the helicases XPB and XPD, and the kinase CDK7 [102], which are required for transcription initiation. TFIIH recruitment requires the Mediator complex which phosphorylates TFIIH and induces CDK7-dependent phosphorylation of serine 5 residues on RNAPII CTD (pRNAPII S5), and RNAPII release from the promoter associated PIC [103]. pRNAPII S5 recruits and activates capping enzymes that modify the 5' end of the nascent RNA. Capping of the RNA is needed to prevent degradation of the RNA by nucleases such as XRN2, and to prevent premature termination of transcription [104]. In addition, pRNAPII S5 recruits the SET1 histone methylase complex that catalyzes H3K4me3 associated with transcription activation [105].

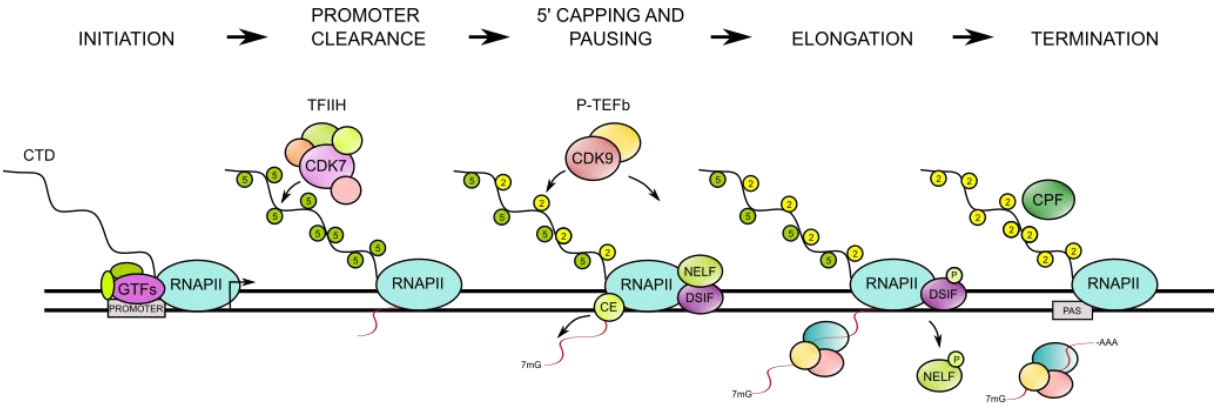

Figure 4: The transcription cycle. The transcription cycle can be divided into steps such as initiation, promoter clearance, 5'Capping and pausing, elongation and termination. The CTD of RNAPII is phosphorylated by e.g. the CDK7 and CDK9 kinases, and the different phospholylation states of the CTD can dictate recruitment of factors involved in the transcription process. Adapted from **[106].**

The exact details of the transition from inactive to active transcription are still unclear, however, it involves formation of the transcription bubble and synthesis of short stretches of RNA. RNAPII frequently pauses 20-60 bases downstream of the transcription start site (TSS): This is called promoter proximal pausing and was originally thought to be a mechanism to keep genes poised and ready for action since promoter proximal pausing is enriched in signal-responsive pathways. However, recently it has become clear that most mammalian genes undergo pausing [107]. Protein complexes such as DSIF (DRB sensitivity factor) and NELF (Negative elongation factor) enforce promoter proximal pausing. The transition into productive elongation requires phosphorylation of the serine 2 residue of RNAPII CTD, and the DSIF and NELF components, mediated by the kinase CDK9 of the P-TEFb complex [108]. Recently, also the PAF1 complex has been shown to be important for pause release, although the details of how it functions remain obscure [108-111].

Serine 2 phosphorylation reaches a plateau as RNAPII approaches the end of the gene [98], and pRNAPII S2 is loaded with factors involved in termination, such as the Cleavage and Polyadenylation Factors (CPF) [99, 112]. RNAPII transcription termination is associated with a slowdown of the polymerase as it transcribes across a poly (A) signal (PAS), and slowdown

may involve the formation of a DNA:RNA hybrid called an R-loop [113]. Two models for how RNAPII is released from chromatin during transcription termination exist. The allosteric model predicts that RNAPII senses the PAS and undergoes a conformational change likely due to CPF recruitment [114]: this model does not depend on cleavage of the transcript. In the other, termed the torpedo model, the transcript is cleaved at the PAS and the downstream transcript is degraded by the XRN2 exonuclease. As XRN2 is faster than the RNAPII, it can acts as a torpedo to displace RNAPII from chromatin [113, 115]. Proper termination of transcription is essential as a failure to do so may produce aberrant mRNAs, cause overlapping transcripts that may activate RNAi pathways, or cause interference between other RNA polymerases or conflicts between transcription and replication [113].

1.3.2 Transcription replication conflicts

Transcription occurs in all cell cycle phases, including S-phase when the replication machinery occupies the DNA template. Although transcription and replication are regulated in both space and time, both processes will occasionally occur at the same locations at the same time causing transcription-replication conflicts (T-R conflicts) [116]. Over the years, it has become clear that transcription is a major source of replication stress and genomic instability [116, 117], a hallmark of cancer [118]. Transcription associated genome instability can be detected as point mutations, transcription associated recombination (TAR) and chromatin rearrangements; both TAR and chromatin rearrangements are dependent on replication [119, 120]. Thus, understanding what causes T-R conflicts and how they are resolved is important in order to understand the underlying mechanisms of carcinogenesis.

Although T-R conflicts are causing genomic instability, not all T-R conflicts are equally harmful. Both the DNA polymerases and the RNA polymerase move along the DNA template in a 3'-5' direction, however, the RNAPII holoenzyme embraces both stands of DNA, meaning the two processes can approach each other either co-directionally or head-on [121]. Head-on conflicts have been shown to be the most detrimental in both bacterial and eukaryotic systems [119, 122-125]. Cells have evolved several strategies to prevent collisions between the two machineries (**Figure 5**). For example, bacterial genes are often co-oriented to

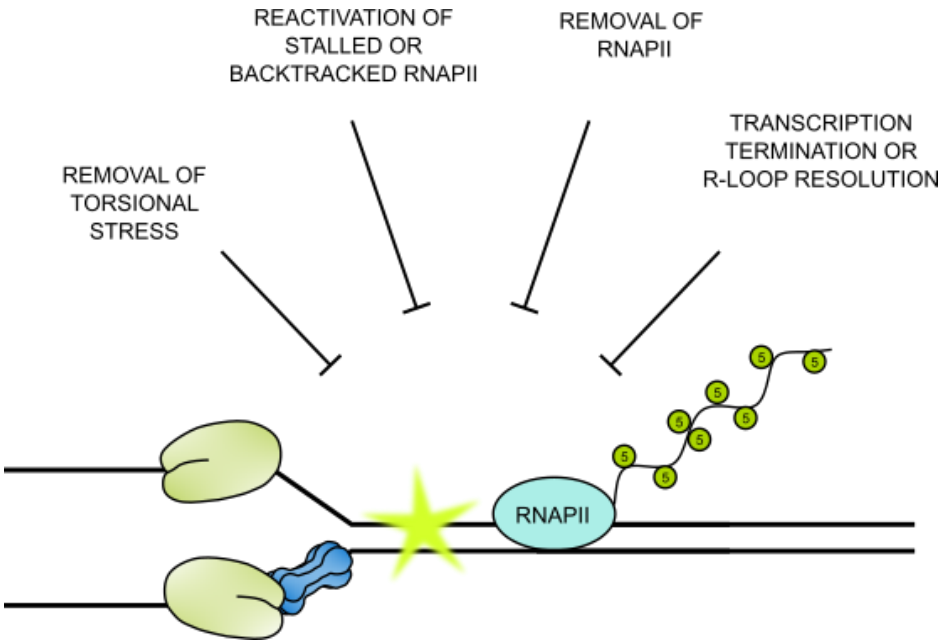

Figure 5: Transcription replication conflicts. A number of different mechanisms are in place to suppress T-R conflicts. See text for detail.

transcribe in the same direction as replication occur [126]. This may be true for human cells as well [127], however, it may not be as important since transcription and replication normally are separated both spatially [128] and temporally [129]. Despite the term collisions, there is relatively little evidence of direct physical contact between the transcription- and replication machineries. The conflict may lie in the topological constraints the DNA is subjected to as the two machineries approach each other. Due to the helical structure of DNA, the opening of the DNA helix will cause positive supercoiling ahead of a moving replication fork. Since DNA is often anchored at fixed sites, such as nuclear pores, or the chromosome scaffold, the torsion cannot simply diffuse [130]. Specialized enzymes called DNA topoisomerases relieve the excessive torsional stress by catalyzing the transient breakage of DNA to allow the single or double strands of DNA to pass each other [131]. Evidence from both yeast and mammalian systems show that topoisomerases are important for preventing T-R conflicts [132, 133]. For example, depletion of Topoisomerase I in

mammalian cells leads to replication fork stalling and DNA breakage particularly in gene rich areas [133].

T-R conflicts may occur in instances where either the transcriptional program or the replication program is altered. Aberrant growth factor induced transcription or oncogene activation may cause transcription outside the normal program, inducing transcription at higher levels than normal, which may subsequently cause replication effects due to T-R conflicts [134-137]. For example, overexpression of the oncogene $HRAS^{V12}$ led to increased transcription, which subsequently resulted in decreased replication fork speed and DNA damage [137]. Vice versa, when replication is perturbed for instance by increased origin activation, transcription may be affected due to T-R conflicts. For example, overexpression of Cyclin E induces origin firing, which leads to reduced replication speed and DNA damage. Treatment with either transcription inhibitors or CDK inhibitors rescued the reduced replication speed and DNA damage, suggesting that Cyclin E-induced origin firing causes more T-R conflicts [134]. Furthermore, oncogenes can cause firing of replication origins that are normally suppressed by transcription in G1. Premature S-phase entry allows firing of these origins while transcription is still ongoing, causing T-R conflicts and DSBs [135].

Deregulated transcription elongation is also a source of transcription replication conflicts. For example, RNAPII may transcribe in both forward and backward direction, a feature associated with its proofreading mechanism [113]. When the RNAPII is in a backtracked state, the enzyme has moved in the opposite direction of transcription, allowing the newly formed 3' end of the RNA strand to be displaced from the active site [138]. This causes RNAPII to arrest in a highly stable configuration, and release requires the transcript cleavage factor TFIIS [139, 140] or GreA/B [141] in eukaryotic or prokaryotic cells, respectively. A backtracked RNAPII can block replication in bacteria [142] and mutation of TFIIS causes enhanced backtracking and DNA damage in human cells [143]. Felipe-Abrio and colleagues [144] showed that mutations in yeast RNAPII that increased its retention on chromatin caused decreased replication fork progression and genome instability. In bacteria, mutants in RNAP that *decrease* replication fork stalling have been described [145, 146], and it has been suggested that this is due to decreased retention of RNAP on chromatin, and thus lower frequency of T-R conflicts.

Structural alterations in the DNA may also prevent RNAPII movement, and promote T-R conflicts. Some DNA sequences are prone to forming secondary structures such as hairpins or G-quadruplexes, that may block transcription [147]. R-loops are stable structures, more stable than dsDNA [148], formed when the newly made RNA reanneals with one of the DNA strands behind the transcribing RNA polymerase [116, 149, 150]. R-loops can have physiological functions in normal transcription, mitochondrial replication and immunoglobulin class switch recombination, however, recently, it has become evident that they may form aberrantly and may be harmful to the cell [150]. The newly formed RNA is usually spliced and packaged co-transcriptionally, and when this process is disrupted, the RNA strand can form an R-loop behind the transcribing RNAP. For example, lack of factors involved in splicing (e.g. ASF/SF2), mRNP particle formation (e.g. THO) and RNA export (e.g. TREX) have all been shown to promote R-loop formation and recombination [150]. R-loops may cause a slowing of the RNA polymerase [151], and thus, if the R-loop is not resolved before the replisome approaches, this will lead to collisions.

Several processes are in place to prevent T-R conflicts, and I have already mentioned a few, such as anti-backtracking and R-loop resolvement. Another important process is proper transcription termination. This was first discovered in bacteria where mutations in the termination factor Rho caused replication dependent DSBs [152]. Later, several 5'end processing factors and transcription termination factors have been shown to prevent T-R conflicts [116] also in eukaryotes. Loss of XRN2, the 5'-3' exoribonuclease involved in transcription termination, led to enhanced replication stress and DSB formation at 3' end transcription pause sites, which were dependent on transcription and R-loops. This suggests proper transcription termination by XRN2 is preventing T-R conflicts [153]. Similar phenotypes have been observed after loss of Sen1, or Senataxin (SETX) as it is called in humans. Sen1/SETX has roles in both R-loop resolvement and transcription termination, and is important for preventing transcription associated genome instability in yeast [154] and humans [155]. SETX can interact with RNAPII and several replication- and repair proteins, and it has been proposed that SETX may be important for resolving T-R conflicts. In addition, SETX can form foci upon treatments that cause replication stress [116]. SETX foci colocalize with the DNA damage markers 53BP1/γH2AX in S/G2 phase, and foci formation depends on

transcription and R-loop formation since treatment with transcription inhibitor α-amanitin or the R-loop resolving enzyme RNAseH1 suppress their formation [156].

Finally, in order to prevent transcription replication conflicts, RNAPII complexes that have been stalled by e.g. DNA lesions need to be removed. One way this may occur is by repairing the lesion and allowing transcription to resume. The TC-NER pathway removes transcription-blocking lesions in a process that involves the Cockayne syndrome proteins A and B (CSA and CSB) [157, 158]. CSB is thought to be important for recruiting other proteins involved in TC-NER, and in addition, structural work from yeast suggest that CSB may act as a 3'-5' ATP-dependent translocase that pulls DNA away from RNAPII to stimulate forward translocation of RNAPII [157]. However, the details of how TC-NER occurs in mammals, and the fate of RNAPII during TC-NER, is not known: it may be displaced, backtracked or even degraded [157-160]. Nevertheless, TC-NER is likely important in preventing T-R conflicts since human fibroblasts deficient in TC-NER factors undergo apoptosis as they enter S-phase with unrepaired UV lesions [161]. RNAPII that is persistently stalled is degraded in a "last resort" pathway involving one or more ubiquitin ligases and the proteasome [162]. Degradation of RNAPII also occurred in response to HU treatment in yeast, and yeast proteasome machinery mutants were deficient in the eviction of RNAPII form chromatin during HU [163]. The recognition of permanently stalled RNAPII may involve the phosphorylation status of the CTD since RNAPII hyperphosphorylated on serine 5 inhibited degradation in yeast [164]. On the other hand, hyperphosphorlation on serine 5 rater promoted degradation of RNAPII in human cells [165].

1.4 Main proteins in this study

1.4.1 PP1

A major regulatory mechanism in cells is the reversible phosphorylation of proteins, and the genome encodes many kinases and phosphatases to perform this task. One of the most abundant phosphatases is Protein Phosphatase 1 (PP1) and, together with PP2A, it accounts for more than 90% of the dephosphorylation events in a cell [166]. PP1 belongs to the superfamily of phosphoprotein phosphatases (PPP) which are recognized by the structure and function of their catalytic core [167]. PP1 is highly conserved and only three genes encode the PP1 isoforms PP1α, PP1β/δ and PP1γ (differential splicing produces the two variants PP1γ1 and PP1γ2). Apart from PP1γ2, which is enriched in the testis, the PP1 isoforms are widely expressed and are involved in a broad range of cellular processes [168]. Although PP1 specifically dephosphorylates serine and threonine residues it does not recognize a consensus sequence around the phosphorylated residue, and the free enzyme is highly promiscuous [166]. To obtain specificity, PP1 relies on a large number of regulatory subunits called “PP1 interacting proteins” (PIPs) [169].

More than 200 different PIPs have been described, and although some only interact with one of the PP1 isoforms, most interact with all [166, 169]. Most of the PIPs interact with PP1 via a conserved RVxF binding motif. The interaction does not alter the conformation or activity of PP1, but rather acts as an anchor for the PIP [170]. A feature of most PIPs is that they are intrinsically disordered and highly unstructured in the unbound state, allowing for interaction with secondary binding motifs located further away on PP1. The interaction with one or more PP1 binding motifs can partially promote the folding of these proteins, and provides specificity for the holoenzyme [171]. The number of PIP molecules vastly outnumbers the amount of PP1 molecules, and PP1 is therefore considered to always be bound to a PIP [169]. Furthermore, since the free PP1 enzyme is highly promiscuous in vitro it is important to identify and study the PIP to address specific functions of PP1.

1.4.2 PNUTS

PP1 NUclear Targeting Subunit (PNUTS) was first identified as a PP1 interacting protein in a yeast two hybrid screen [172]. PNUTS interacts with PP1 through the conserved RVxF motif located in the central region of the protein [173], and as the name implies, targets PP1 to the nucleus. PNUTS is stably expressed throughout the cell cycle, and is located on chromosomes throughout interphase, but released during mitosis [174]. PNUTS has been implicated in several processes in the nervous system [175, 176], and in regulating cell proliferation and apoptosis through inhibiting PP1 mediated dephosphorylation of pRb [177-179] and p53 [180]. PNUTS has also been linked to genome maintenance. Work from our group demonstrated a role of PNUTS in the DNA damage response [181]: depletion of PNUTS enhanced the G2-checkpoint in both unperturbed and irradiated cells, while overexpression inhibited this. PNUTS was recruited to sites of DNA damage and cells depleted of PNUTS also showed increased γH2AX, 53BP1, RAD51, and RPA foci 24h after IR. In addition, PNUTS depleted cells were hypersensitive to IR-induced cell death [181]. PNUTS has also been linked to DNA repair via interactions with PARP1 [182] and Ku70/80 [183].

There are reports involving PNUTS in transcription [184] and RNA processing [185]. The only known substrate of PNUTS-PP1 is pRNAPII CTD S5 [186, 187], and the detrimental effects on drosophila development after deletion of PNUTS [186] likely underscore the importance of proper regulation of CTD dephosphorylation. PNUTS also co-immunoprecipitates with RNAPII in human cells [188]. PNUTS has been shown to interact with factors involved in transcription termination [185, 189, 190], and PNUTS depletion has been shown to cause termination defects [189].

High expression of PNUTS has been identified as a favorable prognostic marker in pancreatic and cervical cancer [191-193].

1.4.3 WDR82

WDR82 is a typical docking protein, consisting of seven WD40 repeats [187]. The WD40 domain is one of the most abundant protein domains, and its name stems from the sequence itself; it usually consists of about 40 amino acids ending with a Tryptophan (W) and Aspartic acid (D). Each WD40 repeat forms a blade-like structure and the seven blades organize into a β-propeller shape with protein interacting surfaces on the top, bottom and sides [194].

The yeast homolog of WDR82, Swd2/CPS35, was first identified as being part of a SET1 containing complex named COMPASS (Complex Proteins Associated with Set1) [195, 196]. SET1 is a histone methyl transferase involved in methylation of histone 3 on lysine 4 (H3K4), a histone mark associated with transcription start sites [197]. Humans have six SET1-containing protein complexes (SETD1A, SETD1B, MLL1, MLL2, MLL3 and MLL4) all involved in H3K4 methylation; however, WDR82 is only associated with SETD1A and SETD1B [198]. In yeast it has been reported that Swd2 requires the PAF1 complex for recruitment of COMPASS to pRNAPII S5 [199], but in humans, WDR82 directly mediates the interaction with SET1DA/B and RNAPII by binding to pRNAPII S5 [200]. In addition to its interaction with COMPASS, Swd2 also interact with the APT (Associated with PTA1) complex that is involved in transcription termination [201]. Recently, a role for WDR82 in transcription termination has also been described in unicellular trypanosomes [190], and in addition, WDR82 has been implicated in transcription termination in mammalian cells, where depletion of WDR82 led to transcription read-through of many non-coding RNAs [189, 202].

WDR82 has also been found to associate with the DDB1-Cul4 ubiquitin ligase [203], which is involved in ubiquitination of proteins involved in a wide range of processes in the cell, including DNA replication, transcription and DNA repair [204]. WDR82 contains a conserved “WDXR” motif shared between many of the DDB1 and Cul4 associated factors (DCAFs) [203]. It is thought that these interactors mediate substrate specificity for the DDB1-Cul4 ubiquitin ligase [204].

A few reports have indicated a role for WDR82 in cancer: WDR82 was identified as a prognostic marker in human colorectal cancer, where low expression levels were associated with tumor progression and reduced overall survival [205]. Low expression levels of WDR82 was also correlated with shorter survival in patients with pancreatic ductal adenocarcinoma [193].

1.4.4 CDC73

CDC73, also called parafibromin, is an evolutionary conserved protein encoded by the HRPT2 gene [206]. Mutations in CDC73 causes hyperparathyroidism-jaw tumor syndrome (HPT-JT) [206] and has been implicated in a number of different cancers including parathyroid- [207], renal- [208], gastric- [209], and breast -carcinomas [210]. Despite extensive research, exactly how loss of CDC73 function causes neoplastic transformation is poorly understood. It has been suggested that it acts as a tumor suppressor to inhibit cyclin D1 and c-myc, and to have an oncogenic role in regulating expression of Wnt signaling genes [211].

CDC73 is part of the PAF1 complex (PAF1C) [212], a conserved protein complex that associates with RNAPII at all stages of the transcription cycle [213]. In yeast, recruitment of PAF1C to chromatin requires the Bur1-Bur2 kinase [214], the DSIF complex [215], and the C-terminal GTP-ase domain of CDC73 [216]. Yeast PAF1C is important for promoting H2B monoubiquitinylation, recruitment of the SET1 complex (COMPASS) and H3K4 trimethylation, factors that have been associated with promoting transcription elongation [199, 217]. Yeast PAFC has also been implicated in 3'end processing [218, 219], and human CDC73 associates with the cleavage and polyadenylation specificity factor (CPSF) and cleavage stimulation factor (CstF) complexes that are required for the maturation of mRNA 3' ends [220]. It has also been shown that PAF1C has roles in suppression of cryptic transcription in yeast [221], and in general transcription in human cells through its role in regulating promoter proximal pausing [109, 222]. A role for human CDC73 in facilitation of homologous recombination repair has been described [223], and yeast PAF1C has been implicated in prevention of T-R conflicts by removal of RNAPII from chromatin [163].

2 Aims of study

The overall aim of this work was to obtain more knowledge about the cellular mechanisms protecting against DNA damage and replication stress. In order to achieve this, we performed three studies addressing biological functions of the PNUTS-PP1 phosphatase complex:

i. Based on previous work in the group where PNUTS was found to have a role in regulating the G2 checkpoint, paper I aimed to further elucidate the molecular mechanisms underlying this phenotype. Specifically, since the G2 checkpoint is regulated by ATR, we addressed 1) whether PNUTS acted upstream of ATR, 2) whether the function of PNUTS depended on binding to PP1, and 3) whether the only known substrate of PNUTS-PP1, namely RNAPII CTD might regulate ATR activation.

ii. During the work of paper I we uncovered a role for PNUTS-PP1 in normal DNA replication. In paper II we aimed to 1) characterize the replication stress phenotype seen after PNUTS depletion, 2) find binding partners of PNUTS-PP1 that might contribute to its role in replication, 3) address whether aberrant dephosphorylation of RNAPII CTD and/or ATR signaling could be involved in the replication stress after depletion of PNUTS or other interacting proteins.

iii. In paper II we found WDR82 to be a strong interactor of PNUTS-PP1, and that this protein complex is important for keeping RNAPII in a dynamic state in order to suppress transcription-replication conflicts. Since inhibition of Wee1 causes massive induction of replication origin firing we hypothesized that cells with low expression of WDR82 would be particularly sensitive to Wee1 inhibition as this would potentially lead to more T-R conflicts. Hence, in paper III we aimed to 1) test whether WDR82 depleted cells were sensitive to Wee1 inhibition, 2) explore if the sensitivity was related to WDR82s role in suppressing T-R conflicts.

3 Summary of papers

3.1 Paper I

Regulation of ATR activity via the RNA polymerase II associated factors CDC73 and PNUTS-PP1.

Previous work from the group had shown a role for the PP1 nuclear targeting subunit, PNUTS, in regulating the G2 checkpoint. Depletion of PNUTS led to an accumulation of cells at the G2-M transition both with and without IR. The G2 checkpoint accumulation was sensitive to caffeine and a CHK1 inhibitor, which suggested that the accumulation was due to an effect on the ATR mediated G2 checkpoint [181].

In paper I we further explored the underlying mechanisms of how PNUTS regulates the G2 checkpoint. We show that PNUTS depletion specifically causes hyperactive ATR- , and not ATM- signaling, as shown by enhanced phosphorylation of ATR targets CHK1 S317/S345 and RPA S33. This effect was dependent on PNUTS binding to PP1, however, PNUTS-PP1 did not dephosphorylate CHK1 or RPA directly. Since RNAPII CTD is the only known substrate for PNUTS-PP1, and RNAPII had been proposed as a DNA damage sensor, we addressed whether dephosphorylation of RNAPII CTD might affect ATR signaling. PNUTS depletion caused higher levels of pRNAPII S5, and reduced dephosphorylation after treatment with an inhibitor against CDK7 (THZ1), the kinase that mediated phosphorylation of the serine 5 residue on RNAPII. Strikingly, THZ1 reduced both pRNAPII S5 and pCHK1 S317 in control cells, which suggested that pCHK1 S317 was dependent on pRNAPII S5. On the other hand, in PNUTS depleted cells both pRNAPII S5 and pCHK1 S317 remained high, which further supported a link between pRNAPII S5 and pCHK1 S317.

Since ATR is known to be activated after replication stalling, we went on to explore the cell cycle dependency of the enhanced ATR signaling after PNUTS depletion. Although PNUTS depleted cells show an accumulation of cells in S-phase, the levels of ATR mediated γH2AX were higher in individual S-phase cells than in control cells, demonstrating that the high ATR activity was not simply a cell cycle effect. In addition, we were able to show that PNUTS depleted cells had enhanced ATR signaling even in non-replicating G1 cells, further strengthening the evidence for non-canonical ATR signaling. Also, the high ATR signaling after PNUTS depletion did not correlate with the levels of DNA damage or loading of RPA,

nor did it correlate with R-loop formation. The ATR activating proteins TOPBP1 and ETAA1 were needed for pCHK1 and pRPA, respectively, after PNUTS depletion, and co-depletion of PNUTS together with both TOPBP1 and ETAA1 could abolish all of the enhanced ATR activity. This suggests ET11A and TOPB1 may direct the activity of ATR towards specific substrates.

In the quest to further elucidate on the mechanistic details of RNAPII mediated ATR signaling we identified CDC73, a RNAPII CTD binding protein that had been vaguely linked to ATR. We showed that co-depletion of CDC73 with PNUTS could prevent the enhanced ATR signaling and suppress the G2 checkpoint accumulation after PNUTS depletion. We also showed that CDC73 could co-IP with both RNAPII and ATR, and that the interaction between CDC73 and RNAPII was dependent on the phosphorylation of RNAPII-CTD.

In summary, we have contributed to more knowledge about a proposed non-canonical ATR signaling pathway involving RNAPII. Our work suggest a model where upon transcription stalling RNAPII becomes hyperphosphorylated and activates ATR. CDC73, which bind hyperphosphorylated RNAPII, is needed for ATR activation via RNAPII, and PNUTS-PP1 can counteract RNAPII mediated ATR activation by dephosphorylating RNAPII S5.

3.2 Paper II

WDR82/PNUTS-PP1 prevents transcription-replication conflicts by limiting RNA polymerase II residence time

During the work with paper I we had seen that PNUTS depleted cells accumulated in S-phase and showed reduced incorporation of the nucleotide analog EdU, which suggested that PNUTS is needed for normal DNA replication. In paper II we elaborated on these findings and showed that expression of an siRNA resistant version of mouse PNUTS could rescue the effect on EdU incorporation after PNUTS depletion, demonstrating that the effects on replication were specific to PNUTS depletion. Expression of a PP1 binding mutant PNUTS could not rescue the effects on EdU incorporation, thus PNUTS depletion suppressed replication in a PP1 dependent manner. Using the DNA fiber assay technique, we showed that the replication fork speed was significantly slower after PNUTS depletion compared to control cells, and PNUTS depleted cells failed to resume replication normally after replication

block. Consistent with enhanced replication stress, ATR signaling remained higher in PNUTS depleted cells compared to control cells six hours after release from replication block.

To understand more about how PNUTS may be involved in normal replication we performed a SILAC-IP and confirmed that, in addition to the PP1 isoforms, WDR82 and TOX4 were strong interactors of PNUTS. Since WDR82 binds directly to pRNAPII S5 [200], the only known substrate of PNUTS [186, 187], we investigated whether WDR82 might also play a role in DNA replication. Interestingly, depletion of WDR82 by siRNA phenocopied PNUTS depletion in that WDR82 depleted cells showed reduced EdU uptake and more cells in S-phase. Also, WDR82 depleted cells had reduced replication fork speed and failed to resume replication normally after release from replication block, compared to control cells. In addition, ATR signaling remained high six hours after release from replication block also in WDR82 depleted cells.

Since PNUTS-PP1 dephosphorylates RNAPII, and the effects of PNUTS depletion on replication depended on PP1, we addressed whether WDR82 was involved in dephosphorylation of RNAPII. Similar as after depletion of PNUTS, WDR82 depletion caused enhanced levels of serine 5 phosphorylated RNAPII CTD, and reduced dephosphorylation of RNAPII CTD in live cells. Through a series of different experiments, we could show that reduced dephosphorylation of RNAPII S5 after PNUTS or WDR82 depletion altered the dynamics of RNAPII. Using a chromatin extraction assay and flow cytometry analysis of pre-extracted cells, we showed that pRNAPII S5 was more stable on chromatin after depletion of PNUTS or WDR82 compared to control cells. Since collisions between transcription and replication is a source of replication stress, and more stable RNAPII may promote collisions, we investigated if the enhanced stability was the cause of the replication stress seen after PNUTS or WDR82 depletion. We could see more proximity between RNAPII and the replication protein PCNA after PNUTS depletion, supporting that transcription-replication conflicts may underlie the replication phenotypes seen after PNUTS depletion. Further supporting this, treatment with the transcription inhibitor THZ1 could (partially) rescue the replication fork speed measured by the DNA fiber assay after PNUTS and WDR82 depletion. Overexpressing RNAseH, the enzyme that removes TRC-promoting R-loops, also partially rescued the EdU uptake after PNUTS depletion.

In paper I we found the RNAPII CTD binding protein CDC73 to be important for RNAPII mediated ATR signaling, and we wondered if CDC73 would have an effect on T-R conflicts in the absence of PNUTS. Remarkably, we found that co-depletion of CDC73 together with PNUTS could partially rescue replication fork speed and EdU incorporation seen after depletion of PNUTS alone. We showed that this was specific for CDC73 since expression of a siRNA resistant version of CDC73 could not enhance the EdU uptake after PNUTS depletion. Co-depletion of CDC73 also suppressed replication effects after depletion of WDR82. We could show that co-depletion of CDC73 together with PNUTS reduced the stability of RNAPII on chromatin compared to in PNUTS depleted cells, without affecting the phosphorylation of RNAPII CTD. In addition, although RNAPII was less chromatin bound after co-depletion of CDC73 with PNUTS, the fraction of RNAPII in the soluble fraction did not increase, which suggested that RNAPII was degraded. This led us to speculate that, in the absence of WDR82/PNUTS-PP1, CDC73 cannot be released from binding to hyperphosphorylated RNAPII, and the binding of CDC73 to RNAPII shields RNAPII from being degraded.

In paper II we have provided new insight into how T-R conflicts can be prevented. We propose a model where WDR82/PNUTS-PP1 normally keeps RNAPII in a dynamic state by controlling dephosphorylation of RNAPII and thus binding of CDC73 to RNAPII CTD. When RNAPII CTD is not dephosphorylated in a normal manner, such as in the absence of WDR82/PNUTS-PP1, CDC73 will continue to bind, preventing degradation of RNAPII from chromatin, potentially by shielding it from factors involved in degradation. The enhanced residency time of RNAPII on chromatin will promote T-R conflicts. Thus, keeping RNAPII in a dynamic state by controlling the phosphorylation status of the CTD is essential for preventing T-R conflicts.

3.3 Paper III

WDR82 protects cancer cells against inhibitors of Wee1 kinase

Previous work in the group have shown that cells treated with inhibitors against Wee1 accumulate DNA damage in S-phase. The exact cause of this DNA damage is still not completely understood, however it is likely linked to the role of Wee1 in suppressing CDK

activity. Since Wee1 inhibitors are in clinical trials for cancer treatment, understanding what factors contribute to the DNA damaging effects of Wee1 inhibitors is important.

In paper II we found that WDR82 is a strong interaction partner of PNUTS-PP1 and that the WDR82/PNUTS-PP1 complex is important for suppressing replication stress, likely by keeping RNAPII in a dynamic state and preventing T-R conflicts. We therefore investigated whether cells depleted of WDR82 might be particularly sensitive to Wee1 inhibition. We found that cells depleted of WDR82 displayed higher levels of the DNA damage marker γH2AX in S-phase as measured by flow cytometry, and showed enhanced DNA damage signaling by immunoblotting. This increased DNA damage was specifically caused by the lack of WDR82 since reintroducing a siRNA resistant version of WDR82 to the cells completely suppressed the DNA damage. Furthermore, as transcription inhibitors could reduce the enhanced DNA damage in Wee1-inhibited, WDR82-depleted cells, it is likely related to the role of WDR82 in suppressing T-R conflicts. In line with this, co-depleting WDR82 with the RNAPII CTD binding protein CDC73 could partially rescue the enhanced DNA damage seen after Wee1 inhibition and WDR82 depletion. In addition, supporting a role for RNAPII phosphorylation in these events, depletion of PNUTS also showed enhanced S-phase DNA damage after Wee1 and CHK1 inhibition.

In summary, we have shown that cancer cells with low levels of WDR82/PNUTS-PP1 are particularly sensitive to Wee1 inhibitors, and we speculate that T-R conflicts may be the underlying cause of this.

4 Discussion

4.1 Non-canonical ATR signaling via RNAPII

In paper I we elaborate on a previously proposed mode of DNA damage signaling via RNAPII [38]. It was previously shown that stalled RNAPII, for example after DNA damaging agents and after microinjection of RNAPII antibodies, could activate p53 [224, 225]. In one study, the authors showed that it occurred in an ATR and RPA dependent manner, as microinjection of ATR or RPA antibodies prevented p53 phosphorylation after RNAPII stalling [224]. The activation of p53 after microinjection of RNAPII antibodies only occurred if the antibodies were against the elongating form of RNAPII, which is phosphorylated on the CTD, and not the non-elongating unphosphorylated form. We have extended these results to show that hyperphosphorylated RNAPII S5, such as in the absence of the phosphatase complex PNUTS-PP1, causes hyperactivated ATR signaling as shown by enhanced levels of pCHK1, pRPA and γH2AX. We also showed that ATR could co-IP with RNAPII, and that the CTD binding protein CDC73 was needed for ATR signaling via RNAPII.

In contrast to the report from Derheimer *et. al.* [224] mentioned above, our results suggest that RPA is not needed for RNAPII mediated ATR signaling in general. In PNUTS depleted cells, the levels of CHK1 phosphorylation was higher than in control transfected cells that had been irradiated with 10 Gy, although RPA loading was lower. In addition, when we depleted RPA70 together with PNUTS, pCHK1 S345 was as high as in the PNUTS depleted cells alone, suggesting that RPA loading is not needed for, or at least does not correlate with, the levels of CHK1 phosphorylation after PNUTS depletion. This is consistent with another report showing that knockdown of RPA70 or RPA32 did not affect pCHK1 S317 levels after HU, UV or IR [226] and that CHK1 can be phosphorylated by ATR in the absence of an interaction with RPA [227]. It has been reported that depletion of RPA can induce human single stranded DNA-binding protein 1 (hSSB1) and its partner INTS3 to initiate ATR signaling [228]. However, triple knockdown of PNUTS, RPA and INTS3 did not abolish the high ATR activity caused by PNUTS depletion (results not shown), supporting an alternative mode of ATR signaling. In addition, there is no correlation between CHK1 phosphorylation and the

amount of ssDNA [77, 229] and a recent report showed that generation of large sections of ssDNA-RPA as a result of inhibition of POLA1 did not elicit an ATR response [230]. Furthermore, there is evidence that ATR-ATRIP exists in a large multi-protein complex which can interact with chromatin in an RPA independent manner [231]. Although we cannot exclude that small amounts of residual RPA contribute to ATR activation after PNUTS depletion it is not clear where the RPA would be located. The transcription bubble contains ssDNA, but the size of it is smaller than what would be needed to accommodate RPA molecules. A transcription bubble is approximately 22 nucleotides in size [232], while RPA covers about 30 nucleotides [233]. In addition, RNAPII almost completely surrounds the transcription bubble, and only about five bases of the single stranded non-template DNA is exposed outside the RNAPII elongation complex [234].

We found that the ATR activating proteins TOPBP1 and ETAA1 are not required for the enhanced ATR signaling in general after PNUTS depletion. Our results rather suggest that TOPBP1 and ETAA1 direct ATR activity towards the specific substrates CHK1 and RPA, respectively. When PNUTS was depleted, ETAA1 co-depletion reduced RPA phosphorylation without affecting CHK1 phosphorylation, and TOPBP1 co-depletion reduced CHK1 phosphorylation without affecting RPA phosphorylation. Only co-depleting ETAA1 together with TOPBP1 reduced both pCHK1 and pRPA. This is in line with previous reports where it was shown that ETAA1 and TOPBP1 likely operate in parallel to stimulate ATR activity [70, 71]. However, TOPBP1 was needed for the ATR dependent phosphorylation of CHK1 after PNUTS depletion, and exactly how TOPBP1 should be activated at sites of transcription stalling is unclear. It is widely accepted that in the context of ATR activation, TOPBP1 is recruited to sites of DNA damage via interactions with the MRN and 9-1-1 complex loaded at 5’ DNA junctions, a structure not present at stalled RNAPII. However, there are reports showing that TOPBP1 can bind DNA in the absence of a 5’ junction to mediate ATR activation towards CHK1 [235] and even RPA [236], which may explain how CHK1 can be activated at stalled RNAPII.

4.2 A role for WDR82 in regulation of ATR signaling

In paper I we show that PNUTS-PP1 phosphatase complex can suppress ATR signaling by dephosphorylating RNAPII CTD. In paper II we find that WDR82 is a strong interaction partner of PNUTS, and that it is important for dephosphorylation of RNAPII CTD in live cells. Our unpublished results show that WDR82 also is important for regulation of ATR signaling. Depletion of WDR82 causes enhanced ATR signaling as seen by increased phosphorylation of ATR targets CHK1 S317/S345 (**Figure 6** A,B), and both pCHK1 S317/S345 and RPA S33 is enhanced after short incubations with thymidine (**Figure 6** A,B). This effect was rescued by expression of an siRNA resistant version of WDR82 (**Figure 6** A, B). Similarly, enhanced ATR signaling after thymidine was also seen after longer exposures to thymidine (24 hr) in paper II. Furthermore, by measuring entry into mitosis after ATR inhibition, we could see that more cells entered mitosis in WDR82 depleted cells compared to control cells. (**Figure 6** C, D). This indicates that more cells were stalled at the G2 checkpoint in the WDR82 depleted cells. In addition, depletion of WDR82 also specifically caused ATR dependent, but replication independent, γH2AX in G2/M (**Figure 7** A, B, C, D) further supporting a role for WDR82 in suppression of ATR signaling. The enhanced ATR signaling seen after depletion of WDR82 is likely mediated via RNAPII, since treatment with transcription inhibitors (**Figure 8** A) or co-depleting the RNAPII CTD binding protein CDC73 (**Figure 8** B) reduces it. To our knowledge, this is the first time WDR82 has been shown to regulate ATR signaling.

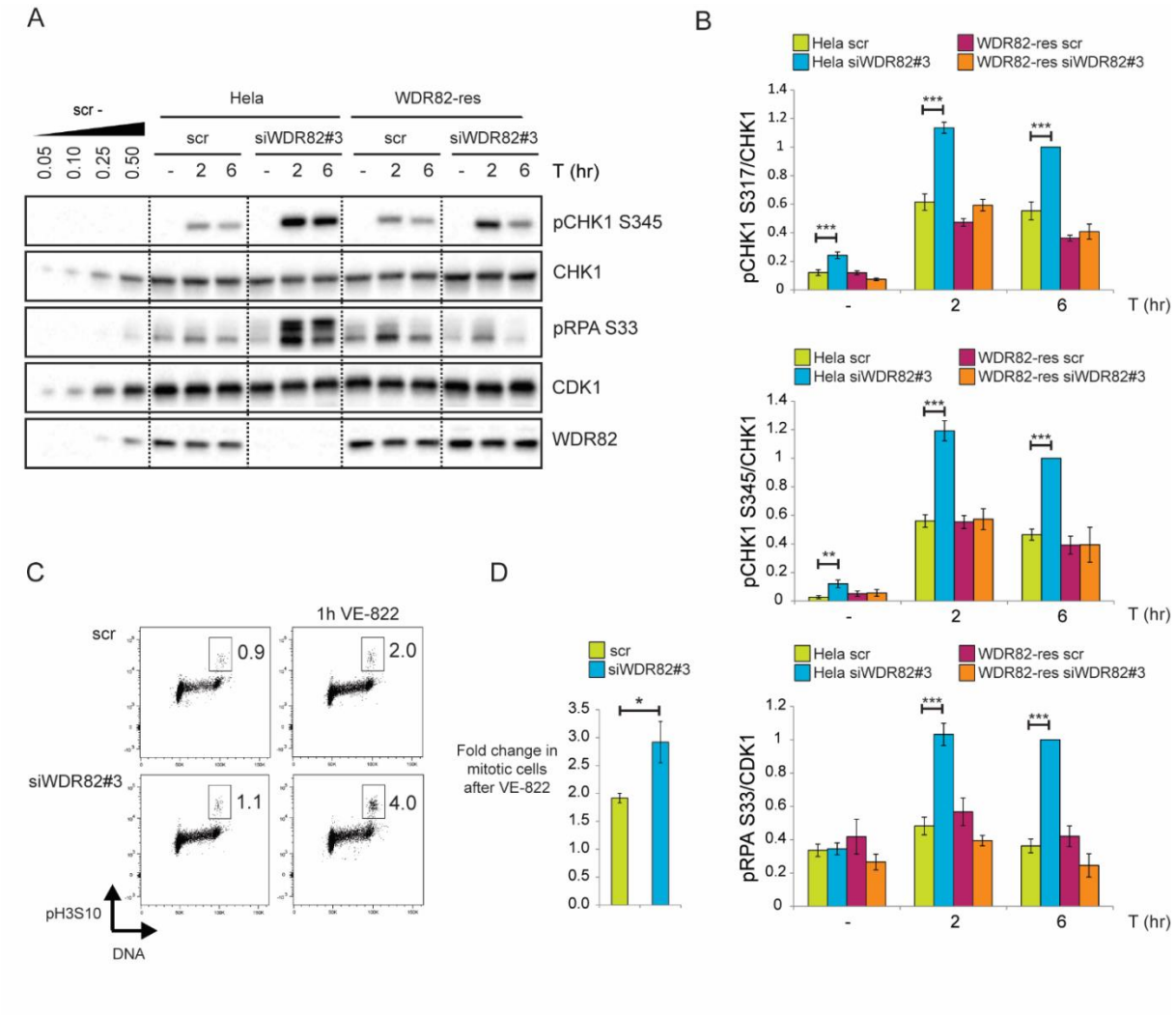

Figure 6: WDR82 depleted cells have enhanced ATR signaling and G2 checkpoint

A) Representative western blot of Hela or Hela expressing siRNA resistant WDR82 (WDR82-res) cells 72h after siRNA transfection with scr or siWDR82#3. Cells were treated or not with thymidine for 2 or 6 hrs. **B)** Quantifications from three independent experiments as in A). P values were calculated by the two-sided two-sample Student's t-test. **C)** Flow cytometry charts showing pH3S10 staining versus DNA content of cells transfected with scr of siWDR82#3 and harvested at 72 h after siRNA transfection with and without 1 h treatment with VE-822. Mitotic cells were selected based on DNA content and high pH3S10 staining as indicated. Numbers indicate percentages of mitotic cells. **D)** Quantifications show fold increase in mitotic cells after 1h VE-822 for each siRNA condition. P values were calculated by the two-sided two-sample Student's t-test.*p<0.05, **p<0.01, ***p<0.001.

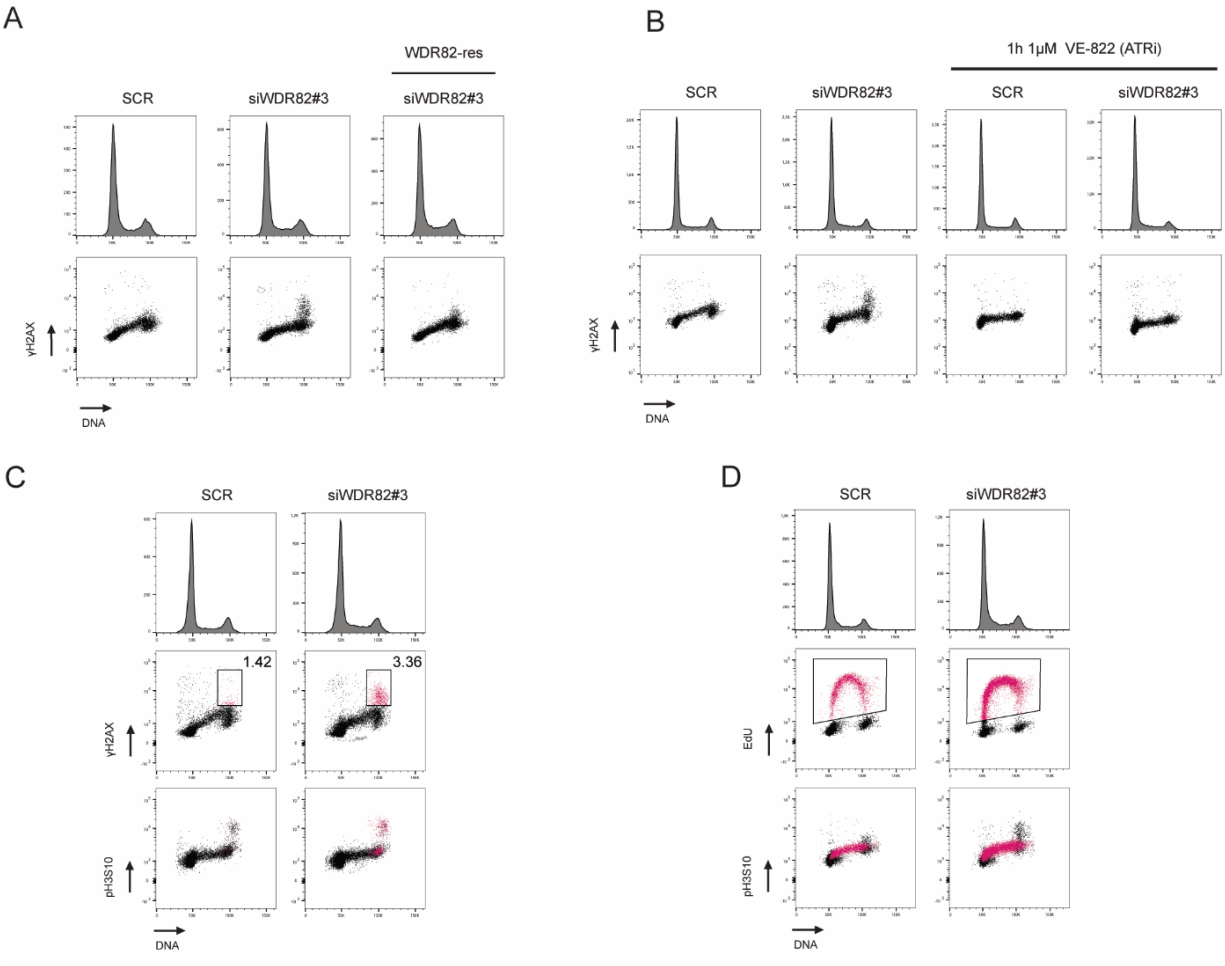

Figure 7: WDR82 depleted cells show ATR dependent, but replication independent, γH2AX staining in G2/M A) Flow cytometry charts showing DNA profiles (upper panel) or γH2AX staining versus DNA content (lower panel) of Hela cells transfected with scr of siWDR82#3 and WDR82-res cells transfected with siWDR82#3. The cells were harvested at 72 h after siRNA transfection. **B)** Flow cytometry charts as in A). Hela cells transfected with scr or siWDR82#3 and harvested 72hrs later. The cells were treated or not with 1 μM of ATR inhibitor VE-822 for 1 hr. **C)** Flow cytometry charts as in A) showing γH2AX or pH3S10 staining versus DNA content (middle and lower panel respectively) of Hela cells transfected with scr of siWDR82#3 and WDR82-res cells transfected with siWDR82#3. The cells were harvested at 72 h after siRNA transfection. Numbers indicate percentage of high level γH2AX cells in gate as indicated. The cells gated in the γH2AX plot are shown in the plot for pH3S10 as pink cells. **D)** Flow cytometry charts as in A) showing EdU incorporation and pH3S10 stained cells versus DNA stain (middle and lower panels, respectively) in Hela cells transfected with scr or siWDR82#3. The cells were harvested 72 hrs after transfection. EdU positive cells are gated and displayed in pink in the pH3S10 plot.

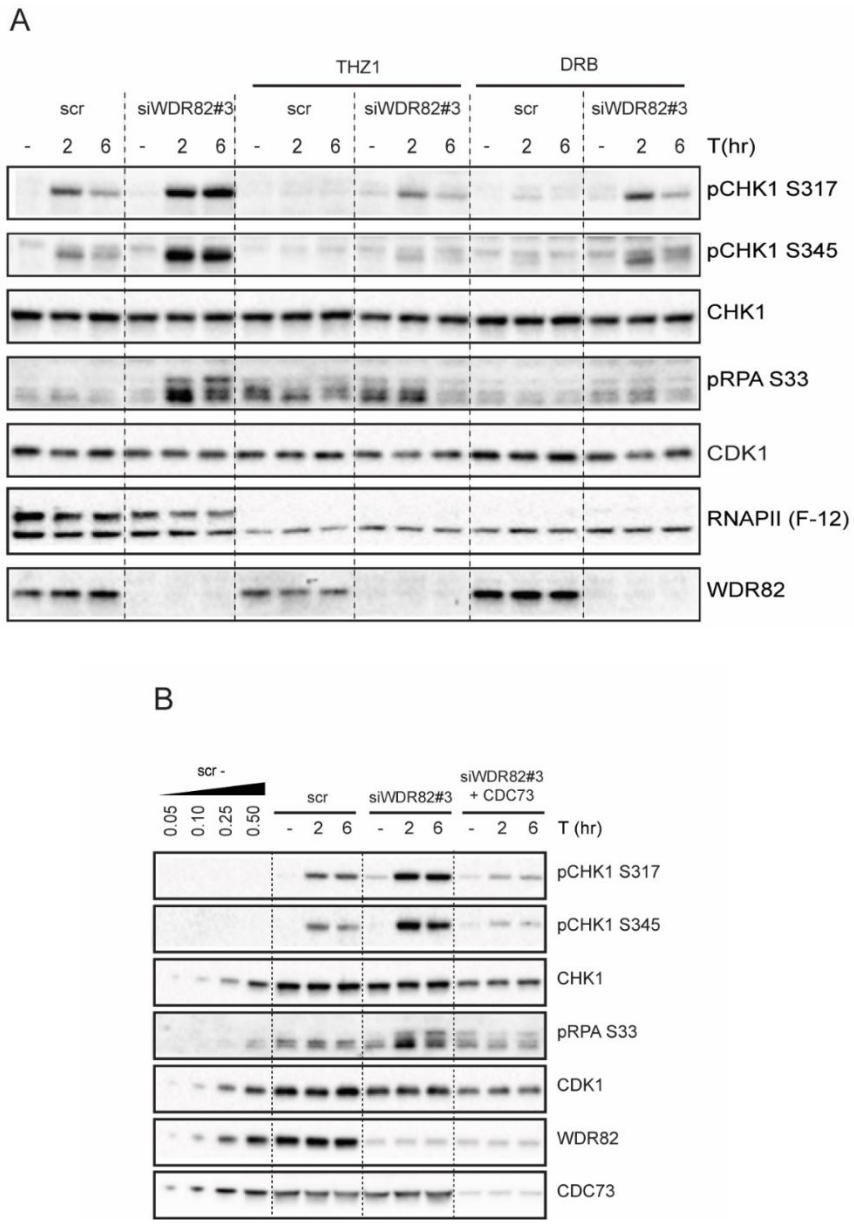

Figure 8: Transcription inhibitors of co-depletion with the transcription-associated protein CDC73 suppress ATR signaling. A) Western blot of cells transfected with scr of siWDR82#3 and harvested after 72 hrs. Cells were treated or not with thymidine for 2 or 6 hrs, and with THZ1 or DRB. Transcription inhibitors were added 30 min prior to addition of thymidine. **B)** Western blot of cell transfected with scr, siWDR82#3 alone or together with siCDC73 and harvested after 72 hrs. The cells were treated or not with thymidine for 2 or 6 hrs.

4.3 WDR82/PNUTS-PP1 are required for normal replication

In paper II we show that WDR82/PNUTS-PP1 are required for normal replication, in addition to their role in suppressing ATR signaling. Cells depleted of either PNUTS or WDR82 had reduced EdU uptake and reduced replication fork speed. A role for WDR82 in replication is consistent with another report, mainly focusing on the WDR5-RbBP5-ASH2L-DPY30 (WRAD) complex, which also showed that WDR82 appears to promote S-phase progression [237]. Depletion of WDR82 led to a reduced percentage of BrdU positive cells, and cells showed mitotic defects such as micronuclei formation and binucliation [237], phenotypes typically associated with replication stress [238]. However, only one siRNA oligo against WDR82 was used in this study and the replication effects were not further verified or explored. In paper II we also showed that cells depleted of WDR82 or PNUTS failed to recover efficiently from replication fork stalling after thymidine. This is consistent with another study [239], where PNUTS was identified as a candidate hit in an siRNA screen for factors required for replication fork stability and/or recovery after stalling by HU. In this study, PNUTS was also found to be associated with collapsed replication forks (but not normal forks) [239].

In addition, WDR82 and PNUTS were found in another screen searching for proteins located at, or in the vicinity of replication forks [240]. Altogether 593 proteins were identified in the screen, and they also performed a secondary screen with these 593 proteins, monitoring hypersensitivity to inhibition of ATR, HU and CTP after siRNA transfection. Interestingly, PNUTS was among 28 proteins scoring at all three criteria. This is highly consistent with our results and with PNUTS having a role in protection against replication stress. More specifically, the first screen used a combination of iPOND and MS, and they found that WDR82 and PNUTS bound the nascent DNA. This was similar to several other proteins involved in RNAPII elongation, and the authors suggested that these may represent proteins needed to prevent T-R conflict. [240]. This is in line with our results from paper II where we found that pRNAPII S5 stability on chromatin was enhanced in the absence of PNUTS or WDR82, and that it was causing T-R conflicts.

4.3.1 T-R conflicts may contribute to the effects of Wee1 inhibition

Inhibition of Wee1 causes DNA damage in S-phase. This can, at least partly, be attributed to enhanced CDK activity and increased origin firing, which in itself may lead to shortage of replication factors and subsequent replication stalling [51, 241]. The high CDK activity after Wee1 inhibition may also directly activate endonucleases that cleave replication fork structures, adding to the DNA damage [242]. Notably, increased origin firing after overexpression of Cyclin E has been shown to cause increased interference between transcription and replication [134]. Whether increased origin firing after Wee1 inhibition causes more T-R conflicts that can add to the S-phase damage is unknown. In paper II we showed that WDR82 protects against replication stress, likely through prevention of T-R conflicts, and in paper III we explored if cells with low levels of WDR82 would be sensitive to inhibition of Wee1. Indeed, we found that WDR82 depleted cells were more sensitive to Wee1 inhibition than control cells. The sensitizing effect could likely be explained by WDR82s role in preventing T-R conflicts since pre-treating with transcription inhibitors, or co-depletion with CDC73, could partially rescue the effects of Wee1i induced DNA damage in WDR82 depleted cells.

4.4 The nature of RNAPII after depletion of WDR82/PNUTS-PP1

Over the years, it has become increasingly appreciated that interference between transcription and replication is a major source of replication stress, and that it can contribute to cancer development. Since the processes of transcription and replication occur on the same template, this execution is highly regulated, and factors that disrupt this regulation may be harmful. As mentioned in section 1.3 (“Transcription as a threat to genome stability”) there are many causes of T-R conflicts, including changes in the replication- or transcriptional programs, alterations in polymerase processivity, and aberrations in the normal RNA processing. In paper I and paper II we show that WDR82/PNUTS-PP1 can dephosphorylate pRNAPII S5. The phosphorylation status of RNAPII-CTD is involved in both RNAPII processivity and also regulates the recruitment of proteins involved in the different

stages of the transcription cycle. In future experiments it would be interesting to look more into the nature of RNAPII after depletion of WDR82/PNUTS-PP1. In the following sections, I will discuss the specific processes that may be affected.

4.4.1 Potential role of WDR82/PNUTS-PP1 in transcription initiation or elongation

As described in chapter 1.3.1 ("The transcription cycle") the process of transcription is regulated in many steps. For transcription to be initiated the polymerase must be recruited to the promoter and several factors operate to shift it into the elongating form. WDR82 and/or PNUTS-PP1 may regulate several aspects of transcription that may affect initiation or elongation when these proteins are missing. For example, one way WDR82 might do this is via its role in histone methylation [200], specifically H3K4me3, which is a histone mark associated with active transcription. However, although there is a strong correlation between this histone mark and active transcription, there is little evidence that H3K4me3 is required for transcriptional activation. Reduced levels of H3K4me3 have no large effects on transcription levels of most genes [243]. Depletion of WDR82 has been shown to reduce H3K4me3 [189, 198], however, we have not been able to see large differences in H3K4me3 levels when immunoblotting whole cell lysates, and there were no correlation between H3K4me3 levels and e.g. ATR activation after WDR82 depletion (results not shown). In addition, PNUTS-PP1 does not bind to SET1, the enzyme that methylates H3K4, and the SET1 complex and PTW/PP1 complex fractionate at different sizes, indicating that these are independent complexes [187]. Although we cannot completely rule out a role for H3K4me3 in RNAPII mediated ATR signaling, or replication stress due to RNAPII retention, it is unlikely that altered H3K4me3 levels can explain the phenotypes we see after depletion of WDR82 and PNUTS.

A feature of the WDR82/PNUTS-PP1 complex, specifically PNUTS, which may influence transcription elongation, is the presence of a TFIIS LW domain in PNUTS [244, 245]. This is not a very common domain, but it is shared with other proteins involved in transcription elongation, including the elongation factors MED26 (part of the mediatior complex), elongin A and the TFIIS elongation factor where the domain lends its name from

[245]. TFIIS is an elongation factor that is involved in release of backtracked RNAPII (see 1.3), and depletion or mutation of TFIIS has been shown to cause genome instability (e.g. [143]). The TFIIS LW domain is what is called Domain I in TFIIS, and although this domain has been shown to be involved in transcription, it is not needed for the transcript cleavage function of TFIIS, which is important for the release of backtracked RNAPII [246]. Rather, it has been suggested to be a "docking platform" for other proteins involved in transcription elongation [247]. Although the physiological function of this domain in PNUTS is unknown, it binds TOX4 [187], which was one of the strongest hits from the SILAC-IP in paper II. Interestingly, TOX4 has been shown to co-IP with several of the proteins in the PAF1 complex, including CDC73 [248]. Since depletion of TOX4 strongly reduces PNUTS levels and PNUTS depletion almost completely depletes TOX4 ([187] and data not shown) we have not been able to study the independent functions of TOX4 in relation to ATR signaling (paper I) and replication stress (paper II). Studies of PNUTS lacking the TFIIS LW domain may be able to give clues to how TOX4 is functioning and whether its interaction with the PAF1 complex is relevant, and would thus be an interesting topic for further investigation.

4.4.2 Potential role of WDR82/PNUTS-PP1 in transcription termination

Transcription termination is important to prevent interference between transcription and replication, and lack of factors involved in transcription termination has been shown to cause replication stress and transcription dependent DNA damage [153]. Transcription termination defects may also cause interference between converging RNAPII machineries [249, 250]. WDR82/PNUTS-PP1 and their yeast homologs have been shown to cause transcription termination defects in both yeast and vertebrates [189, 251-254], but whether the lack of dephosphorylation of RNAPII in the absence of WDR82/PNUTS-PP1 is influencing termination directly is not clear. It may be that aberrant dephosphorylation of RNAPII S5 prevents loading of factors needed for transcription termination. For example, different termination factors are loaded on serine 2 phosphorylated RNAPII [97], and one possibility is that loading of these are disrupted in the case when RNAPII S5 is not dephosphorylated.

Transcription termination differs somewhat in yeast and vertebrates, and the mechanism for termination is different depending on the type of transcript. However, in general, and as mentioned before (1.3.1), transcription termination involves loading of 3' processing factors, such as the Cleavage and polyadenylation factor complex (CPF in yeast, CPSF in human), RNAPII transcription across a polyadenylation signal (PAS), and release of RNAPII from chromatin. In yeast, the homologs of WDR82/PNUTS-PP1 interact with the CPF as part of a sub-complex of the called APT (for Associated with Pta1) [185, 251-253]. Both PNUTS and PP1 have also been shown to co-IP with factors in the human 3' processing complex [255]. It is possible that, in addition to causing a defect in RNAPII S5 dephosphorylation, depletion of WDR82 or PNUTS also cause transcription termination defects related to its interaction with CPF/APT that may underlie the replication stress we see in paper II.

One mechanism for transcription termination ("the torpedo model", see 1.3.1) involves slowdown of RNAPII as it traverses the PAS, and release of RNAPII by the exonuclease XRN2. PNUTS-PP1 has been implicated in the slowdown of RNAPII, an effect attributed to dephosphorylation of the elongation factor Spt5 (component of DSIF) [254]. Transcription could not terminate properly when Spt5 was not dephosphorylated. However, the authors do not show conclusive evidence that PNUTS-PP1 dephosphorylates Spt5 directly, and have not considered pRNAPII S5 as a target for dephosphorylation, and thus, it cannot be ruled out that it is involved.

4.4.3 Potential role of WDR82/PNUTS-PP1in removal of RNAPII from chromatin

RNAPII may stall for a number of different reasons, and how the stalling is resolved depends on the context. As mentioned above, a backtracked polymerase may be released by the action of TFIIS, or RNAPII molecules that have encountered a transcription-blocking lesion may be tackled by TC-NER. CSB is needed for TC-NER, although the exact role of CSB during TC-NER is elusive ([157] and see 1.3.2.). RNAPII that cannot be repaired by TC-NER or otherwise resume transcription will eventually be targeted for degradation. This is thought to be a last resort mechanism for removal of RNAPII, which is independent of TC-NER [162].

Interestingly, the PTW/PP1 complex associates with CSB after UV-damage [256]. Ubiquitination and degradation of RNAPII is completely blocked by phosphorylated RNAPII S5 in yeast [164], and thus, dephosphorylation of RNAPII by WDR82/PNUTS-PP1 may be a way of promoting degradation when TC-NER fails. In support of this, in paper II we found that RNAPII was more stable on chromatin after depletion of PNUTS or WDR82. Lack of dephosphorylation of the RNAPII CTD enhances the binding of CDC73 to RNAPII and we speculate this may somehow shield RNAPII from factors involved in degradation. The reasoning behind this is that co-depletion of CDC73 with PNUTS reduced the fraction of chromatin bound RNAPII without increasing RNAPII levels in the soluble fraction, suggesting that it can be removed and degraded from of chromatin in the absence of CDC73. In addition, in paper I we showed that pRNAPII S5 can activate ATR signaling in a CDC73 dependent manner. The yeast homolog of CSB, Rad26, is phosphorylated by the ATR homolog Mec1 upon UV-damage, and the phosphorylation site is required for TC-NER in yeast [257]. Interestingly, CDC73 has been shown to be required for TC-NER in yeast [258], and thus, controlling the phosphorylation status of RNAPII by WDR82/PNUTS-PP1 may control binding of CDC73 to RNAPII to prevent degradation of RNAPII while TC-NER is attempted. Based on this, it is tempting to speculate on a model where stalled RNAPII becomes hyperphosphorylated and attracts CDC73 to promote ATR signaling (**Figure 9**).

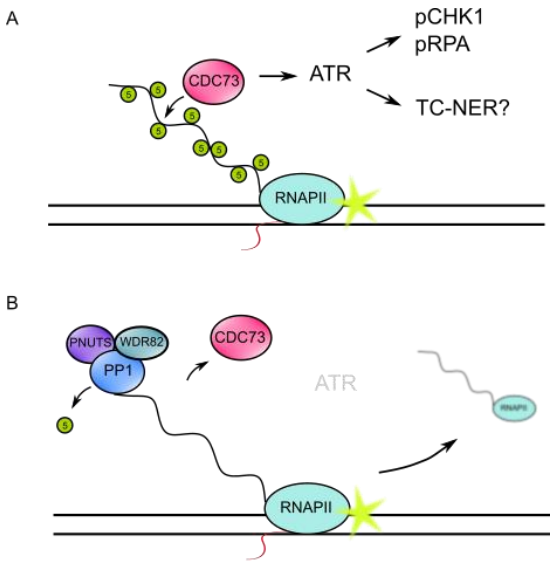

Figure 9: Model for how WDR82/PNUTS-PP1 regulates ATR signaling and counteract T-R conflicts.
Se text for details.

Activated ATR can promote TC-NER by phosphorylation of Rad26/CSB. In the case where TC-NER is not needed (such as when RNAPII stalling is due to e.g. T-R conflicts?) or TC-NER fails, RNAPII may be dephosphorylated, CDC73 released, and RNAPII degraded (**Figure 9** B). This could also "shut off" the ATR signaling pathway. More work is needed to establish if this model is correct.

4.5 Implications for cancer and cancer treatments

As mentioned in the introduction, both cancer and ageing are associated with a failure to protect the genome from DNA damage. Classical DNA damage response proteins have been considered to be tumor suppressor proteins, since their loss leads to genomic instability and tumorigenesis [259]. In contrast, ATR is essential for cell viability [26, 56, 57, 260], and ATR is almost never mutated in cancer [259]. In fact, ATR-Seckel mice, which have severely reduced levels of ATR, are actually resistant to developing tumors [261-264]. On the other hand, evidence suggest that tumor cells are highly dependent on ATR to tackle the high levels of replication stress these cells experience. Accordingly, inhibitors against ATR are explored as cancer treatment, either as mono-treatment or as an adjuvant to chemotherapy or IR [259, 265]. However, it is important to know how ATR is functioning, and what the consequences of inhibiting it might be, before implementing ATR inhibitors in the clinic. In paper I we have provided more knowledge about a non-canonical mode of ATR activation via RNAPII. We showed that PNUTS/PP1, and WDR82 (this thesis) suppress RNAPII mediated ATR signaling also in non-replicating cells, which may be relevant when it comes to using ATR inhibitors as cancer therapy. Most cells in the body do not replicate at any given time [266], and the outcome of ATR activation in replicating versus non-replicating cells differ. For example, while the effects of ATR activation after replication stress is promoting genome stability by e.g. activation of cell cycle checkpoints, controlling origin firing and stabilizing replication forks [24, 63], ATR activation after RNAPII stalling in non-cycling cells can lead to apoptosis [267, 268].

In paper II we established that the phosphorylation status of RNAPII can affect the residency time of RNAPII on chromatin, and that dephosphorylation of RNAPII S5 could

possibly promote degradation and prevent T-R conflicts. It has become increasingly clear that T-R conflicts are a major source of replication stress, and that this may promote tumorigenesis [116, 117]. Knowledge about factors that prevent replication stress in this way, such as WDR82 or PNUTS, might be useful when determining the efficacy of treatment. As such, in paper III we showed that cells with low levels of WDR82 or PNUTS might be particularly sensitive to treatment with a Wee1 inhibitor. Wee1 inhibitors are in clinical trials, and there is need to find good biomarkers for determining which patients may benefit from the treatment. For example, low expression of WDR82 was demonstrated in a cohort of colorectal cancer patients [205]. Our results may suggest that patients with low expression of WDR82 or PNUTS could benefit from treatment with a Wee1 inhibitor. In addition, it is important to understand the basic mechanisms underlying the S-phase effects of Wee1 inhibition. T-R conflicts may add to the effects already described such as depletion of replication factors, nuclease activation etc. (**Figure 10**). Furthermore, high expression of WDR82 correlated with higher survival in pancreatic cancer and high expression of PNUTS is a favorable prognostic marker in pancreatic and cervical cancer [191-193], suggesting that an important function of these proteins is to suppress replication stress and thereby reduce aggressiveness of these cancers.

As mentioned in section 1.4.4, CDC73 is a known tumor suppressor, but it has also been described to have oncogenic capacities in some cases. The exact reason for why this is not known. We found that CDC73 is important for ATR signaling and that it prevented degradation of RNAPII from chromatin after RNAPII stalling, both of which may be relevant for cancer development. Since mutations in CDC73 causes hyperparathyroidism-jaw tumor syndrome (HPT-JT) [206] and a number of different cancers [207-210], more knowledge about the diverse functions of this protein is needed.

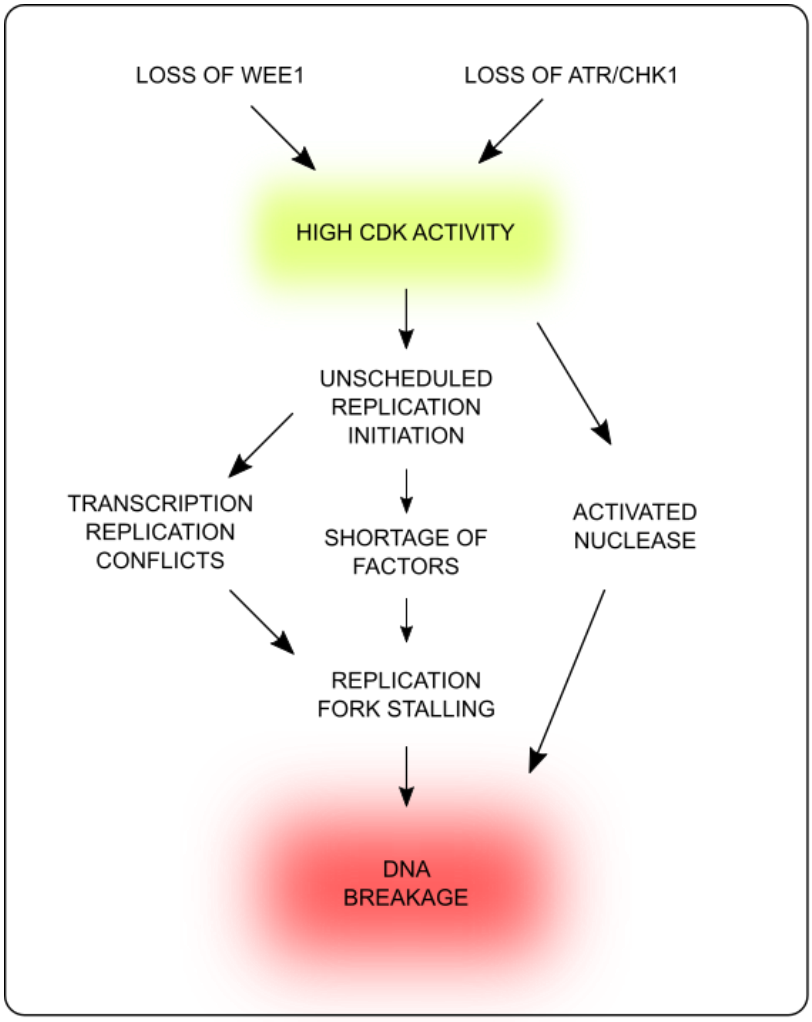

Figure 10 Transcription replication conflicts can add to the replication stress and S-phase DNA damage after Wee1 inhibition.

5 Concluding remarks

Cancer development is a multistep process associated with replication stress and increased genomic instability, and thus, the DNA damage response can act as a barrier for cancer development. However, since cancer cells have high levels of replication stress, they also rely on factors in the DDR to survive. For example, cancer cells have been shown to rely on ATR for survival [259]. Understanding the underlying causes of replication stress, and how the cells deal with it, is important to understand cancer development and to develop effective treatments.

We have provided more insight to non-canonical ATR signaling (paper I), and shown that the phosphorylation status of RNAPII can trigger activation of ATR in a CDC73 dependent manner. More work is needed to fully understand the role of CDC73 in this context, and to find out how ATR phosphorylates the downstream targets in the absence of classical ATR activating structures. This mode of ATR signaling is likely important for the many non-cycling cells in the body and should be offered more attention. Importantly, understanding how ATR operates in non-cycling cells is essential to evaluate the potential for ATR inhibitors in clinic.

We have also shown that the phosphorylation status of RNAPII can determine the stability of RNAPII on chromatin (paper II), and shown that dephosphorylation of RNAPII by WDR82/PNUTS-PP1 is needed to keep RNAPII in a dynamic state. The consequences of faulty dephosphorylation, such as in the absence of WDR82 or PNUTS, is enhanced replication stress by T-R conflicts. The enhanced replication stress can be exploited for cancer treatment, and we show that cells with low levels of WDR82 are particularly sensitive to Wee1 inhibition (paper III).

In summary, we have contributed to more knowledge about the basic mechanisms of replication stress, how the DDR kinase ATR is activated, and how we may use this information for cancer treatment.

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Regulation of ATR activity via the RNA polymerase II associated factors CDC73 and PNUTS-PP1

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ABSTRACT

Ataxia telangiectasia mutated and Rad3-related (ATR) kinase is a key factor activated by DNA damage and replication stress. An alternative pathway for ATR activation has been proposed to occur via stalled RNA polymerase II (RNAPII). However, how RNAPII might signal to activate ATR remains unknown. Here, we show that ATR signaling is increased after depletion of the RNAPII phosphatase PNUTS-PP1, which dephosphorylates RNAPII in its carboxy-terminal domain (CTD). High ATR signaling was observed in the absence and presence of ionizing radiation, replication stress and even in G1, but did not correlate with DNA damage or RPA chromatin loading. R-loops were enhanced, but overexpression of EGFP-RNaseH1 only slightly reduced ATR signaling after PNUTS depletion. However, CDC73, which interacted with RNAPII in a phospho-CTD dependent manner, was required for the high ATR signaling, R-loop formation and for activation of the endogenous G2 checkpoint after depletion of PNUTS. In addition, ATR, RNAPII and CDC73 co-immunoprecipitated. Our results suggest a novel pathway involving RNAPII, CDC73 and PNUTS-PP1 in ATR signaling and give new insight into the diverse functions of ATR.

INTRODUCTION

The ataxia telangiectasia mutated and Rad3-related (ATR) kinase is a master regulator of DNA-damage and replication-stress signaling coordinating DNA repair, cell cycle checkpoint and cell-death pathways (1). Understanding how ATR is activated is therefore a critical issue in biomedical research. The canonical pathway for ATR activation is initiated by the presence of single-stranded DNA (ssDNA) coated by RPA (ssDNA-RPA) (2). ssDNA-RPA at sites of DNA damage recruits ATR via its obligate binding partner ATRIP (2,3). Full activation of ATR is further facilitated by TOPBP1 (1). A large amount of evidence supports an important role for the canonical pathway in ATR activation (e.g. reviewed in (4)) However, there is also evidence suggesting the existence of alternative pathways (5), which are less well understood.

In one proposed alternative pathway the cell takes advantage of its transcription machinery to activate ATR (6,7). This was proposed based on the finding that upon stalling, elongating RNAPII could induce ATR-dependent P53 phosphorylation (7). RNAPII might thus act as a sensor for DNA damage (6). In fact, RNAPII is a recognised sensor in transcription-coupled repair where it recruits DNA-repair factors to sites of damage (8,9). The discovery of pervasive transcription outside protein coding genes (10), suggests that RNAPII might be scanning a majority of the genome and makes an involvement of RNAPII in sensing DNA damage and activating ATR conceivable (6). However, such an upstream role of RNAPII in ATR activation has yet to gain wide acceptance, perhaps because the factors involved in signaling between stalled RNAPII and ATR remain unknown.

During the transcription cycle, RNAPII becomes reversibly phosphorylated on the carboxy-terminal domain (CTD) of its largest subunit. Phosphorylation of specific residues in the CTD heptapeptide repeats, e.g. Ser 2 (S2) and Ser 5 (S5), is associated with specific phases of the transcription cycle. This is thought to contribute to a CTD 'code', in which combinations of post-translational modifications on the CTD can be 'written' and 'read' to regulate association with transcription and RNA processing factors (11). Interestingly, increased phosphorylation of the CTD has been observed after ultraviolet radiation and camp-

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tothecin in human cells (12,13) and is tightly connected to RNAPII stalling (14,15). Notably, RNAPII stalling can also occur after other types of stress, e.g. upon head-on collisions between RNAPII and the replication fork (16–18) or following ssDNA breaks or cyclopurines such as formed after IR (8,19–21). Furthermore, several proteins that interact with the phosphorylated CTD were required for resistance to ionizing radiation (IR) or doxorubicin in *Saccharomyces cerevisiae* (22). Based on these findings, one possibility would therefore be that RNAPII responds to stress by signaling via its CTD.

We previously discovered that siRNA-mediated depletion of the Protein Phosphatase 1 Nuclear Targeting Subunit (PNUTS) activates a G2 checkpoint in unperturbed cells and prolongs the G2 checkpoint after IR, but the underlying molecular mechanisms remained to be identified (23). Interestingly, PNUTS is one of the most abundant nuclear regulatory subunits of PP1 (24,25), and RNAPII CTD is the only identified substrate of PNUTS-PP1 (26). PNUTS-PP1 dephosphorylates RNAPII S5 (CTD) in vitro (27) and depletion of PNUTS causes enhanced RNAPII S5 phosphorylation (pRNAPII S5) in human cells (28). Because RNAPII, as described above, has a proposed role in ATR activation and ATR is a crucial player in the G2 checkpoint, we addressed whether PNUTS-PP1 might suppress ATR signaling. Our results show that ATR signaling increases after PNUTS depletion in a manner not simply correlating with DNA damage, R-loops or RPA chromatin loading. The increased ATR signaling rather appears to depend upon CTD phosphorylation, which is counteracted by PNUTS-PP1. Furthermore, the known phospho-CTD binding protein, CDC73, is required for the high ATR signaling, and ATR, RNAPII and CDC73 co-immunoprecipitates.

MATERIALS AND METHODS

Cell culture and treatments

Human cervical cancer HeLa and osteosarcoma U2OS cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (Life Technologies). The cell lines were authenticated by short tandem repeat profiling using Powerplex 16 (Promega) and regularly tested for mycoplasma contamination. HeLa BAC cells stably expressing EGFP mouse pnuts were a generous gift from the laboratory of Tony Hyman (http://hymanlab.mpi-cbg.de/bac_viewer/search.action).

To generate the flag-CDC73 cell lines, CDC73 (Addgene plasmid # 11048) was amplified using the primers aggctttaaaggaaccaattcagtcgactgGAATTCGGATCC ACCA (Cdc73 entry fwd) and aagaaagctgggtctagata tctcgagtgcTCAGAATCTCAAGTGCG (Cdc73 entry rev) and cloned into BamH1–Not1 cut pENTR1A using Gibson cloning (NEB E5510S). To generate the siRNA-resistant constructs, silent mutations were introduced in the siRNA target site using the Quick Change Lightning kit (Agilent 210518). The mutagenic primers were: CATCAGATGAAAAGAAGAAGCAGGGA-T GCCAGAGGGAAAATGAAACTCTAATACA and TGTATTAGAGTTTCATTTTCC-CTCTGGCATCCCT GCTTCTTCTTTTCATCTGATG. The construct was cloned into the lentiviral expression vector pCDH-eF1-GW-IRES-puro by Gateway cloning (Thermo-Fisher Scientific 11791020). HeLa cells were transduced and cells carrying the transgene were selected with 0.5 μg/ml puromycin.

Cells were irradiated in a Faxitron x-ray machine (160 kV, 6.3 mA, 1 Gy/min). Thymidine (Sigma-Aldrich) was used at 2 mM, Hydroxyurea was used at 80 μM, ATR-inhibitors VE-821 (Axon Medcem) and VE-822 (Selleck Biochem) at 10 and 1 μM respectively, CDK7-inhibitor THZ1 (ApexBio) at 1 μM, CDK9-inhibitor DRB (Sigma-Aldrich) at 100 μM, XPB-inhibitor triptolide (Sigma-Aldrich) at 1 μM and translational inhibitor cycloheximide (Sigma-Aldrich) at 10 μg/ml.

siRNA and DNA transfections

Wildtype and RAXA (mutated in the 'RVXF' (^{398}SVTW^{401}) motif: V399A, W401A) full-length EGFP PNUTS DNA constructs containing 14 silent mutations in the domains targeted by siPNUTS (#1 and #2) were synthesized by Geneart and cloned into pGLAP3 (siPNUTS #2 is also called siPNUTS). pEGFP-RNaseH1 was a kind gift from Robert Crouch. Sequences of siRNA oligonucleotides can be found in supplementary Table S1. siRNA was transfected using Oligofectamine or RNAimax (Life technologies), and plasmid DNA with Fugene HD (Promega) or Attractene (Qiagen). Experiments were performed 65–72 h after siRNA transfection unless otherwise stated.

Western blotting and antibodies

For quantitative western blotting, cells were resuspended in ice-cold TX-100 buffer (100 mM NaCl, 50 mM Tris pH 7.5, 2 mM $MgCl_2$, 0.5% TX-100) containing 100 U/ml Benzonase (Sigma-Aldrich). After 1 h incubation on ice, Lane Marker Reducing Sample Buffer (Pierce Biotechnologies) was added and samples were boiled (95°C, 5 min). Criterion TGX gels (BioRad) and nitrocellulose membranes (BioRad) were used for separation and transfer respectively. Antibodies used are found in supplementary Table S2. Blots were imaged in a Chemidoc MP (BioRad) using chemiluminescence substrates (Supersignal west pico, dura or femto; Thermo Scientific). Quantifications were performed and images processed in Image Lab 4.1 (BioRad) software. Range of detection was verified by including a dilution series of one of the samples (see, e.g. Figure 1B) and excluding saturated signals. The resulting standard curve allowed accurate quantification. To blot for total protein after detection of a phosphorylated protein, membranes were stripped using ReBlot Plus Mild Antibody Stripping Solution (Millipore).

Cell sorting and flow cytometry

For cell sorting and flow cytometry with EdU labeling, cells were labeled for 1 h with 2 μM EdU and fixed in 70% ethanol. EdU was labeled with the Click-iT Plus EdU Alexa Fluor 488 Flow Cytometry Assay Kit (Thermo Fisher), and DNA with FxCycle Far Red. Cells were sorted with a

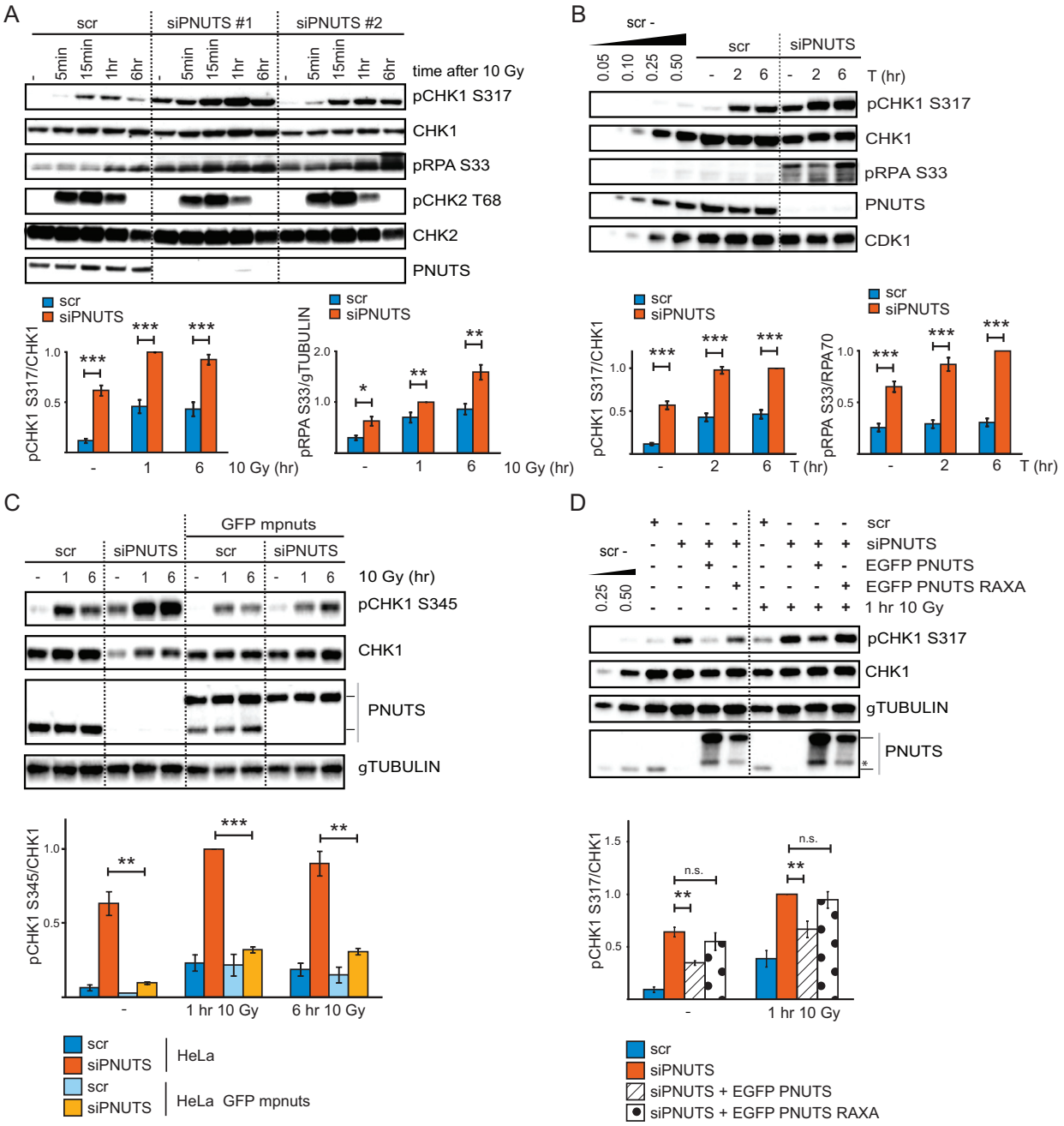

Figure 1. PNUTS-PP1 suppresses ATR signaling. (**A**) Western blot analysis of ATR and ATM signaling events in control scrambled siRNA transfected (scr) or PNUTS siRNA transfected (siPNUTS #1 and siPNUTS #2) HeLa cells, without IR or at indicated times after 10 Gy. Cells were harvested at 72 h after siRNA transfection. Bottom bar charts show quantification of pCHK1 S317 relative to CHK1 and pRPA S33 relative to γTUBULIN levels for siPNUTS #2, hereafter called siPNUTS ($n = 8$). (**B**) Western blot analysis of untreated cells or at 2 or 6 h after addition of thymidine to cells siRNA transfected as in A) (scr and siPNUTS). Bottom bar charts show quantification of pCHK1 S317 relative to CHK1 and pRPA S33 relative to RPA70 levels ($n = 10$). (**C**) Western blot analysis of HeLa cells or HeLa BAC clones stably expressing EGFP mouse pnuts (mpnuts) transfected with scr or siPNUTS (specifically targets human PNUTS), without IR or at 1 or 6 h after 10 Gy. Lines to the right of the western blot indicate migration of human endogenous PNUTS (lower band) and EGFP mpnuts (upper band). Bottom bar chart shows quantification of pCHK1 S345 relative to CHK1 levels ($n = 3$). (**D**) Western blot analysis of HeLa cells transfected with scr or siPNUTS. At 24 h post transfection, the indicated samples were transfected with wild type EGFP PNUTS or PP1-binding deficient EGFP PNUTS RAXA. Cells were harvested 48 h later without further treatment (–) or 1 h after 10 Gy. Lines to the right of the western blot indicate migration of endogenous PNUTS (lower band) and EGFP PNUTS/EGFP PNUTS RAXA (upper band), asterisk indicates what is likely EGFP PNUTS/EGFP PNUTS RAXA degradation products. Bar chart shows quantification of pCHK1 S317 relative to CHK1 ($n = 3$). Error bars indicate standard error of the mean (SEM) and statistical significance was calculated by the two-tailed Student's two sample t-test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

BD FACSAria Cell Sorter (BD Biosciences) using FlowJo software. Sorted cells were analyzed by western blotting as above. For flow cytometry analysis of RPA loading, we used a similar assay as one previously shown to detect end resection (29). Cells were pre-extracted, fixed and labeled as in (30) using anti-RPA70 antibodies (Cell Signaling). For flow cytometry analysis of γH2AX, samples were fixed and labeled as in (31). For simultaneous monitoring of EGFP-RNaseH1 with γH2AX and DNA, cells were fixed with 1% formalin in PBS for 1hr on ice, washed in PBS and resuspended in 70% ethanol. Samples were labeled with γH2AX antibody as in (30,31), but secondary antibody used was anti-mouse AlexaFluor568 (Thermo Fisher). In experiments in Figures 3E, F and 4C, barcoding of sets of four samples with pacific blue was performed as previously described (30) to eliminate variation in antibody staining between the individual samples. For analysis, a LSRII flow cytometer (BD Biosciences) was used with Diva or FlowJo software.

Immunofluorescence

R-loops were detected as described previously (32). Briefly, U2OS cells were depleted for PNUTS and CDC73 using standard siRNA transfection for 72 h. siRNAs targeting the firefly luciferase were used as controls. After 72 h, cells were fixed and permeablized with 100% ice-cold methanol and acetone for 10 and 1 min on ice, respectively. Incubation with S9.6 antibody (ENH001, Kerafast) was followed by incubation with fluorochrome-conjugated antibodies Dy488 (Bethyl Laboratories). All the washing steps were done with PBS containing 0.05% (vol/vol) Tween 20. The intensity of the nucleoplasmic staining is plotted. At least, 50 cells from three independent experiments were scored.

For detection of RPA chromatin loading by immunofluorescence, HeLa cells were pre-extracted in detergent buffer (20 mM HEPES, pH 7.4; 50 mM NaCl; 1.5 mM $MgCl_2$: 300 mM sucrose; 0.5% Triton X-100) for 5 min on ice prior to fixation with 4% paraformaldehyde. Cells were stained with anti-RPA32 in PBS-AT (PBS with 0.5% Triton X-100 and 1% BSA), followed by anti-mouse Alexa Fluor 568 (Thermo Fisher). All washing steps were done with PBS containing 0.01% (vol/vol) Tween 20. To stain DNA, cells were incubated briefly with Hoechst 33342. Mowiol (4-88, Sigma) was used for mounting. Cells were examined with a Zeiss LSM 710 confocal microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany) equipped with an Ar-Laser Multiline (458/488/514 nm), a DPSS-561 10 (561 nm), a Laser diode 405–30 CW (405 nm), and a HeNe-laser (633 nm). The objective used was a Zeiss plan-Apochromat 63×NA/1.4 oil DICII. Image processing and analysis were performed with basic software ZEN 2011 (Carl Zeiss MicroImaging GmbH, Jena, Germany) and Imaris 7.7.2 (Bitplane AG, Zürich, Switzerland). Average intensity of RPA staining per nuclei (based on Hoechst 33342) was determined. In total, >130 cells for each condition from three independent experiments were analyzed.

Immunoprecipitation experiments

For immunoprecipitations, cells were lyzed in TX-100 buffer (see under western blotting) containing 100 U/ml Benzonase (Sigma-Aldrich). Lysates were precleared and anti-CDC73 (Bethyl) or anti-pATR T1989 (GeneTex) or anti-RNAPII (F-12, Santa Cruz Biotechnologies) or anti-pCHK2 T68 (used as control antibody, from Cell Signaling) were added. Dynabeads (protein G; Life technologies) were used to isolate antibody-bound complexes.

Statistics

All experiments, except when otherwise stated, were performed three times or more. Error bars represent standard error of mean (SEM). *P*-values were calculated with the two-tailed Student's one or two sample *t*-tests or the Mann–Whitney test.

RESULTS

PNUTS inhibits ATR signaling in a PP1-dependent manner

In our previous work (23), we observed increased phosphorylation of CHK1 and RPA32 at late timepoints (2-24 h) after IR in PNUTS depleted HeLa cells. As CHK1 and RPA32 are ATR targets (33,34), we addressed whether ATR signaling was affected specifically. Indeed, depletion of PNUTS with two different siRNA oligonucleotides caused increased IR-induced phosphorylation of the ATR substrates CHK1 S317 and RPA S33, but not of the ATM substrate CHK2 T68 (Figure 1A). Phosphorylation of CHK1 and RPA were increased both at early (5min-1h) and late (6h) timepoints after IR, as well as in the absence of IR (Figure 1A), suggesting a general role for PNUTS in suppressing ATR signaling. In agreement with this notion, pCHK1 S317 and pRPA S33 were higher also during thymidine-induced replication stress in PNUTS-depleted cells (Figure 1B). Similar results were found in U2OS cells (Supplementary Figure S1A), and the effect was clearly ATR-mediated, as the ATR inhibitor VE-821 inhibited the increased CHK1 phosphorylation after IR and thymidine (Supplementary Figure S1B,C). Inhibition of ATR activity was not a general effect after depletion of a PP1 regulatory subunit because knockdown of another abundant nuclear regulatory subunit, NIPP1 (24), did not increase CHK1 S317 or RPA S33 phosphorylation (Supplementary Figure S1D). Furthermore, the increased ATR signaling was not due to off-target effects of the siRNA oligonucleotides, since expression of mouse pnuts-EGFP to near endogenous levels abrogated the increased CHK1 phosphorylation after depletion of human PNUTS, both in the absence and presence of IR (Figure 1C).

To address the importance of PP1 for the inhibitory effects of PNUTS on ATR signaling, siRNA-resistant wild type and PP1-binding deficient PNUTS were overexpressed in cells depleted for endogenous PNUTS. Wild type PNUTS, but not the PNUTS-RAXA mutant deficient for PP1-binding (25), partially abrogated increased CHK1 phosphorylation in the absence of exogenous stress and after IR or thymidine (Figure 1D and Supplementary Figure S2A), showing that PP1-PNUTS binding is important for the negative effect of PNUTS on ATR signaling. Higher expression levels of the PNUTS RAXA mutant did not alter these results (Supplementary Figure S2B).

ATR substrates CHK1 or RPA are not direct targets of PNUTS-PP1

Potentially, PNUTS-PP1 could counteract ATR signaling by generally dephosphorylating ATR substrates, as is the case for *Saccharomyces cerevisae* PP4 and the ATR homologue Mec1 (35). To address this, we added the ATR inhibitor VE-822 after induction of ATR signaling by IR. If PNUTS-PP1 directly dephosphorylates CHK1 and RPA, depletion of PNUTS should cause delayed removal of pCHK1 S317 and pRPA S33 after addition of the ATR inhibitor. However, both pCHK1 S317 and pRPA S33 declined at a similar rate in cells transfected with control siRNA and PNUTS siRNA (Figure 2A), showing that phosphatase activity against these substrates is similar under these conditions. Furthermore, overexpression of PNUTS did not decrease pCHK1 S317 or pRPA S33 relative to control transfected cells (Figure 1D and data not shown). These results strongly suggest PNUTS-PP1 does not directly dephosphorylate these ATR targets. To further verify this finding, we also examined pCHK1 S317/S345 and pRPA S33 after addition of the ATR inhibitor to thymidine-treated cells transfected with control siRNA and PNUTS siRNA (Supplementary Figure S2C). Decline of pCHK1 S317 and pCHK1 S345 occurred similarly also under these conditions, consistent with the notion that CHK1 is not a direct substrate of PNUTS-PP1. On the other hand, pRPA S33 declined less in PNUTS-depleted cells in the presence of thymidine (Supplementary Figure S2C). As pRPA S33 declined similarly in cells transfected with control and PNUTS siRNA after IR (Figure 2A), this most likely implies that another kinase contributes to pRPA S33 in PNUTS-depleted cells after prolonged replication stress (thymidine 16h). ATR-independent phosphorylation of pRPA S33 has e.g. been reported in the presence of hydroxyurea (HU) in combination with ATR inhibitor (36). Altogether, these results suggest that PNUTS-PP1 does not suppress ATR signaling by generally counteracting phosphorylation of its downstream substrates.

Reduced dephosphorylation of RNAPII-CTD is likely promoting the high ATR signaling in cells depleted for PNUTS

As the RNAPII CTD is the only known direct substrate of PNUTS-PP1 (26,27), and RNAPII has a proposed role in ATR activation (6,7), we addressed whether dephosphorylation of RNAPII CTD is involved in the effects of PNUTS depletion on ATR signaling. We first verified that higher levels of pRNAPII S5 could be observed after depletion of PNUTS in HeLa cells (Figure 2B). We next added THZ1, a specific inhibitor of CDK7, the kinase mediating phosphorylation of RNAPII S5 (CTD) (37,38), to cells transfected with control siRNA or PNUTS siRNA during thymidine-induced replication stalling. To allow a robust activation of ATR signaling before inhibition of CDK7, thymidine was added 2 h prior to THZ1. Remarkably, both pRNAPII S5 and pCHK1 S317 were reduced upon addition of THZ1 to cells transfected with control siRNA (Figure 2C, lanes 11–13), and both pRNAPII S5 and pCHK1 S317 remained high in PNUTS-depleted cells (Figure 2C, lanes 14–16), suggesting that pCHK1 S317 depends on RNAPII CTD phosphorylation. Notably, the levels of pRNAPII S5 were reduced also when measured relative to total RNAPII after THZ1 in control siRNA transfected cells (Figure 2D). Also, while the ATR inhibitor VE-822 reduced pCHK1 S317 equally in cells depleted for PNUTS and cells transfected with control siRNA (Supplementary Figure S2C), the CDK7 inhibitor THZ1 only reduced pCHK1 S317 in cells transfected with control siRNA (Figure 2C), thus ruling out the possibility that THZ1 should directly inhibit ATR kinase.

The finding that pRNAPII S5 levels remained high in PNUTS-depleted cells after THZ1 treatment (Figure 2C) is consistent with a major role of PNUTS-PP1 in mediating the dephosphorylation of this residue (Figure 2C, compare lanes 14–16 with lanes 11–13). Moreover, depletion of another pRNAPII S5 phosphatase, SSU72 (39,40), also increased ATR signaling (Supplemental Figure S3A), supporting a role for pRNAPII S5 in ATR signaling. In addition, pRNAPII S2 and S7, two other phosphorylation sites on the RNAPII CTD also correlated with ATR signaling, as they were less reduced in PNUTS siRNA compared to control siRNA transfected cells after THZ1 (Supplemental Figure S3B). pRNAPII S2 and S7 may therefore also depend upon pRNAPII S5, and/or be direct targets of PNUTS-PP1. Interestingly, the effects of PNUTS-PP1 appeared to be most pronounced on pRNAPII S5, as pRNAPII S2 and S7 declined more than pRNAPII S5 after THZ1 in PNUTS siRNA treated cells, with average fold changes of 0.45 and 0.68 respectively, versus 0.97 at 4 h after THZ1 (Figure 2C and supplemental Figure S3B). Also, in contrast to pRNAPII S5 (Figure 2B) neither pRNAPII S2 nor S7 were significantly increased 72 h after PNUTS siRNA compared to control siRNA transfection (results not shown). Nevertheless, we cannot exclude a role for pRNAPII S2 and/or S7 in the high ATR signaling after depletion of PNUTS, and conclude that ATR signaling correlates with RNAPII CTD phosphorylation in general under these conditions.

To confirm the correlation between ATR signaling and RNAPII CTD phosphorylation, we added THZ1 to IR-treated cells. Similarly as observed during replication stress, pRNAPII S5 and pCHK1 S317/S345 were reduced after THZ1 in cells transfected with control siRNA (Supplementary Figure S3C, see charts and compare lanes 3–4 with 9–10). And again, pRNAPII S5 and pCHK1 S317/S345 remained higher in cells depleted for PNUTS (Supplementary Figure S3C, see charts and compare lanes 7–8 with 11–12). An inhibitor of translation, cycloheximide, did not reduce pRNAPII S5 and pCHK1 S317/S345 after IR neither in control nor in PNUTS-depleted cells (Supplementary Figure S3C, compare lanes 3–4 with 13–14 and lanes 7–8 with 15–16), suggesting the effects of THZ1 on ATR signaling are independent of *de novo* protein production (via transcription and translation). To further explore the correlation between RNAPII CTD phosphorylation and ATR signaling, THZ1 was added prior to IR. Consistent with a link between transcription and ATR, pCHK1 S317 was suppressed by THZ1 in HeLa cells (Supplementary Figure S3D). The effects of THZ1 on ATR signaling were likely mediated by RNAPII because similar effects were also obtained with 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) which inhibits transcription elongation via RNAPII (reviewed in (41,42)) and

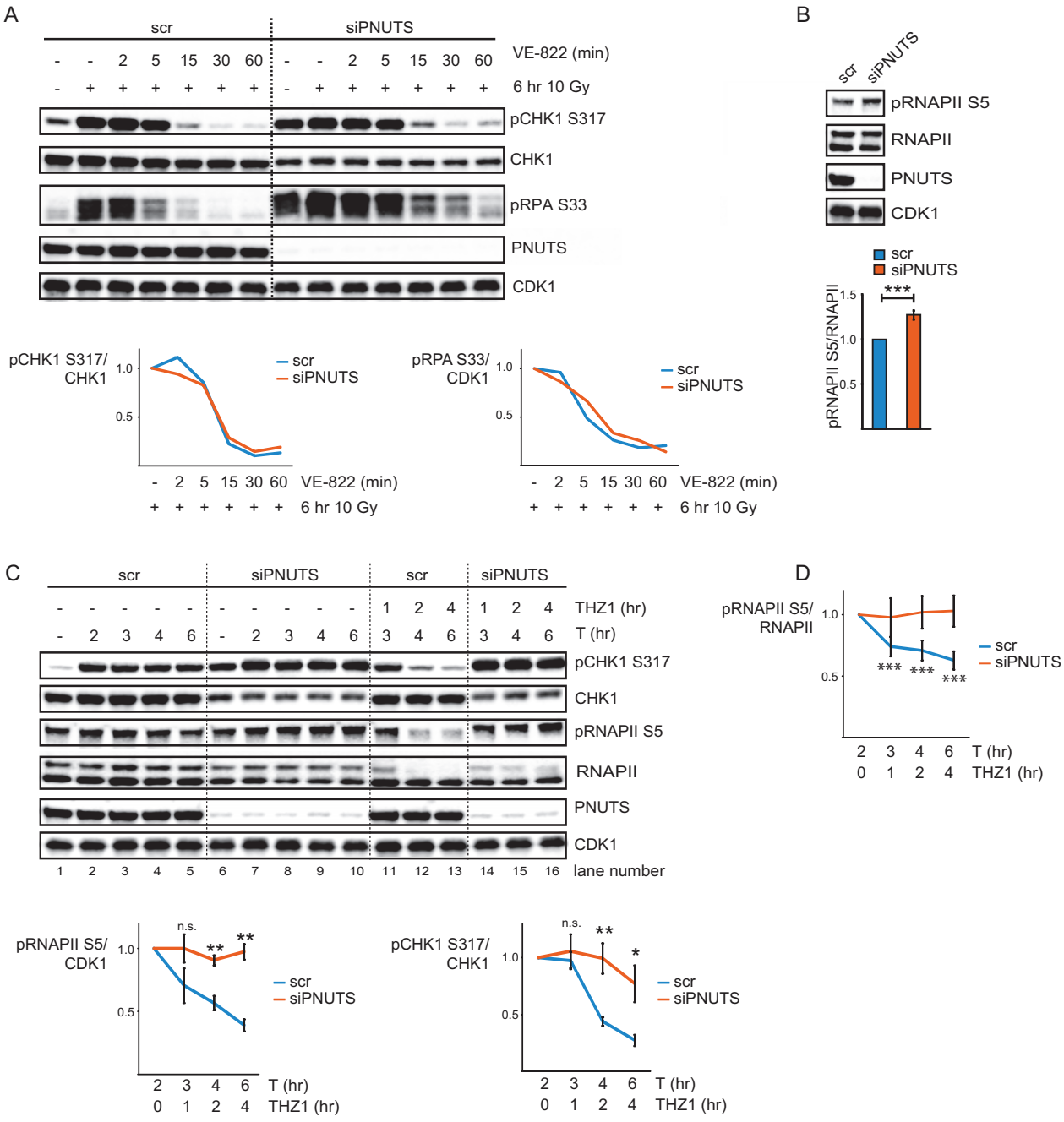

Figure 2. PNUTS-PP1 likely suppresses ATR signaling by dephosphorylating pRNAPII CTD. (**A**) Western blot analysis of scr or siPNUTS transfected cells without IR or 6 h after 10 Gy. VE-822 was added for 2, 5, 15, 30 or 60 min to indicated samples 6 h after 10 Gy. Charts show fold changes for VE-822-treated samples relative to the 10 Gy 6 h sample, for respective siRNA oligos from quantifications of pCHK1 S317 relative to CHK1 and pRPA S33 relative to CDK1. Experiment was performed 2 times with similar results. (**B**) Western blot analysis of scr and siPNUTS cells at 72 h after transfection. Bottom bar chart shows quantification of pRNAPII S5 relative to RNAPII ($n = 14$). ***$P < 0.001$ based on two-tailed Student's two sample t-test. (**C**) Western blot analysis of scr or siPNUTS transfected HeLa cells treated with thymidine for 2, 3, 4 and 6 h. THZ1 was added at 2 h after thymidine to the indicated samples. The bottom charts show fold changes for THZ1 and thymidine samples relative to the 2 h thymidine sample, for the respective siRNA oligonucleotides ($n = 4$) from quantifications of pRNAPII S5 relative to CDK1, and pCHK1 S317 relative to CHK1. Statistical significance was calculated from fold changes in scr versus siPNUTS samples at indicated timepoints by the two-tailed Student's two sample t-test. (**D**) Chart showing fold changes as in (C) from quantifications of pRNAPII S5 relative to RNAPII. Statistical significance was calculated with a two-tailed one sample t-test asking whether fold change after THZ1 was different from 1 (when the initial value prior to addition of THZ1 was set to 1) at the indicated timepoints for the respective siRNA oligonucleotides ($n = 4$). Note that the fold change after THZ1 in the siPNUTS transfected cells was not significantly different from 1 at any of the timepoints tested. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Error bars represent SEM.

triptolide, which leads to the degradation of RNAPII (43). Notably, DRB and triptolide lead to reduced global levels of pRNAPII S5 (Supplementary Figure S3D). Also, translational inhibitor cycloheximide did not reduce pCHK1 S317 when added prior to IR (Supplementary Figure S3D). Collectively these results support a connection between RNAPII-driven transcription, RNAPII CTD phosphorylation and ATR signaling and suggest that PNUTS-PP1 inhibits ATR activity by dephosphorylating pRNAPII CTD.

Enhanced ATR signaling occurs in G1 and in individual S-phase cells after depletion of PNUTS

ATR plays a major role in regulation of DNA replication and is known to be active in S-phase even in the absence of exogenous stress (reviewed in (44)). Potentially, high ATR signaling might therefore simply reflect a larger number of S-phase cells. As γH2AX in S-phase is ATR-dependent (45), we addressed this issue by simultaneously assessing γH2AX levels and cell-cycle position in individual cells after transfection with PNUTS siRNA- or control siRNA. ATR-dependent γH2AX levels in individual S-phase cells were higher after PNUTS depletion (Figure 3A). Therefore, higher ATR signaling following depletion of PNUTS cannot simply be explained by more cells in S-phase.

On the other hand, an accumulation of cells in S-phase could be observed after transfection with PNUTS siRNA (Supplementary Figure S4A), indicating effects on replication. We therefore compared ATR signaling after PNUTS depletion with the ATR signaling resulting from treatment with hydroxyurea (HU), a drug that is thought to activate ATR primarily by causing replication stress. HeLa cells treated with 80 μM HU for 24 h showed similar levels of replication stalling and percentage of cells in S-phase compared to PNUTS-depleted cells 48 h after siRNA transfection, as measured by uptake of the nucleoside analog EdU (Supplementary Figure S4B and S4E). However, pCHK1 S317 and S345 were clearly higher in the PNUTS depleted cells (Supplementary Figure S4C and D), strongly suggesting that the high ATR activity after depletion of PNUTS is not caused by replication stress alone.

Interestingly, previous studies have suggested that blockage of elongating RNAPII is sufficient to induce ATR signaling in human cells (7), and ATR has been shown to be activated in G1-phase (46,47), when replication does not occur. We reasoned that signaling via phosphorylated RNAPII CTD might be a mechanism permitting ATR activation in G1. To address this issue, cells in G1- and S-phases of the cell cycle were sorted based on EdU incorporation and DNA content (Figure 3B). Remarkably, pCHK1 S317 was higher in both G1- and S-phase after depletion of PNUTS, with and without IR (Figure 3C). To validate the purity of the G1-population following sorting, thymidine, which specifically targets S-phase cells, was added for 30 min after EdU labeling (Supplementary Figure S4F). Induction of pCHK1 S317 and presence of CYCLIN A could only be detected in the S-phase population (Supplementary Figure S4F), confirming that the populations were pure. These results suggest increased ATR signaling can also occur in the absence of replication following depletion of PNUTS.

ATR signaling does not correlate with DNA damage or RPA loading after depletion of PNUTS

ATR is also well known to be activated by DNA double strand breaks, such as caused by IR (48). We therefore next compared PNUTS-depleted cells with IR-treated control siRNA transfected cells to address whether the high ATR activity after PNUTS depletion could correlate with DNA-damage. Higher levels of DNA damage markers pATM S1981, pDNAPK S2056, pCHK2 T68 and γH2AX, but lower levels of pCHK1 S317, were observed in IR-treated control cells (1 and 6 h after 10 Gy) compared to PNUTS-depleted cells (Figure 3D,E). Furthermore, the lack of DNA-damage signaling in PNUTS-depleted cells was not caused by a reduced ability to activate ATM or DNAPK, as this occurred normally after IR (Supplementary Figure S4G). The high ATR activity in PNUTS-depleted cells is therefore not likely caused by DNA damage.

RPA-ssDNA is a primary signal for ATR activation (e.g. reviewed in (4)), and can be assessed by measuring the amount of RPA loaded onto chromatin. We therefore compared the levels of RPA loading in non-treated cells tansfected with PNUTS siRNA and IR-treated cells transfected with control siRNA. Although pCHK1 S317 was higher in non-treated PNUTS-depleted cells compared to IR-treated control siRNA transfected cells 6 h after 10 Gy, RPA loading was lower (Figure 3F and G compared to 3D). This suggested a lack of correlation between ATR signaling and RPA loading after depletion of PNUTS. To further explore this, we co-depleted PNUTS and RPA70, an essential component of the RPA complex (reviewed in (49)). Remarkably, in cells co-depleted for PNUTS and RPA70 ATR-dependent pCHK1 S345 was as high as in cells depleted for PNUTS alone (Supplementary Figure S5A,B). High pCHK1 S345 was dependent on depletion of PNUTS, as higher pCHK1 S345 was observed in cells depleted for PNUTS and RPA70 compared to cells depleted for only RPA70 (Supplementary Figure S5B). As expected, co-depletion of RPA70 with PNUTS strongly reduced pRPA S33 (Supplementary Figure S5A,B). The high pCHK1 S345 was not caused by residual chromatin-bound RPA in the RPA70 and PNUTS co-depleted cells, as these cells had reduced RPA chromatin loading, but similar amounts of pCHK1 S345 compared to cells depleted for PNUTS alone 6 h after 10 Gy (Supplementary Figure S5C). Therefore, although our results do not exclude a contribution, they clearly show that the high ATR signaling after depletion of PNUTS is not correlated with enhanced amounts of RPA-ssDNA.

R-loops are formed after depletion of PNUTS but likely play a minor role in the high ATR signaling

As R-loops recently have been proposed to play a role in ATR activation (50), we next addressed whether they might play a role in the increased ATR signaling after depletion of PNUTS. Interestingly, increased amounts of R-loops could be observed in cells transfected with PNUTS siRNA compared to cells transfected with control siRNA both by immunofluorescence and dot blotting using the S9.6 antibody (Figure 4A and Supplementary Figure S5D). Moreover, moderate levels of EGFP-RNaseH1 overexpression caused

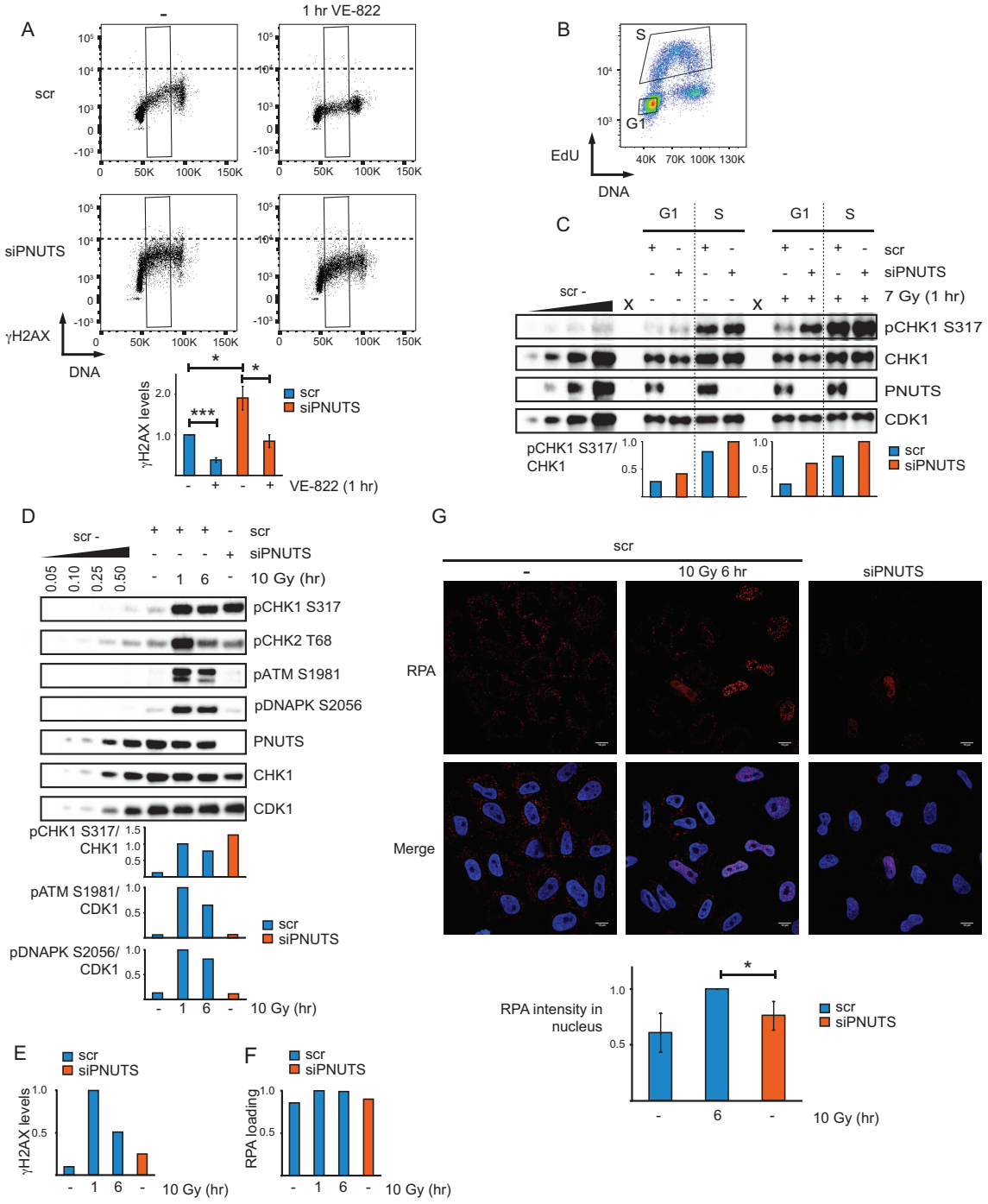

Figure 3. High ATR signaling after PNUTS depletion is present in individual cells, does not correlate with DNA damage markers and can occur in G1-phase. (**A**) Flow cytometry charts showing γH2AX versus DNA staining of individual scr and siPNUTS transfected cells with and without VE-822 for 1 h. S-phase cells were gated based on DNA content as indicated (black boxes). Quantifications show average median γH2AX levels in S-phase ($n = 3$). *$P < 0.05$, ***$P < 0.001$ based on two-tailed two sample Student's t-test. (**B**) Cell sorting was performed by flow cytometry into G1- and S-phases based on EdU incorporation and DNA content as indicated. (**C**) Western blot analysis and quantifications of sorted (as in B) scr and siPNUTS transfected HeLa cells. Cells were harvested at 48 h after siRNA transfection, with and without IR (harvested at 1 h after 7 Gy). Irradiation was performed immediately prior to addition of EdU. One representative image is shown, with X indicating empty lanes. Quantifications were performed on images with different exposure times for the non-irradiated and irradiated samples (due to their different intensities), and normalized to the respective siPNUTS S-phase sample. The experiment was performed three times, two at 72 h and one at 48 h after siRNA transfection with similar results. (**D**) Western blot analysis of DNA damage markers for scr (without IR or 1 and 6 h after 10 Gy) and siPNUTS transfected cells 48 h after siRNA transfection. (**E**) Bar chart showing median levels of γH2AX from flow cytometry analysis from cells harvested in parallel with samples from the same experiment in D. The samples were barcoded with pacific

a partial reduction in ATR-dependent γH2AX in S-phase in cells transfected with PNUTS siRNA, but not in cells transfected with control siRNA (Figure 4B,C). However, in the whole cell population γH2AX levels were similar (Figure 4C), and upon higher levels of EGFP-RNaseH1 overexpression, γH2AX levels increased in all phases both in PNUTS siRNA and control siRNA transfected cells (data not shown). R-loops may therefore contribute to, but are not likely to be the major underlying cause, of the high ATR signaling after depletion of PNUTS.

High ATR signaling does not strictly require common ATR activators after depletion of PNUTS

We further addressed the involvement of other known key upstream ATR activating proteins, namely TOPBP1 and ETAA1. Though pCHK1 S345 was reduced, ATR-dependent pRPA S33 was not reduced in cells co-depleted for TOPBP1 and PNUTS compared to cells depleted for PNUTS alone, in the absence or presence of IR (Figure 5A and B). Thus, in PNUTS depleted cells TOPBP1 is required for the high ATR-mediated phosphorylation of CHK1 S345, but not of RPA S33. Notably, transfection of TOPBP1 siRNA alone did not greatly alter pRPA S33 (Figure 5B and Supplementary Figure S5E), confirming that the enhanced pRPA S33 in cells co-depleted for PNUTS and TOPBP1 was dependent on PNUTS depletion. Conversely, upon co-depletion of PNUTS with ETAA1, pRPA S33 was reduced, but pCHK1 S345/S317 was not greatly altered, compared to cells depleted for PNUTS alone (Figure 5C and D). Again the enhanced pCHK1 S317/S345 was dependent on PNUTS depletion, as pCHKS317/S345 was much lower in cells depleted for ETAA1 alone compared to cells transfected with PNUTS siRNA (Figure 5C and D). Triple depletion of PNUTS, ETAA1 and TOPBP1 suppressed both pCHK1 S317/S345 and pRPA S33 (Figure 5C and D). Together, these results are in agreement with recent findings suggesting that TOPBP1 is required for pCHK1 S317/S345 and ETAA1 for pRPA S33 (45,51). We conclude that neither TOPBP1 nor ETAA1 appear to be required for PNUTS-dependent ATR activity in general, but rather play essential downstream roles in the phosphorylations of specific substrates such as CHK1 and RPA, respectively.

To further characterize known ATR regulators following depletion of PNUTS, we closely compared their levels in cells transfected with PNUTS or control siRNA 24 or 48 h after siRNA transfection. Levels of ATR and ATRIP were not detectably altered (Supplementary Figure S5F). However, we found that ETAA1 was increased in PNUTS-depleted cells compared to cells transfected with control siRNA, particularly at 48 h after siRNA transfection (Supplementary Figure S5F). Upon close examination, CLASPIN and TOPBP1 were also slightly increased at 48 h (Supplementary Figure S5F). The co-depletions of PNUTS with ETAA1 or TOPBP1 nevertheless suggest that the ATR signaling can occur independently of either of these factors, though they are required for downstream phosphorylations (Figure 5A–D). Also, after IR, CLASPIN levels were downregulated, but pCHK1 S317 was higher in PNUTS-depleted cells relative to cells transfected with control siRNA (Supplementary Figure S6A), suggesting CLASPIN is not essential for enhanced ATR signaling upon PNUTS downregulation. The increased levels of ETAA1, CLASPIN and TOPBP1 are thus not likely the cause behind the high ATR signaling after depletion of PNUTS. However, their upregulation may be a consequence as ATR was recently shown to promote the transcription and protein stability of certain factors (52).

pRNAPII-CTD interacting protein CDC73 is required for the high ATR signaling and the G2 checkpoint after depletion of PNUTS

Our results showing a connection between RNAPII CTD phosphorylation and ATR signaling (Figure 2B,C and Supplementary Figure S3) suggest that the CTD may be acting as a signaling platform for ATR activity. We therefore searched for factors that might participate in signaling from phosphorylated RNAPII CTD towards ATR. In the literature, we identified three proteins, BRCA1, PRP19 and CDC73, that associate with hyperphosphorylated RNAPII and have been linked to ATR (53–58). We found that co-depletion of BRCA1 or PRP19 with PNUTS did not reduce the high ATR signaling (data not shown). However, co-depletion of CDC73 with PNUTS reduced both pCHK1 S317/S345 and pRPA S33, but not pRNAPII S5, in the presence or absence of IR (Figure 5E). The reduction in pCHK1 S345 phosphorylation after co-depletion was observed with several siRNA oligonucleotides against CDC73 (four out of five) (Supplementary Figure S6B). Furthermore, expression of siRNA resistant Flag-CDC73 partially rescued the effects on pCHK1 S317/S345 and pRPAS33 downregulation after co-depletion of CDC73 with PNUTS (Figure 5E), excluding siRNA off-target effects. The reduction in ATR signaling after co-depletion of CDC73 with PNUTS was not due to indirect cell cycle effects, because γH2AX in individual S-phase cells was significantly reduced under these conditions compared to cells depleted for PNUTS alone (Figure 6A and Supplementary Figure S6E).

We previously found that depletion of PNUTS activates an endogenous G2 checkpoint in unperturbed cells (23). As the G2 checkpoint depends upon ATR and its downstream target CHK1 (59), and co-depletion of CDC73 suppressed ATR signaling after depletion of PNUTS (Figures 5E and 6A), we addressed whether co-depletion of CDC73 might also suppress activation of the endogenous G2 checkpoint. For this purpose, we measured entry into mitosis after addition of VE-822 to siRNA-transfected cells. In agreement

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blue and mixed prior to staining to minimise sample to sample variation. The experiment in (E) compared to (D) was performed two times with similar conditions and results. (**F**) Bar chart showing median levels of RPA loading from flow cytometry analysis of pre-extracted cells from the same experiment as in (D). Samples were barcoded as in (E). The experiment in (F) compared to (D) was performed three times with similar conditions and results. (**G**) Immunofluorescence analysis of pre-extracted cells treated as in (D), but harvested at 72 h after siRNA transfection. Bottom bar chart shows average intensity of nuclear RPA staining from three independent experiments. *$P < 0.05$ for using two-tailed one sample Student's *t*-test (to test if RPA values in siPNUTS sample was different than 1, which we had set scr 10 Gy 6 h sample to). >130 cells were scored per condition in total. Error bars represent SEM.

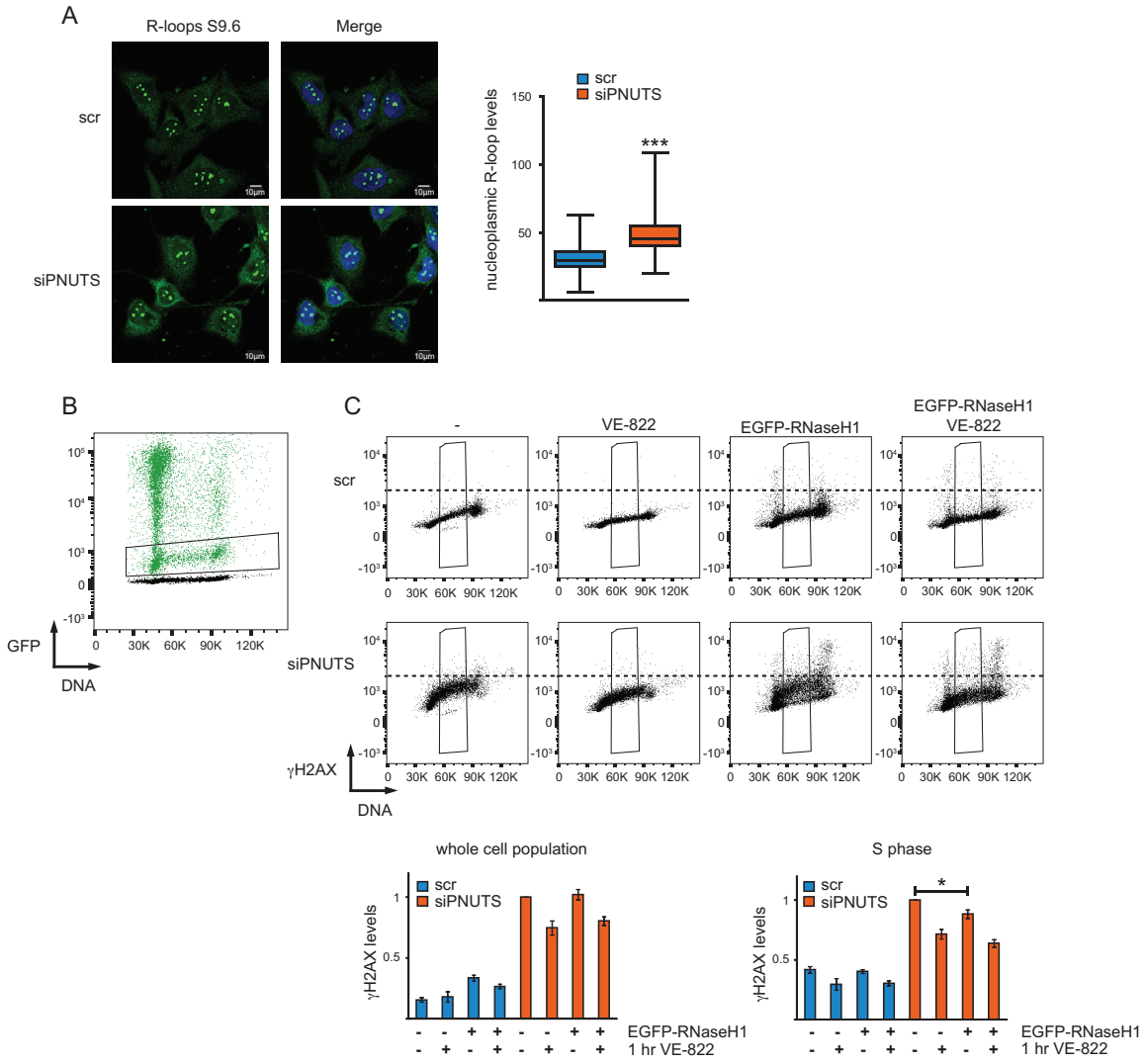

Figure 4. Depletion of PNUTS promotes R-loops, but overexpression of EGFP-RNaseH1 has only minor effects on ATR signaling. (**A**) Immunofluorescence analysis of R-loops in PNUTS depleted and control siRNA transfected cells at 72 h after siRNA transfection. The intensity of the nucleoplasmic staining is plotted. At least 50 cells from three independent experiments were scored. ***$P < 0.001$, by the Mann–Whitney test. (**B**) Representative flow cytometry chart showing GFP intensity versus DNA content. PNUTS depleted and control siRNA transfected cells were transiently transfected with EGFP-RNaseH1 at 24 h after siRNA transfection, and harvested at 72 h after siRNA transfection. Chart shows overlay of EGFP-RNaseH1 transfected (green) and non-EGFP-RNaseH1 transfected cells (black). Cells with moderate levels of EGFP-RNaseH1 expression were selected as indicated (black box). (**C**) Flow cytometry chart showing γH2AX staining versus DNA content in PNUTS depleted or control siRNA transfected cells with and without VE-822 for 1 h and with and without transient EGFP-RNaseH1 overexpression (selected for moderate levels of GFP expression as shown in B). Samples treated with the same siRNA oligonucleotides, were barcoded with pacific blue and mixed prior to staining as in 3E). Quantifications show relative, median γH2AX levels in the whole cell population or in the selected S-phase cells ($n = 3$). Error bars represent SEM. *$P < 0.05$ using two-tailed Student's *t*-test. Note that VE-822 reduces γH2AX in S-phase less than in Figure 3A, this is likely due to differences in the fixation protocol (required to preserve GFP intensity), which prolonged incubation time after wash-out of VE-822.

with our previous results using caffeine and a CHK1 inhibitor (23), after addition of VE-822, more cells transfected with PNUTS siRNA entered into mitosis compared to cells transfected with control siRNA (Figure 6B). Remarkably, co-transfection of CDC73 siRNA suppressed this effect (Figure 6B). Notably, to ensure that only entry into mitosis from cells arrested in G2 phase was being assessed, we added VE822 for only 1 h, a time point well below the average duration of G2, which is ∼3 h in HeLa cells (60), and we also only counted cells with a *4C* DNA content (Figure 6B).

We next addressed whether co-depletion of CDC73 might also influence R-loops, which we found to be increased after depletion of PNUTS (Figure 4A and Supplementary Figure S5D). Interestingly, we found that the levels of R-loops were reduced after co-depletion with CDC73 compared to cells treated with PNUTS siRNA alone (Supplementary Figure S6C). As CDC73 plays a role in transcription, this supports our hypothesis that the enhanced levels of R-loops after de-

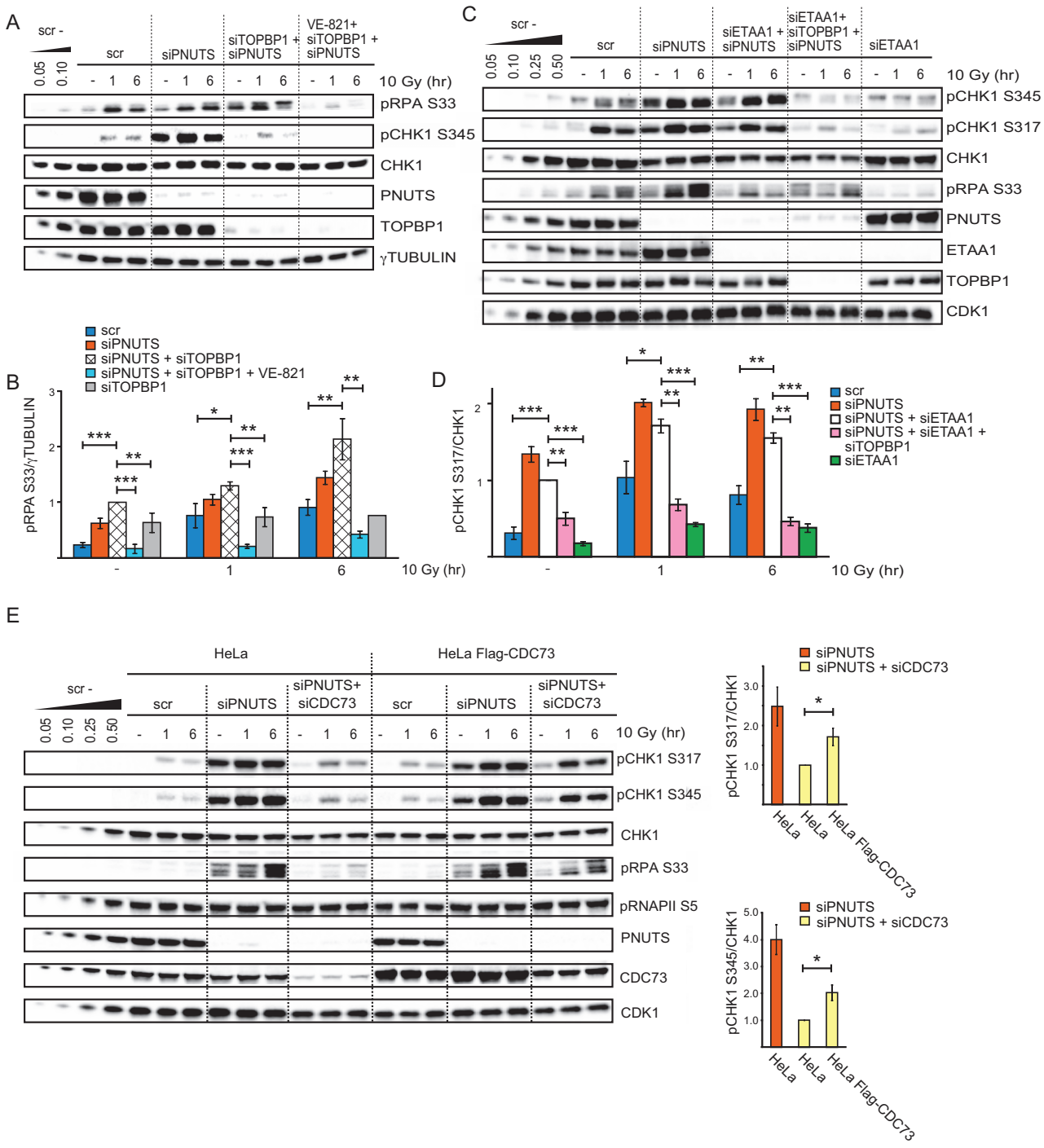

Figure 5. CDC73, but not TOPBP1 nor ETAA1, is required for high ATR-dependent phosphorylation of both CHK1 and RPA after PNUTS depletion. (**A** and **B**) Western blot and quantifications ($n = 3$) from cells transfected with scr, siPNUTS, and siRNA against TOPBP1 (siTOPBP1) harvested at 72 h after siRNA transfection and 1 and 6 h after 10 Gy. VE-821 was added 30 min prior to 10 Gy. For the siTOPBP1 10 Gy 6 h sample error bar was emitted in the quantifications as experiment was performed two times. Western blot for siTOPBP1 alone is shown in Supplementary Figure S5E. (**C** and **D**) Western blot and quantifications ($n = 3$) from cells transfected with scr, siPNUTS, siTOPBP1 and siRNA against ETAA1 (siETAA1) harvested at 48 h after siRNA transfection and 1 and 6 h after 10 Gy. (**E**) Western blot analysis and quantifications of scr, siPNUTS or CDC73 siRNA (siCDC73) transfected HeLa cells or HeLa cells stably expressing siRNA-resistant Flag-CDC73 treated with IR (10Gy) as indicated. Bar charts show quantification of pCHK1 S345 and pCHK1 S317 versus CHK1 levels at 6 h after 10 Gy ($n = 3$). Error bars indicate SEM and statistical significance was calculated by the two-tailed Student's two sample *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

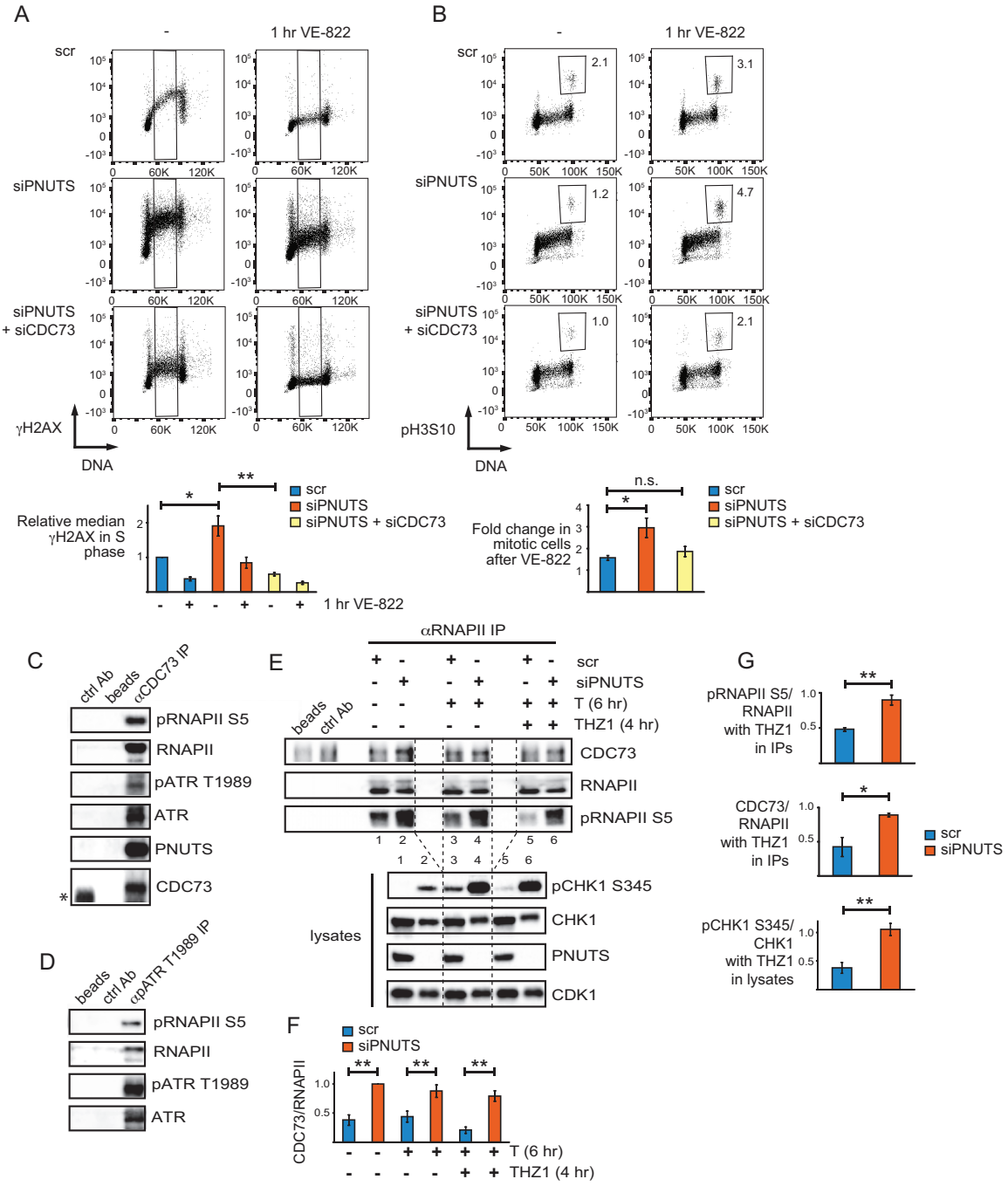

Figure 6. CDC73 is required for high ATR signaling in S-phase and activation of the endogenous G2 checkpoint after PNUTS depletion, and interacts with ATR and RNAPII. (**A**) Flow cytometry charts showing γH2AX staining versus DNA content as in 3A) of scr, siPNUTS or siPNUTS and siCDC73 transfected cells harvested at 72 h after siRNA transfection with and without 1 h treatment with VE-822. Quantifications show relative median γH2AX levels in indicated S-phase cells (black box). ($n = 3$) $^*P < 0.05$, $^{**}P < 0.001$ based on two-tailed two sample Student's *t*-test. (**B**) Flow cytometry charts showing phosphohistone H3 Ser 10 (pH3S10) staining versus DNA content of cells treated as in 6A). Mitotic cells were selected based on DNA content and high pH3S10 staining as indicated. Numbers indicate percentages of mitotic cells. Quantifications show fold increase in mitotic cells after 1h VE-822 for each siRNA condition. $^*P < 0.05$, based on two-tailed two sample Student's *t*-test. (**C**) Western blot analysis of immunoprecipitations from HeLa cell lysates, using a control antibody (ctrl Ab), no antibody (beads) or anti-CDC73 antibodies (αCDC73 IP). *Indicates IgG band from the control antibody, which migrated slightly faster than CDC73 in the western blot. (**D**) Western blot analysis of immunoprecipitations as in C), but using anti-pATR T1989 antibodies (αpATR T1989 IP). (**E**) Western blot analysis of immunoprecipitations as in (C), but using RNAPII antibodies recognizing both the phosphorylated and the non-phosphorylated RNAPII (αRNAPII IP). Immunoprecipitations were performed on lysates from scr and siPNUTS transfected cells harvested at 72 h after siRNA transfection, with and without thymidine for 6 h and THZ1 for 4 h. Upper western blot shows immunoprecipitations, and lower blot shows corresponding lysates. (**F**) Bar chart showing quantifications from three independent experiments performed such as E, of CDC73 relative to RNAPII in

pletion of PNUTS are also caused by effects on transcription. Altogether, these results suggest that CDC73 plays an important role in ATR activation that is counteracted by PNUTS, and are consistent with a role for CDC73 in signaling from phosphorylated RNAPII CTD to ATR.

CDC73 interacts genetically with the ATR homologue Mec1 in *Saccharomyces cerevisiae*, and a physical interaction has been proposed but not previously shown (57). Furthermore, RNAPII is a known interacting partner of CDC73 (61,62), and in *S. cerevisae* it was shown that CDC73 binds the RNAPII CTD in a phosphorylation-dependent manner (62). To examine CDC73, ATR and RNAPII interactions in HeLa cells, we performed co-immunoprecipitation (co-IP) experiments of endogenous proteins. Indeed, co-IPs using a CDC73 antibody pulled down RNAPII, pRNAPII S5, ATR and pATR T1989 (Figure 6C). As pATR T1989 is thought to be an autophosphorylation site (63), this indicates that catalytically active ATR associates with CDC73. Interestingly, PNUTS and PP1 were also detected in the CDC73 co-IPs (Figure 6C and Supplementary Figure S6D). We verified that the immunoprecipitations were specific by using lysates from cells depleted of CDC73, which pulled down less ATR and RNAPII (Supplementary Figure S6D). Furthermore, the depletion of CDC73 was only partial and significant amounts of CDC73 were present in the co-IPs from cells transfected with CDC73 siRNA (Supplementary Figure S6D, CDC73-high exposure), which may explain the residual ATR and RNAPII pulled down under these conditions. Next, we performed ATR co-IPs to address whether ATR and pRNAPII S5 could physically associate. To enrich for active ATR in these experiments, we used pATR T1989 antibodies. This efficiently pulled down ATR and faint bands corresponding to pRNAPII S5 and RNAPII could also be detected, suggesting an interaction in live cells (Figure 6D). Moreover, to address whether hyperphosphorylation of the RNAPII CTD after depletion of PNUTS might promote binding to CDC73, we performed RNAPII co-IPs using an antibody that recognizes both the phosphorylated and non-phosphorylated forms of RNAPII. Indeed, more CDC73 was pulled down in RNAPII immunoprecipitates after depletion of PNUTS compared to control siRNA transfected cells (Figure 6E). In these experiments we also induced replication stress with thymidine and added THZ1. In line with our results showing that THZ1 reduced RNAPII CTD phosphorylation and ATR signaling in control-, but not in PNUTS-depleted cells (Figure 2C), immunoprecipitated RNAPII was less phosphorylated and less CDC73 was pulled down in the control-, but not in the PNUTS-depleted cells after THZ1 treatment (Figure 6E, F lanes 3 versus 5 and 4 versus 6 and Figure 6G). Of note, in these experiments we measured pRNAPII S5, but other CTD-phosphorylation sites, such as S2 or S7 may play a role but are not shown here. Also, all the co-IPs were performed after treatment with the endonuclease benzonase, strongly suggesting that the interactions were not mediated by DNA. Altogether these results suggest that CDC73, ATR and RNAPII may interact in live cells, and that CDC73 interacts with the RNAPII CTD in a phosphorylation-dependent manner also in humans. These results thus strongly support a role for phosphorylated RNAPII and CDC73 in the high ATR activity after PNUTS depletion.

DISCUSSION

ATR kinase plays a central role in signaling after DNA damage and replication stress. Here, we show for the first time that the RNAPII phosphatase PNUTS-PP1 suppresses ATR signaling. Furthermore, we have identified a well-known RNAPII binding protein, CDC73, as a novel factor mediating ATR activation via the RNAPII CTD and being required for the high ATR signaling in PNUTS-depleted cells. Our results suggest that ATR signaling is restrained by PNUTS-PP1 mediated dephosphorylation of RNAPII CTD, and thus support a role for RNAPII in ATR signaling. Moreover, our results support recent findings that TOPBP1 and ETAA1 may direct ATR activity towards different substrates. Altogether, based on these results we propose a new model for ATR activation via CDC73, RNAPII and PNUTS-PP1 (Figure 7).

Interestingly, this model is in line with previous reports showing that perturbation of transcription can induce ATR activation in the absence of DNA damage and prior to detection of replication-stress (7,64). We envision that signaling to ATR by pRNAPII CTD via CDC73 may be a general event that occurs upon RNAPII stalling, regardless of context. As Mec1 was shown to promote removal of RNAPII at sites of transcription-replication conflict (57), viewed in light of our results, ATR activity might thus promote removal of stalled RNAPII also outside of S-phase. This is likely important, because stalled RNAPII could create an obstacle for further transcription in a region which might e.g. contain an essential- or tumor suppressor gene. In agreement with prolonged RNAPII stalling being detrimental to the cell, it has been shown to be a strong signal for apoptosis (65).

In addition to the high ATR signaling, depletion of PNUTS also caused an accumulation of cells in S-phase and decreased EdU uptake (Supplementary Figure S4A,B,E), indicating increased replication stalling. These effects might be expected as stalled RNAPII and R-loops after PNUTS depletion may create obstacles for the replication fork (reviewed in (66)), and the high ATR activity likely also contributes to slowing down replication (reviewed in (44)). Nevertheless, our results strongly suggest that the high ATR activity after depletion of PNUTS cannot simply be caused by canonical signaling via enhanced replication stress. First

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western blots from RNAPII immunoprecipitations. (**G**) Bar charts showing fold changes of THZ1 and thymidine treated samples relative to samples treated with thymidine alone for respective siRNA oligonucleotides from quantifications of western blots from three independent experiments performed such as (E). pRNAPII S5 relative to RNAPII and CDC73 relative to RNAPII values were from the immunoprecipitations, and pCHK1 S345/CHK1 values were from the corresponding lysates. For quantifications of CDC73 from immunoprecipitations, background (value of band in beads alone), was substracted during the quantifications. $*P < 0.05$, $**P < 0.01$ based on the two-tailed Student's two sample *t*-test. Error bars represent SEM.

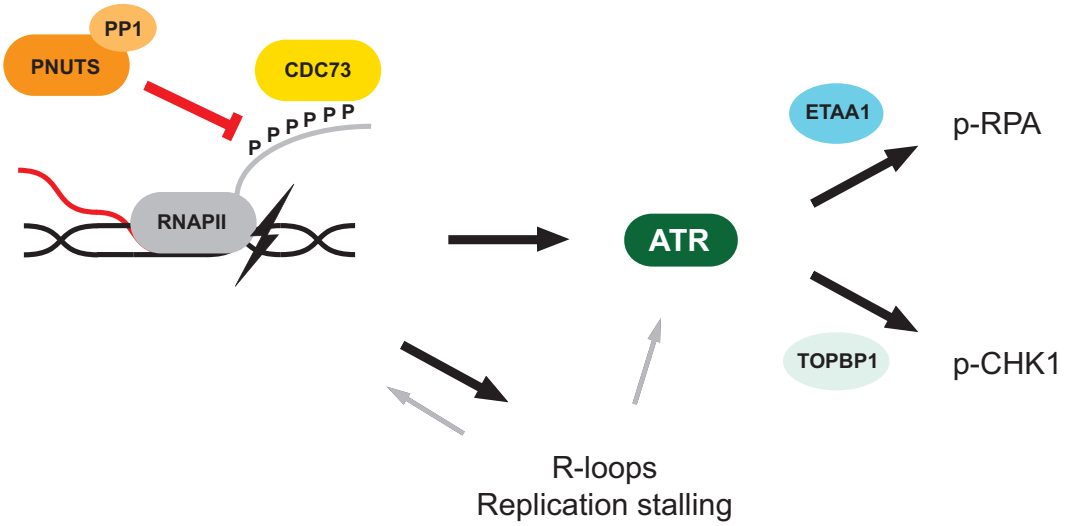

Figure 7. Model for regulation of ATR signaling via PNUTS-PP1, the phosphorylated CTD of RNAPII and CDC73. We envision that stalling of elongating RNAPII, caused by DNA damage or other obstacles (e.g. reviewed in (8)), causes hyperphosphorylation of the RNAPII CTD (see main text for details) which increases its binding to CDC73. Once bound to the RNAPII CTD, CDC73 either directly or indirectly activates ATR. PNUTS-PP1 suppresses ATR activity by dephosphorylating the RNAPII CTD, thus reducing the binding of CDC73 to RNAPII CTD and activation of ATR. R-loops formed under these conditions may also contribute to ATR signaling, but are likely to play a minor role. Furthermore, during S-phase the stalled RNAPII, R loops and ATR activity likely also cause replication stalling, which may further contribute to induce ATR signaling through canonical activation or potentially via further increasing RNAPII stalling in a positive feedback loop. Our results also indicate that TOPBP1 and ETAA1 can direct the ATR activity towards pCHK1 S317/345 and pRPA S33, respectively. Altogether our model is consistent with the model originally proposed by the groups of Sancar and Ljungman, where RNAPII signals the presence of DNA damage by stalling as it encounters an obstacle during transcription elongation (6,7).

of all, it was also observed in the absence of replication in G1-phase after PNUTS depletion (Figure 3B, C) and was higher than expected compared to ATR signaling induced by HU-generated replication stress (Supplementary Figure S4B–E). In addition, the high ATR activity did not correlate with RPA-ssDNA (Figure 3D, F, G and Supplementary Figure S5A–C), which is considered to be the main signal for replication stress-induced ATR activity (44). Furthermore, suggesting that it is rather RNAPII phosphorylation which is important for the high ATR signaling after depletion of PNUTS, short-term incubation with the CDK7-inhibitor THZ1 reduced both RNAPII phosphorylation and ATR signaling in control siRNA but not in PNUTS siRNA transfected cells (Figure 2C and Supplementary Figure S3B,C). Moreover, RNAPII and CDC73 may be directly involved in ATR signaling as they were found to interact with ATR (Figure 6C, D). Phosphorylation of the RNAPII CTD was also important for the interaction between CDC73 and RNAPII (Figure 6E–G), and co-depletion of CDC73 with PNUTS strongly reduced ATR signaling (Figures 5E and 6A). Altogether, our results thus point to a signaling pathway involving ATR, RNAPII and CDC73 which is continuously counteracted by PNUTS-PP1. On the other hand, in S-phase, canonical signaling from stalled replication forks may also contribute to promoting ATR activation after depletion of PNUTS (see model in Figure 7). Still, it is tempting to speculate that replication stalling after depletion of PNUTS may further enhance RNAPII stalling and thus create a positive feedback loop by increasing RNAPII/CDC73-mediated ATR activity (see model in Figure 7).

Interestingly, R-loops were enhanced after depletion of PNUTS and suppressed by co-depletion of CDC73 (Figure 4A and Supplementary Figure S5D). However, EGFP-RNaseH1 only partially suppressed ATR-dependent γH2AX in S-phase cells transfected with PNUTS siRNA (Figure 4C), suggesting that R-loops may contribute to the high ATR activity but likely play a minor role. As R-loops were recently shown to cause ATR activation at centromeres in mitosis by a mechanism proposed to involve RPA-ssDNA (50), one speculation could be that depletion of PNUTS causes small amounts of ssDNA-RPA associated with R-loops, and that the resulting structure may confer some specificity which enhances ATR signaling. On the other hand, there is an intimate connection between stalled RNAPII and R-loops (67). It was recently shown that overexpression of RNaseH1 can cause release of stalled RNAPII, suggesting that R-loops can promote RNAPII stalling (32). Therefore, another possibility might be that R-loops might contribute to ATR signaling by leading to stalling of RNAPII and subsequent RNAPII CTD phosphorylation.

We found that RNAPII CTD phosphorylation was required for, but did not strictly correlate with, ATR signaling (e.g. Supplementary Figure S3D—compare lanes 1 and 2, pCHK1 S317 versus pRNAPII S5). However, RNAPII CTD phosphorylation is a frequent event during the normal transcription cycle. The most studied phosphorylation sites are S5 and S2, and in brief, studies have shown that phosphorylation on S5 is high at the start of the gene and thereafter gradually decreases, while inversely, phosphorylation on S2 increases throughout the gene (Reviewed in (37,68)). The widespread presence of S2 and S5 RNAPII CTD phos-

phorylation implies that a strict linear correlation with ATR activation is unlikely, as it would suggest that ATR becomes activated merely as a consequence of normal transcription. Thus, it is likely that only a subpopulation of pRNAPII CTD is responsible for signaling to ATR. Supporting this, only stalling of the elongating form of RNAPII caused increased ATR signaling (7). As elongation is associated with phosphorylation on S2 and phosphorylation on S5 is enhanced upon RNAPII stalling, e.g. at sites of UV damage or at splice sites located at gene-internal regions (14,69), one conceivable mechanism is that dual S2 and S5 phosphorylation might be required for signaling to ATR. Supporting this, CDC73 bound more tightly to dually- than to singly-phosphorylated pRNAPII CTD in vitro (62). Nevertheless, the situation is likely to be more complex, as the human CTD contains 52 heptapeptide repeats and different modifications, and combinations of these, exist (68).

Of note, in the alternative splicing response to UV, pRNAPII CTD was proposed to occur downstream of ATR activation, and ATR activation to occur independently of transcription in HaCaT cells (70). These results may appear to be contradictory to ours. However, we did not detect any reduction in pRNAPII S5 after ATR inhibition during replication stress in HeLa cells (Supplementary Figure S2C) suggesting ATR is not always upstream of RNAPII CTD phosphorylation. Furthermore, the differing results may be explained by the existence of several pathways for ATR activation acting in parallel, e.g. via RNAPII, via ssDNA-RPA, and via unknown pathways. The contribution from each pathway is likely to vary between cell types and with different stresses.

Our results point to a new role for CDC73 in ATR activation. CDC73 is a component of the PAF1 complex, including PAF1, CTR9, LEO1, RTF1 and WDR61, involved in all stages in RNAPII transcription (61). However, CDC73 does not appear to be essential for transcription as its depletion in HeLa cells was found to both up and down-regulate mRNA expression (71). In *S. cerevisae* CDC73 was found to act downstream of Mec1 in collisions of transcription and replication (57). Our results suggest CDC73 in association with RNAPII can also act upstream of ATR activation. Interestingly, CDC73 is also a well known tumor suppressor gene. It is currently not clear how CDC73 acts as a tumor suppressor, though roles in Wnt signaling, regulation of P53 and CYCLIN D levels and homologous recombination repair have been suggested (72–75). ATR activity protects genome integrity by stabilizing stalled forks during replication stress and promoting DNA repair and checkpoint activation (76). In addition, ATR activity can promote apoptosis in non-cycling cells, which implies the majority of cells in humans (77). Therefore, CDC73 could potentially protect against cancer by promoting RNAPII-mediated ATR activity, leading to cell death in non-cycling cells with DNA damage. Consistent with this interpretation, PNUTS, which counteracts CDC73 in ATR activation, is a putative proto-oncogene (78).

In conclusion, this work sheds light upon a previously proposed pathway for ATR activation via the RNAPII machinery. We have identified novel factors involved, including CDC73, the phosphorylated CTD of RNAPII and PNUTS-PP1. Future studies are likely to uncover more details into this understudied and highly relevant pathway for ATR activation.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Author contributions: H.B.L. performed most of the experiments. H.B.L. and R.G.S. conceived the project and analyzed the results. L.E.S. and G.E.R. performed experiments and contributed to the analysis. B.G. constructed HeLa cells expressing siRNA resistant CDC73. L.T.M. contributed conceptually with regards to PP1 and to the development of cells expressing GFP-PNUTS. S.C.S., J.C.S. and S.F.A. performed experiments related to R-loops and contributed conceptually regarding R-loops. H.B.L. and R.G.S. wrote the paper. All the authors contributed to revision of the paper.

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SUPPLEMENTARY DATA:

Supplementary figure legends:

Figure S1. A) Western blot analysis and quantifications of scr and siPNUTS transfected U2OS cells at 2 or 6 hr after addition of thymidine. Bar chart under the western blot shows results from the same experiment. Bar charts to the right show quantification from 5 independent experiments of pCHK1 S317 relative to CHK1 or pRPA S33 relative to γTUBULIN or CDK1. **B)** Western blot analysis and quantifications (n=3) of scr and siPNUTS transfected HeLa cells at 1 or 6 hr after 10 Gy. VE-821 was added 30 min prior to IR. **C)** Western blot analysis and quantifications (n=3) of siPNUTS HeLa cells at 2 or 6 hr after thymidine. VE-821 was added 30 min prior to thymidine. For A-C) Error bars indicate SEM and statistical significance was calculated by the two-tailed students two sample t-test. *p<0.05, **p<0.01, ***p<0.001. **D)** scr, siPNUTS cells or cells transfected with siRNA against NIPP1 (siNIPP1) at 1 or 6 hr after 10 Gy. Bar chart under the western blot shows results from the same experiment. Experiment was performed two times with similar results.

Figure S2. A) Western blot analysis and quantifications from experiment as in 1D, 6 hr after thymidine. Bar chart shows quantification of pCHK1 S317 relative to CHK1 (n=3). Error bars indicate standard error of the mean (SEM) and statistical significance was calculated by the two-tailed students two sample t-test. ***p<0.001 **B)** Western blot analysis and quantifications from experiment performed as in 1D). Bar chart below shows quantification from the same experiment. **C)** Western blot analysis of scr or siPNUTS transfected cells without or with thymidine (16hr T). VE-822 was added for 2, 5, 15, 30, or 60 min to indicated samples 16hr after addition of T. Charts show fold changes after VE-822 treatement at various timepoints vs T 16 hr alone, for respective

siRNA oligos from quantifications from western blot of pCHK1 S317/CHK1, pCHK1 S345/CHK1 and pRPA S33/CDK1. Experiment was performed two times with similar results.

Figure S3. A) Western blot analysis and quantifications (n=3) of HeLa cells 72 hr after siRNA transfection with scr or siRNA against SSU72 (siSSU72) with or without 2 or 6 hr thymidine. **B)** Western blot analysis and quantifications (n=3) from experiments performed as in 2C). The charts to the right show fold changes for THZ1 and thymidine samples 4 hr after THZ1, relative to the 2 hr thymidine sample for the respective siRNA oligos for pRNAPII S2 or pRNAPII S7 relative to CDK1. **C)** Western blot analysis of scr or siPNUTS transfected cells with or without IR (10 Gy, 1 or 2 hr). THZ1 or cycloheximide was added 10 min after IR to the indicated samples. Samples with or without THZ1/CHX were collected together to allow direct comparison. The right charts show fold changes of THZ1 + 10 Gy relative to 10 Gy alone at the same timepoints after IR for the respective siRNA oligonucleotides for pRNAPII S5 relative to CDK1 and pCHK1 S317/S345 relative to CHK1 levels (n=3). For pCHK1 S345 and pCHK1 S317, statistical significance was calculated using the two-tailed one sample students t-test, testing whether the fold change after THZ1 for the respective siRNA oligonucleotides was different from one. Notably, using this test, no statistical significance could be detected after THZ1 treatment for neither pCHK1 S317 nor S345 for the siPNUTS samples. For pRNAPII S5, statistical significance was calculated using the two-tailed two sample students t-test, comparing fold change after THZ1 between siPNUTS and scr transfected samples. *$p<0.05$, **$p<0.01$. **D)** Representative western blot and resulting quantification from HeLa cells with or without transcriptional inhibitors THZ1, DRB, triptolide or translational inhibitor cycloheximide. Inhibitors were added to cells 60 min prior to 10 Gy and samples were harvested at 15, 30 and 60

min. CDC25A levels verify effects of cycloheximide on a short-lived protein. Quantifications beneath western blot shows results from the same experiment. The experiment was performed three times under resembling conditions with similar results.

Figure S4. A) Histograms from the experiment in 3A, showing cell cycle profiles based on DNA content. **B)** Flow cytometry charts showing EdU incorporation versus DNA content of HeLa cells with or without 80 μM HU for 24 hr and siPNUTS transfected cells 48 hr after siRNA transfection. **C)** Western blot from same experiment as in B). **D)** Bar charts show quantifications from western blots from three independent experiments such as B). Statistical significance was tested using the two-tailed two sample t-test, ***$p<0.001$, *$p<0.05$. **E)** Bar charts show quantifications from flow cytometry analysis from three independent experiments such as B). Statistical significance was tested using the two-tailed two sample t-test, but siPNUTS transfected cells were not found to be significantly different from 80 μM HU samples when EdU incorporation or percentage cells in S phase was compared. **F)** Western blot and quantifications from experiment as in 3B. Cells were labeled with EdU for 1 hr, followed by 30 min incubation with thymidine, harvested and sorted. **G)** Western blot analysis and quantifications of scr or siPNUTS transfected cells without or with IR (10 Gy, 1 or 6 hr)). (n=7 for pATM S1981, and n=5 for pDNAPK S2056).

Figure S5 A) and B) Representative western blots of cells transfected with scr, siPNUTS, and siRNA against RPA70 (siRPA70) harvested at 72 hr after siRNA transfection and 1 and 6 hr after 10 Gy. VE-821 was added 30 min prior to 10 Gy. (n=3). **C)** Immunofluorescence analysis and quantifications (n=3) of pre-extracted cells treated as in A) and B). Quantifications of pCHK1 S345/CHK1 are from western blots of samples treated in parallel with the immunoflourescence samples. Statistical significance was tested using the two-tailed two sample t-test**,** **$p<0.01$ **D)** Dot blots

from scr or siPNUTS transfected cells harvested at 72 hr after siRNA transfection showing R-loops (n=5). **E)** Western blots of cells transfected with scr, siPNUTS, and siTOPBP1 harvested at 72 hr after siRNA transfection and 1 and 6 hr after 10 Gy. **F)** Western blot analysis of scr or siPNUTS transfected cells at 24 and 48 hr after siRNA transfection.

Figure S6 A) Western blot analysis of scr or siPNUTS transfected cells at 72 hr after siRNA transfection, without and with IR (10 Gy, 6h). **B)** Western blot and quantifications from representative experiment of cells transfected with scr, siPNUTS, and five different oligonucleotides against CDC73, siCDC73 #1, #2, #3, #4 and #5 harvested 48 hr after siRNA transfection. (n=3). siCDC73 #2 is also called siCDC73. **C)** Immunofluorescence analysis of R-loops in cells transfected with scr, siPNUTS and scr or siPNUTS and siCDC73 at 72 hr after siRNA transfection. The intensity of the nucleoplasmic staining is plotted. At least 50 cells from three independent experiments were scored. Statistical significance was determined using the Mann-Whitney test. **D)** Western blot from immunoprecipitation experiment performed as in 6C on lysates from cells transfected with scr or siCDC73 #1, at 72 hr after siRNA transfection. **E)** Histograms from the experiment in 6A) showing cell cycle profiles based on DNA content.

Supplementary Materials and Methods:

Dot Blot

U2-OS cells were transfected with control, PNUTS, harvested after 72 hours and lysed in lysis buffer (100 mM NaCl, 10 mM Tris pH 8.0, 25 mM EDTA pH 8.0, 0.5% SDS) with 140 µg/ml Proteinase K, at 37ºC overnight. Nucleic acids were extracted using standard phenol-chloroform extraction and re-suspended in DNase/RNase-free water. Nucleic acids were fragmented using a restriction enzymes cocktail, containing 20U of each of the following restriction enzymes: EcoRI (FD0274, Thermo Scientific), BamHI (FD0054, Thermo Scientific), HindIII (FD0504, Thermo Scientific), Bsp1407I (FD0933, Thermo Scientific) and XhoI (FD0694, Thermo Scientific). Then, half of each sample was digested with 40U of RNaseH enzyme (MB08501, NZYTech) to serve as negative control, for about 48 hours at 37ºC. Digested nucleic acids were purified with phenol-chloroform extraction, re-suspended in DNase/RNase-free water and quantified. DNA samples were diluted to a concentration of 10 µg/mL and 100 µL of solution (1 µg DNA) was loaded per well, into a positively charged nylon membrane (RPN203B, GE Healthcare), using the Bio-Dot® Microfiltration System (1703938, Bio-Rad). The DNA was cross-linked through UV irradiation (UV Stratalinker 2400, Stratagene) and membranes were blocked for 1 hr at room temperature with 5% milk in PBS 1x containing 0,05% Tween 20. Incubation with the primary antibodies (anti-dsDNA (sc-58749, Santa Cruz) and S9.6 (ENH001, Kerafast)) was performed at 4ºC overnight, followed by incubation with secondary antibody (anti-Mouse-HRP (170-6516, Biorad)) for 1 hour at room temperature. Detection was achieved using enhanced chemiluminescence substrates (RPN2209, GE Healthcare).

Figure S1
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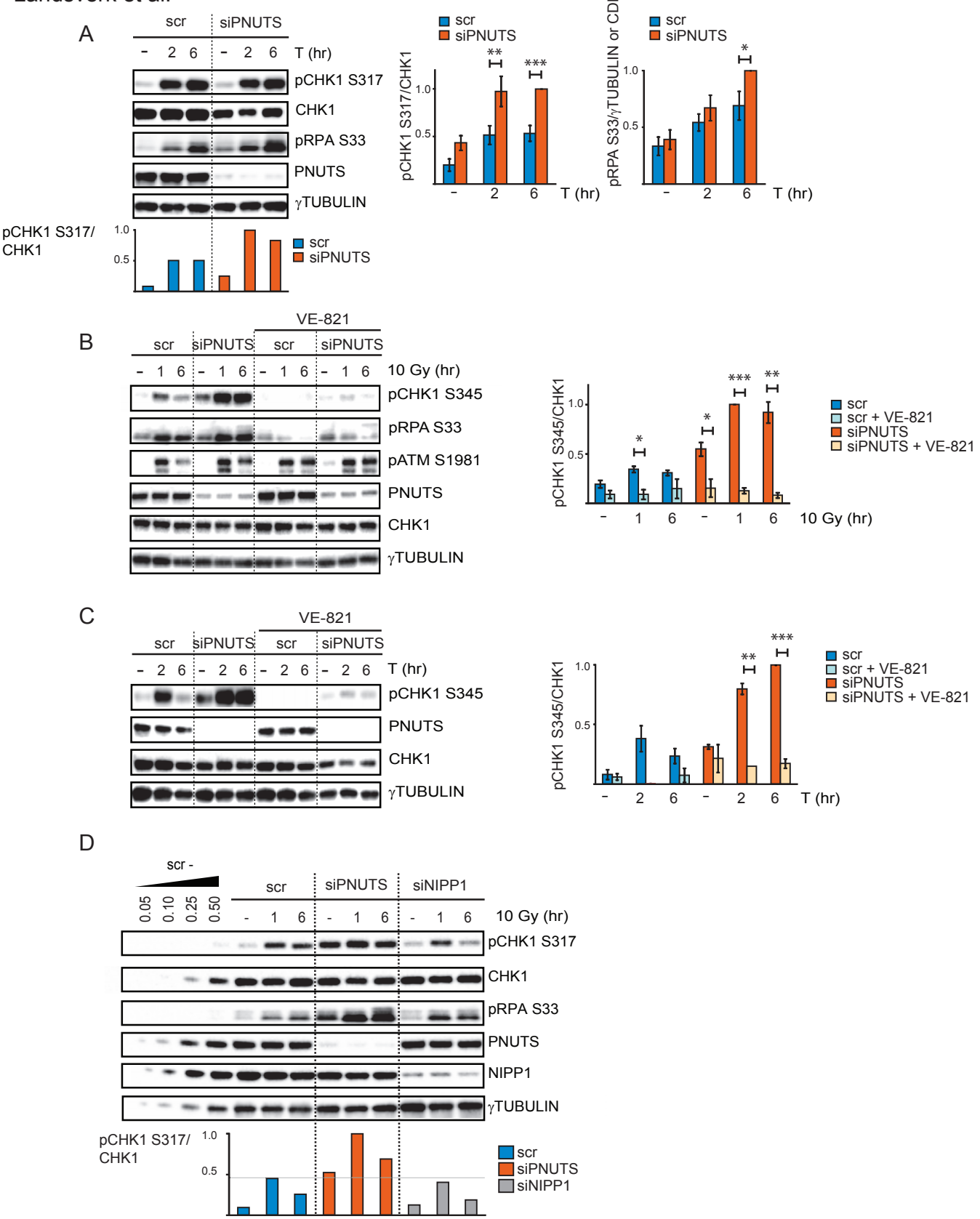


Figure S2
Landsverk et al.

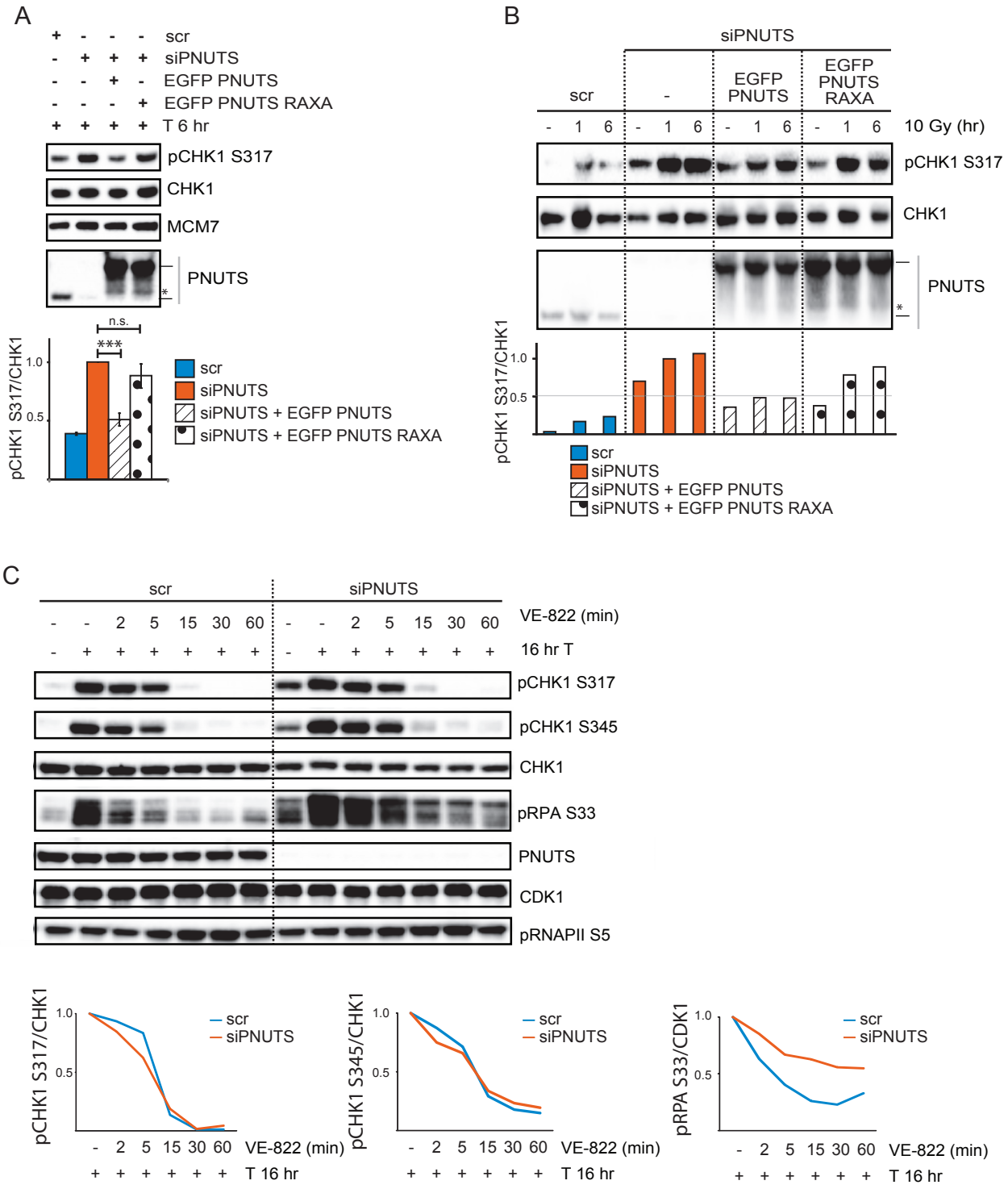
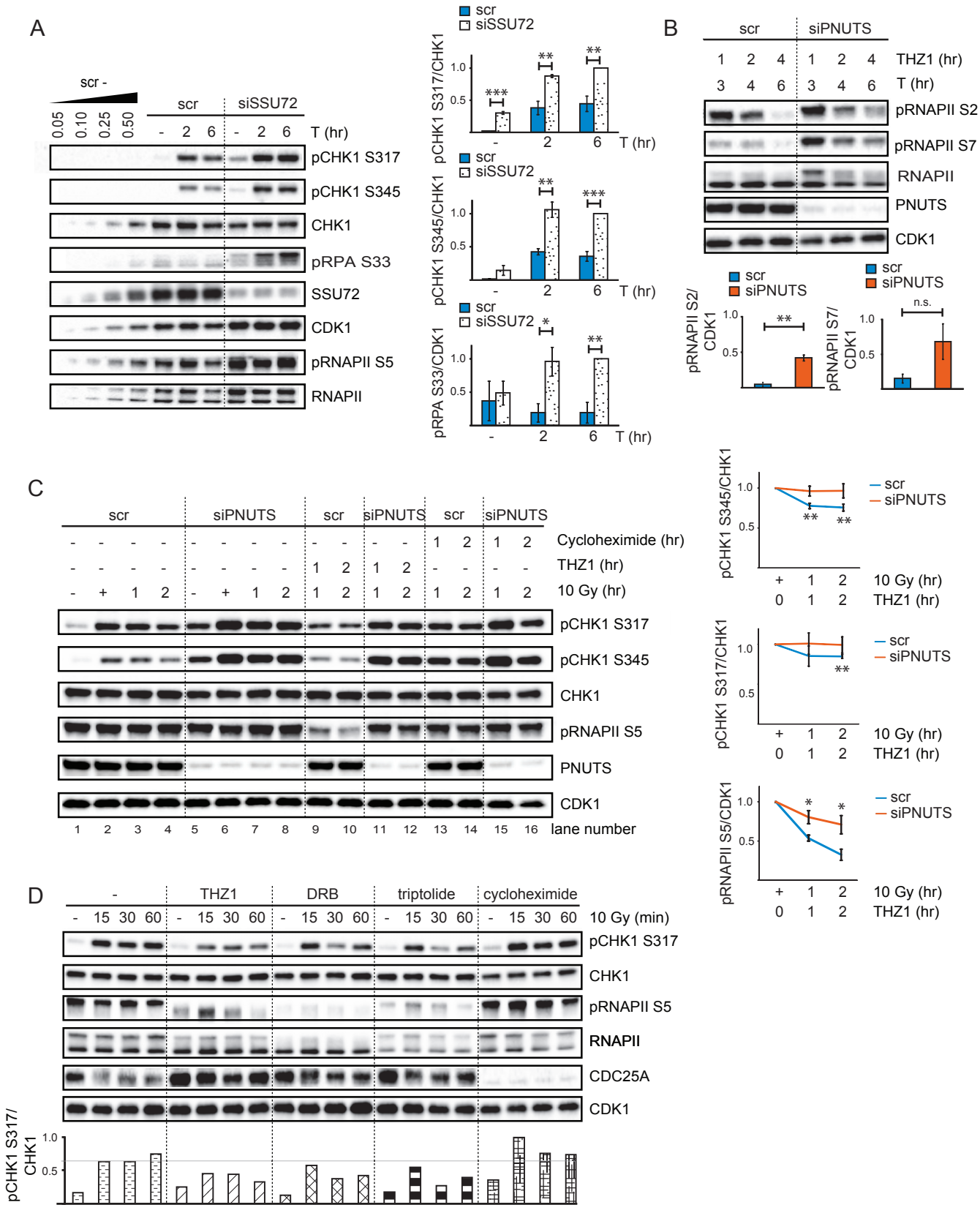


Figure S3
Landsverk et al.



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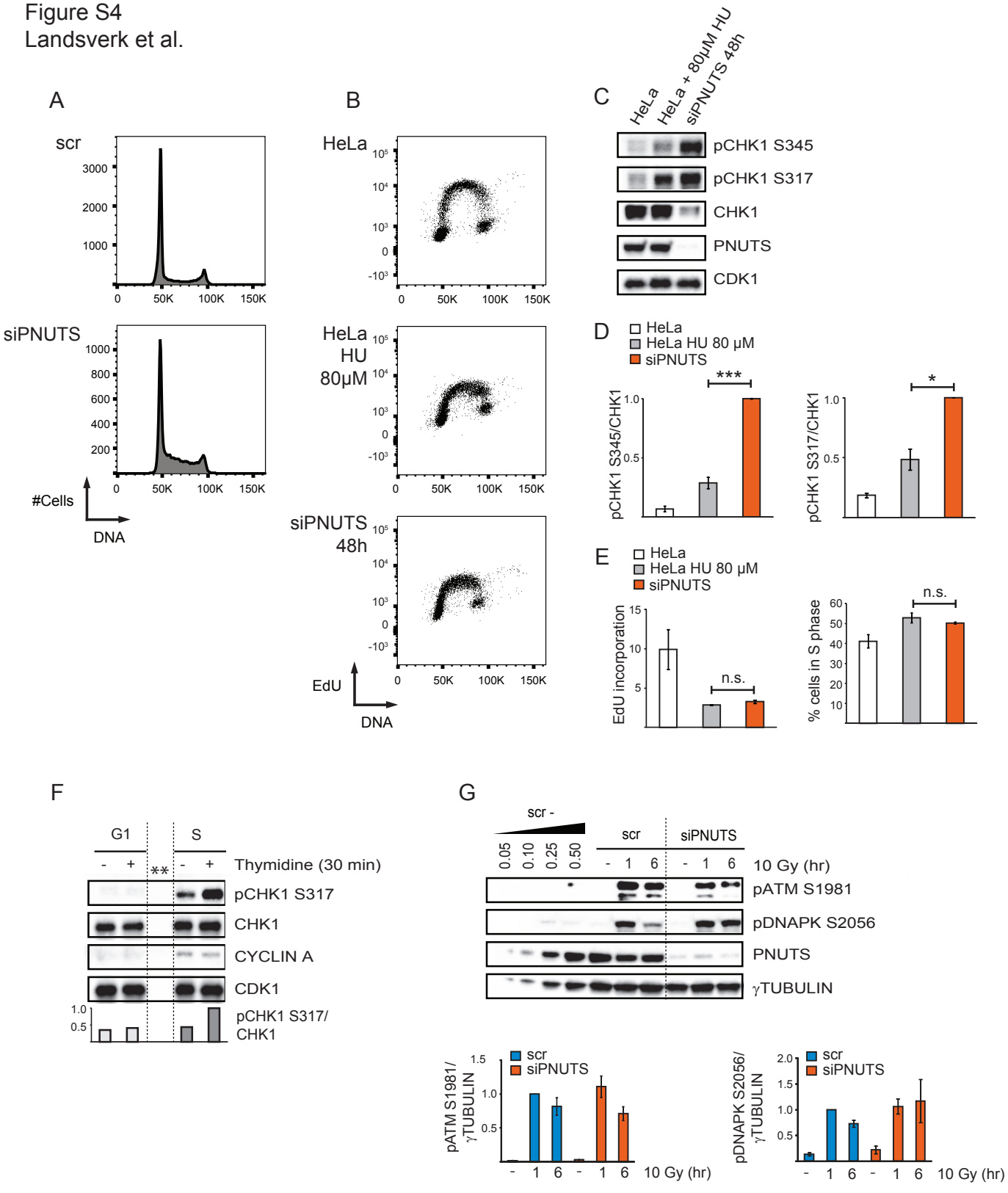
Landsverk et al.



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Landsverk et al.

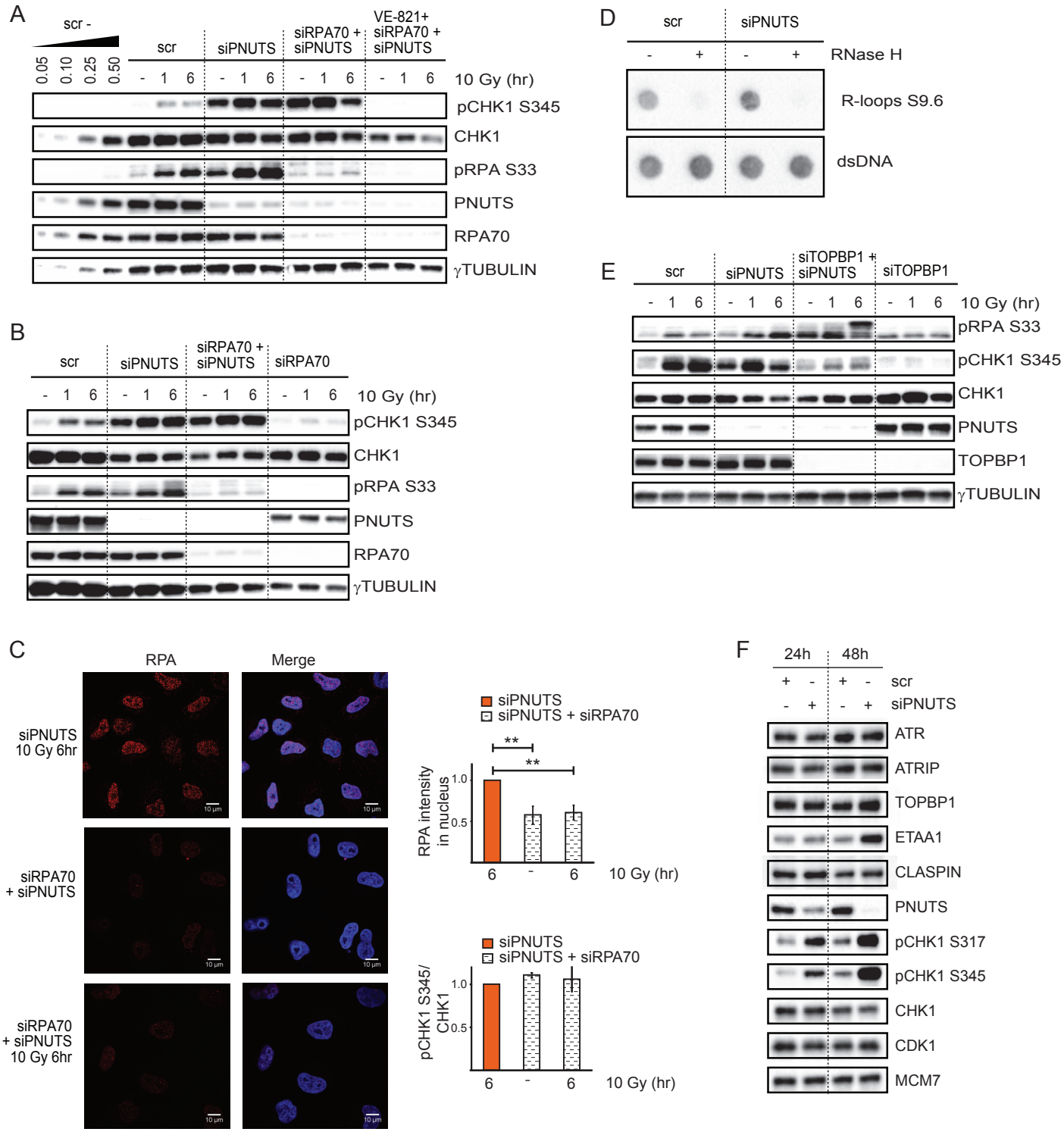

Figure S6
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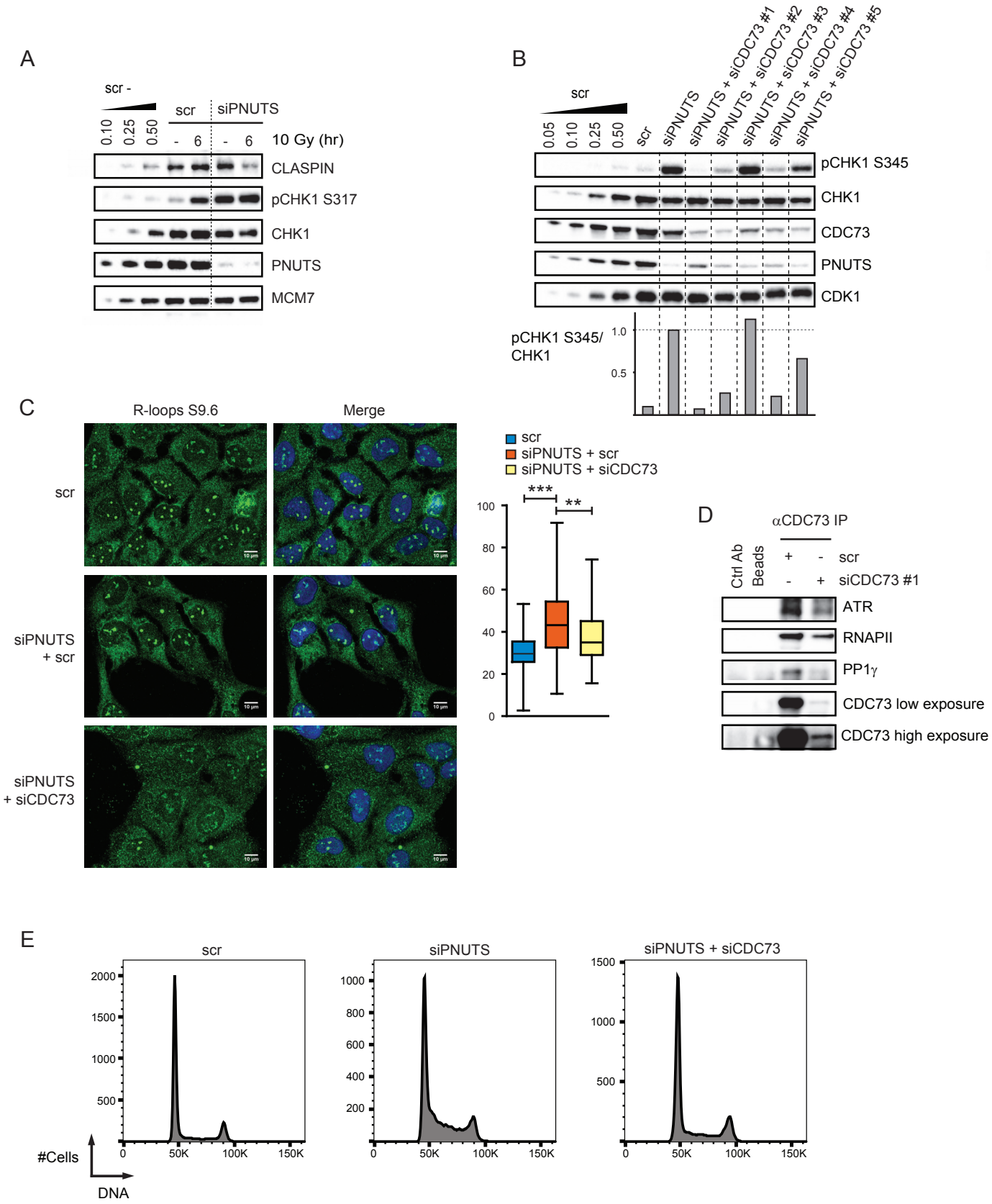

Table S1. siRNA oligonucleotide sequences

Name of siRNA	Sequence	Manufacturer	Reference
Scr (scrambled control siRNA of siPNUTS #1)	GGUUUCUGUCAAAUGCAAACGGCUU	Thermo Fisher (stealth siRNA)	(1)
siPNUTS #1	GGUGGUUUCUGACAAGUACAACCUU	Thermo Fisher (stealth siRNA)	(1)
siPNUTS #2 (also called siPNUTS)	GCAAUAGUCAGGAGCGAUA (silencer select s328)	Thermo Fisher	
siCDC73 #1	AAGCGUCAACAUCGGCAAGUA	Sigma-Aldrich	(2)
siCDC73 #2 (also called siCDC73)	AAACAAGGUUGUCAACGAGAA	Sigma-Aldrich	(2)
siCDC73 #3	CUGAACAGAUUAGGUCUUU (SASI_Hs01_00126024)	Sigma-Aldrich	
siCDC73 #4	GGAUCUCGAACACCCAUUA	Sigma-Aldrich	(3)
siCDC73 #5	CUAUCAAGACUGAUCUAGA	Sigma-Aldrich	(3)
siRPA70	GGACAAGUUCUUUCCUCUUAUUG	Sigma-Aldrich	(4)
siTOPBP1	AGACCUUAAUGUAUCAGUA	Sigma-Aldrich	(5)
siNIPP1	GGAACCUCACAAGCCUCAGCAAAUU	Thermo Fisher (stealth siRNA)	(6)
siETAA1	GAGCAAAACAAGAGGAAUU	Sigma-Aldrich	(7)
siSSU72	GGAGCUUCCUGUUGUUCAU (SASI_Hs01_00024012)	Sigma-Aldrich	

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3. Herr P, *et al.* (2015) A genome-wide IR-induced RAD51 foci RNAi screen identifies CDC73 involved in chromatin remodeling for DNA repair. *Cell discovery* 1:15034.
4. Liu S, *et al.* (2011) ATR autophosphorylation as a molecular switch for checkpoint activation. *Molecular cell* 43(2):192-202.
5. Kousholt AN, *et al.* (2012) CtIP-dependent DNA resection is required for DNA damage checkpoint maintenance but not initiation. *The Journal of cell biology* 197(7):869-876.
6. Minnebo N, *et al.* (2013) NIPP1 maintains EZH2 phosphorylation and promoter occupancy at proliferation-related target genes. *Nucleic acids research* 41(2):842-854.
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Table S2. Antibodies used

Antibody target	Manufacturer	Reference	Use
PNUTS	BD Biosciences		WB
phosphoCHK1 Ser317	Cell Signaling Technology		WB
phosphoCHK1 Ser345	Cell Signaling Technology		WB
phosphoATM Ser1981	Cell Signaling Technology		WB
phosphoCHK2 Thr68	Cell Signaling Technology		WB
ATR	Cell Signaling Technology		WB
CHK1		(1)	WB
γTUBULIN (GTU-88)	Sigma		WB
CDK1 (sc-54)	Santa Cruz Biotechnology		WB
RNAPII (F-12)	Santa Cruz Biotechnology		WB
MCM7 (DCS-141)	Santa Cruz Biotechnology		WB
phospho RPA32 Ser33	Bethyl		WB
phosphoDNAPK S2056	Abcam		WB
phosphoRNAPII S5 (3E8)	Millipore		WB
phosphoRNAPII S7 (4E12)	Millipore		WB
phosphoRNAPII S2 (3E10)	Millipore		WB
CDC73	Bethyl		WB, IP
phosphoATR Thr1989	GeneTex		WB, IP
ETAA1		(2)	WB
R-loops (S9.6, ENH001)	Kerafast		IF, Dotblot
NIPP1 (sc-393991)	Santa Cruz Biotechnology		WB
TOPBP1	Abcam		WB
CLASPIN	Cell Signaling		WB
ATRIP	Millipore		WB
γH2AX	Abcam		FC
phosphoHISTONE H3 S10	Millipore		FC
CDC25A (DCS-120)	Santa Cruz Biotechnology		WB
PP1γ (sc-6108)	Santa Cruz Biotechnology		WB
CYCLIN A (sc-751)	Santa Cruz Biotechnology		WB
RPA70	Cell Signaling Technology		WB, FC
Double stranded DNA (sc-58749)	Santa Cruz Biotechnology		Dotblot
RPA32 (MABE286)	Millipore		IF
SSU72 (D3I2D)	Cell Signaling		WB

WB: Western blotting, FC: flow cytometry, IF: immunofluorescence microscopy

Peroxidase-conjugated secondary antibodies were from Jackson Immunoresearch.

Alexa-conjugated secondary antibodes were from Thermo Fisher.

1. Sorensen CS*, et al.* (2003) Chk1 regulates the S phase checkpoint by coupling the physiological turnover and ionizing radiation-induced accelerated proteolysis of Cdc25A. *Cancer Cell* 3(3):247-258.
2. Haahr P*, et al.* (2016) Activation of the ATR kinase by the RPA-binding protein ETAA1. *Nature cell biology* 18(11):1196-1207.