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Review

The p21 and PCNA partnership

A new twist for an old plot

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Abbreviations: TLS, translesion DNA synthesis; NER, nucleotide excision repair; pol, polymerase; CDK, cyclin dependent kinase; PCNA, proliferating cell nuclear antigen; IDCL, interdomain connecting loop; PIP box, PCNA interacting protein motif; CPD, cyclobutane pyrimidine dimmers; TT, thymidine dimmers; BP-G, benzo[a]pyrene-guanine; XPV, xeroderma pigmentosum variant; ATR, ataxia telangiectasia and Rad3-related; Gadd45, growth arrest and DNA damage gene 45; DDB2, damage-specific DNA binding protein 2; CRL4, cullin RING ubiquitin ligase containing cullin CUL4; CDT2, chromatin licensing and DNA replication factor 2; hprt, hypoxanthine-guanine phosphoribosyltransferase

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The contribution of error-prone DNA polymerases to the DNA damage response has been a subject of great interest in the last decade. Error-prone polymerases are required for translesion DNA synthesis (TLS), a process that involves synthesis past a DNA lesion. Under certain circumstances, TLS polymerases can achieve bypass with good efficiency and fidelity. However, they can also in some cases be mutagenic, and so negative regulators of TLS polymerases would have the important function of inhibiting their recruitment to undamaged DNA templates. Recent work from Livneh's and our groups have provided evidence regarding the role of the cyclin kinase inhibitor p21 as a negative regulator of TLS. Interestingly, both the cyclin dependent kinase (CDK) and proliferating cell nuclear antigen (PCNA) binding domains of p21 are involved in different aspects of the modulation of TLS, affecting both the interaction between PCNA and the TLS-specific pol η as well as PCNA ubiquitination status. In line with this, p21 was shown to reduce the efficiency but increase the accuracy of TLS. Hence, in absence of DNA damage p21 may work to impede accidental loading of pol η to undamaged DNA and avoid consequential mutagenesis. After UV irradiation, when TLS plays a decisive role, p21 is progressively degraded. This might allow gradual release of replication fork blockage by TLS polymerases. For these reasons, in higher eukaryotes p21 might represent a key regulator of the equilibrium between mutagenesis and cell survival.

Introduction

Even without excessive exposure to DNA damaging agents, DNA damage occurs with a daily frequency of ~10,000 hits/cell.[1] To ensure the maintenance of genomic integrity several molecular networks have evolved to coordinate cell cycle control and lesion removal. The cyclin-dependent kinase inhibitor p21[waf/cip1] is an important effector of cell cycle arrest after many genotoxic insults. In response to DNA damage the interaction of p21 with CDKs and PCNA is crucial for cell cycle withdrawal.[2] Intriguingly, despite effective activation of upstream signals such as checkpoint kinases ATR and CHK1, p21 does not accumulate after UV irradiation. In fact, UV induces p21 proteolysis in many cell types, indicating a negative role of p21 in the cellular response to UV light.[3-7] However, CDK inhibition still occurs after UV exposure in a manner that is independent of p21.[7] This suggests that the reduction in p21 levels is more relevant to the other main target of p21, the proccesivity factor PCNA.

PCNA is a ring shaped trimeric complex with essential roles in DNA synthesis associated with both DNA replication and repair.[8-10] PCNA forms a sliding platform required for the processivity of DNA polymerases δ and ε during DNA replication.[11] PCNA also participates in several forms of DNA repair including nucleotide excision repair (NER) and TLS.[10] p21 interacts with the interdomain connecting loop (IDCL) of PCNA which is the same region bound by DNA polymerases and other proteins involved in chromatin organization.[10,12] Remarkably, p21 binds to the IDCL with much higher affinity than any other known PCNA interacting proteins.[13] Further, in vitro experiments demonstrate that p21 is potentially capable of precluding PCNA interaction with many factors including pol δ.[14] In a similar fashion to replicative polymerases, TLS polymerases interact with the IDCL of PCNA and therefore p21 could also block the interaction between PCNA and TLS polymerases.

It is not yet clear whether p21 levels in cells are sufficient to displace such a broad range of PCNA partners. In addition, the amount of p21 available for PCNA interaction could also be conditioned by other events such as p21 sequestration by CDK/cyclins or modifications in chromatin accessibility, etc. In this respect we have recently shown that PCNA interaction with TLS polymerases might be more sensitive to changes in p21 levels than replicative

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polymerases.[15] The biological implications of our findings will be discussed below.

Are p21 Levels Sufficient to Prevent DNA Replication, Nucleotide Excision Repair and Translesion Synthesis in Cells?

Two main targets of p21 are associated with its negative effect on DNA synthesis. The N terminal domain of p21 contains a CDK binding motif followed by a cyclin interacting element. The PCNA interacting protein motif (PIP box) is located within its C terminus and partially overlaps with a nuclear localization signal and a second cyclin binding motif.[2] The contribution of its CDK-interacting domain to cell cycle arrest is broadly accepted but there is still controversy regarding the role of its PCNA-interacting domain in blocking DNA synthesis in cells.

PCNA is a highly abundant protein, especially during S-phase, and so it is not clear whether p21 levels would ever be sufficient to titrate PCNA. In fact, while the p21/PCNA ratio might never exceed 1:1 in vivo,[16,17] much higher ration of p21 to PCNA (as high as 10:1 or more) were required to block DNA replication[18-20] and DNA synthesis associated with nucleotide excision repair (NER)[17,21-23] in vitro. Conversely in vivo, several reports showed little or null effect of the p21/PCNA interaction on DNA replication[17,24-28] while only a few studies suggest that p21/PCNA interaction in cells might result in the CDK-independent arrest of the cell cycle.[29,30] The effect of p21 on NER in vivo has also been the subject of strong debate. Some groups have observed no effect of p21 on NER[31-35] while others have reported an inhibitory effect on NER when p21 is ectopically overexpressed.[7,23] Intriguingly, two groups have reported that while the deletion of p21 in cells did not alter NER efficiency, the upregulation of p21 (that results from the removal of specific genes such as Gadd45 and DDB2) does impair NER.[36,37] This suggests that, in certain scenarios, endogenous modulation of p21 levels might be sufficient to negatively regulate the DNA synthesis associated with NER.

The inconsistency between these many reports might have arisen from the different amounts of p21 used which becomes quite hard to evaluate. Also, the different p21 constructs used such as just the N- or C-termini of the protein might have missed biologically relevant interactions. Moreover, the majority of previous reports that investigated p21's impact on DNA replication and repair have not taken into consideration the fact that p21 is actively degraded after UV irradiation. Therefore, the physiological effect of p21 on UV-associated DNA synthesis processes is currently difficult to predict.

We have recently tried to clarify this issue. To do so we compared constructs expressing wild-type p21 and a series of full-length p21 mutants which resist UV increased proteolysis due to an N terminal tag of 6 Myc epitopes.[3,38] We assessed in parallel the role of the CDK and PCNA binding domains of p21 in different PCNA-driven DNA synthesis processes including DNA replication, NER and TLS both before and after UV irradiation.[15] We showed that p21 levels were similar to the ones observed after p53-dependent upregulation of endogenous p21 following DNA damaging treatments. Under identical experimental settings, p21 binding to PCNA did not affect DNA replication and NER but it impair events associated with TLS such as: (a) UV-dependent formation of nuclear foci containing the TLS-specific polymerase, pol η and (b) UV-dependent interaction of pol η with chromatin-associated PCNA. These observations are consistent with our previous findings indicating that failure to down-regulate p21 impairs also TLS-associated PCNA ubiquitination.[3] Importantly as well, others have shown that p21 expression prevents TLS associated mutagenesis in unstressed cells.[39] Our findings do not rule out that higher levels of p21 might eventually impair all types of PCNA-associated DNA synthesis but they suggest that TLS is the PCNA-regulated process that is more sensitive to changes in p21 levels. In fact, both TLS efficiency and pol η/PCNA interaction are modulated by endogenous p21 in cycling cells.[15,39]

TLS Activation and Regulation

While replicative DNA polymerases are stopped at DNA lesions, the specialized TLS polymerases have evolved to incorporate nucleotides opposite damaged DNA. In mammalian cells, these polymerases are pol η, pol ι, pol κ and Rev 1 in the Y-family and pol ζ in the B-family. They have all conserved catalytic domains in their N-terminus while their less conserved C-terminus is involved in protein-protein interactions that are significant for their recruitment to stalled forks at DNA lesions. All TLS polymerases are characterized by poor processivity, relaxed fidelity and lack of 3'–5' proofreading activity.[40]

As mentioned before, TLS polymerases are not always mutagenic. There are at least three examples that suggest that TLS polymerases might be able to synthesize past DNA lesions accurately and efficiently. This was shown for pol η bypass of the major UV-photoproduct, cyclobutane pyrimidine dimmers (CPD),[41,42] pol κ bypass of benzo[a]pyrene-guanine (BP-G)[43,44] and pol η bypass of cisplatin-GG lesions.[45] In line, the *Xeroderma pigmentosum* variant (XPV) disease which is defective in pol η expression, is characterized by predisposition to skin cancer that presumably results from a more mutagenic bypass of CPDs by other TLS polymerases (reviewed in ref. 46). However, given the high number of DNA damaging events that can arise after different insults, it is unlikely that all DNA lesions could accurately be bypassed by a specialized TLS polymerase. It is also possible, given their overlap of functions,[40,46,47] that different TLS polymerases compete for the access to a specific type of DNA lesion in cells. Moreover, their activity must be tightly controlled to ensure that they act only at DNA lesions and not on undamaged template. Taken together, these observations suggest that negative regulators of TLS polymerases might be central for the control of spontaneous mutagenesis in cells.

PCNA ubiquitination, which can be induced by a broad range of genotoxic agents,[3,48-52] was shown to be associated with the activation of TLS and to be essential for post-UV cell survival in *S. cerevisiae*.[52,53] UV-induced PCNA mono-ubiquitination on lysine 164 depends on the accumulation of DNA photolesions[54] and on the activity of the Rad6-Rad18 E2-E3 ligases. Subsequently, the Mms2-Ubc13 and Rad5 proteins are in charge of the non-degrading lys 63-linked polyubiquitination of PCNA.[52] While monoubiquitination of PCNA has been linked to error-prone repair, PCNA polyubiquitination seems to direct error-free damage removal. In mammals, the major modification of PCNA after UV exposure is monoubiquitination. Monoubiquitinated PCNA was reported to have a much higher affinity than unmodified PCNA for Pol η.[48,55-57] This is in line with the identification of ubiquitin binding motifs in TLS polymerases[55,56] that might contribute to the increased interaction of PCNA and TLS polymerases after UV irradiation. Moreover,

USP1, a deubiquitinating enzyme (DUB) for PCNA, is inactivated by autocleavage after UV irradiation[58] thus re-enforcing the link between PCNA ubiquitination and the recruitment of TLS polymerases to DNA lesion.

The exact function of PCNA ubiquitination in TLS is not yet clear. While one report suggests that PCNA ubiquitination stimulates pol η and REV 1 bypass of abasic sites,[59] another report showed no stimulation of pol η, REV 1 and pol ζ by ubiquitinated PCNA.[60] The latter report suggested that the real function of PCNA ubiquitination is to promote the disassembly of factors that prevent pol η recruitment to replication foci. In agreement, more recently it was shown that PCNA ubiquitination might prevent pol δ reloading at DNA lesions.[61] Together, these results show that increased PCNA ubiquitination might be only one aspect of the process necessary to achieve efficient TLS. Importantly as well, blocked replication forks might not be the only triggers for PCNA ubiquitination since this post-translational modification of PCNA was observed in human cells arrested in G_1 and G_2,[3] and in *S. pombe* cells held in G_2.[62]

The exact timing of lesion bypass by TLS polymerases is also under current investigation. While the current model associates PCNA ubiquitination with stalled replication forks and envisages that polymerases must switch at such DNA structures new evidence validates the gap-filling model that was proposed decades ago.[63] In fact, electron microscopy data showed that short patches of ssDNA were left behind the leading and lagging strand of replication forks. The increase in the number of gaps observed in cells lacking TLS polymerases supported their involvement in the post-replicative filling of those gaps.[64] Moreover, a central role of PCNA ubiquitination in the filling of post-replicative gaps but not in the restoration of blocked replicating forks has been demonstrated in DT40 cells.[65] Therefore, TLS at or behind the replication fork might be controlled by partially independent mechanisms and might be favored by different scenarios.

How does p21 Regulate TLS?

Several lines of evidence indicate that p21 could act as a negative regulator of TLS polymerases both before and after DNA damage. In fact, p21 was shown to modulate TLS-dependent mutagenesis, pol η/PCNA interaction and PCNA ubiquitination, as will be discussed below.

TLS-dependent mutagenesis. To analyze the effect of p21 in gap-filling opposing a DNA lesion, Dr. Livneh and colleagues set up a plasmid based assay that can quantitatively measure TLS outside the context of the chromatin.[44] This technology is based on the introduction into cells of gapped plasmids carrying a site-specific damaged nucleotide at the gap region. TLS is required to fill-in the plasmid and make it suitable to transform bacteria after purification from cell extracts. Those plasmids do not replicate in mammalian cells and therefore, the number of bacterial colonies obtained depends directly on TLS efficiency. The percentage and nature of mutations can also be analyzed and their link to TLS can be established.

Taking advantage of this assay, Livneh and colleagues showed that the tumor suppressor p53 and its transcriptional target p21 inhibits TLS frequency and makes it more accurate.[39,66] A construct containing only the C-terminal PCNA binding region of p21 was sufficient to control TLS-associated mutagenic load. By contrast, the CDK-interacting N-terminus of p21 had no effect on this assay thus suggesting that CDK inactivation is not required for the repression of mutagenic TLS. Similar results were obtained with three specific lesions: cisplatin-GG known to be bypassed mainly by pol η, BP-G known to be bypassed mainly by pol κ and abasic sites which, in the context of these assay are bypassed by aphidicolin-sensitive polymerases[66] but can also be bypassed by other polymerases.[67,68] This suggests that p21 might regulate TLS polymerase recruitment to a broad range of DNA lesions in cells.

Remarkably, UV irradiation did not affected TLS any further in this experimental system. This might depend on the fact that UV irradiation is not needed to create the gap associated to the lesion in this assay. However, it is clear that other signals such as PCNA ubiquitination and USP1 autocleavage are missing in the absence of DNA damage. Thus, in unstressed cells, endogenous p21 may prevent the loading of TLS polymerases that is independent of PCNA ubiquitination to DNA gaps opposing a lesion.

Evidence linking p21 to the repression of chromosomal mutagenesis has been previously provided. In fact, increased spontaneous mutation frequency was reported in $p21^{-/-}$ versus $p21^{+/+}$ cells by exploring the hypoxanthine phosphoribosyltransferase (*hprt)* lucus inactivation.[69] While the contribution of TLS to this phenotype was not yet explored, the results obtained by Dr. Livneh and colleagues suggest that in unstressed cells, p21 might repress mutagenesis by avoiding the loading of TLS polymerases at the wrong sites on DNA.

Pol η/PCNA interaction. Pol η is localized in the nucleus of both unstressed and UV irradiated cells. In a small fraction of cells (approximately 10%) pol η relocalizes into nuclear structures (foci) that are associated with replication factories in S phase. After UV irradiation the great majority of nucleoplasmic pol η reorganizes into chromatin-associated foci at sites of un-repaired DNA damage.[70] In all cases, a striking colocalization of PCNA and pol η was observed[70] and in fact, this correlated with increased recruitment of pol η to chromatin bound PCNA.[48] Pol η re-localization into foci is likely to be crucial for its function since mutants that were unable to do so failed to correct the sensitivity of XPV cells to UV light.[70]

We analyzed the effects of wild-type p21 and stable p21 mutants lacking its CDK or PCNA binding domains on these TLS-associated events. Initially, we observed that pol η focus formation was delayed in cells expressing wild type p21. Strikingly, increased pol η foci formation correlated time-wise with p21 degradation. Accordingly, stable p21 expression impaired both pol η focus formation and pol η/PCNA interaction at all times and this ability depended on the p21/PCNA interaction.[15] In agreement with previous findings, the binding of p21 to PCNA did not influence PCNA/pol δ interaction both before and after UV irradiation.[26,31,32] Further, the p21/PCNA interaction was neither sufficient to induce cell cycle arrest nor to block NER dependent DNA synthesis.[15] Thus, p21 association with PCNA greatly impairs TLS-associated events without displacing replicative polymerases from DNA synthesis factories.

Interestingly, while TLS polymerases interact mainly with the IDCL of PCNA,[71,72] multimeric replicative polymerases (pol ε and δ) utilize different interacting motifs of PCNA which results in multi-site interactions.[73-77] Thus, the high affinity of p21 for the IDCL of PCNA[13] might displace monomeric TLS polymerases more efficiently than replicative polymerases. Strikingly, the ability of p21 to block pol η recruitment to stalled replication sites may have

critical consequences for the cell. In fact, stabilized p21/PCNA interaction after UV exposure resulted in highly altered DNA topology revealed by high levels of phosphorylated-H2AX and increased cell death.[15]

By performing similar analysis in $p21^{+/+}$ and $p21^{-/-}$ HCT116 cells, we observed increased levels of endogenous PCNA/pol η interaction and pol η foci formation in unstressed $p21^{-/-}$ cells.[15] In agreement with Livneh and colleagues,[39] our findings suggest that during unstressed DNA replication p21 might prevent the mutagenesis triggered by uncontrolled activity of TLS polymerases. Our results indicate that after UV irradiation, the progressive reduction in p21 levels might allow cell survival associated with the gradual loading of TLS polymerases onto damaged DNA.

PCNA ubiquitination. By analyzing the effect of different genotoxic treatments on both the levels of p21 and PCNA ubiquitination we observed that p21 dowregulation and efficient PCNA ubiquitination did take place simultaneously.[3] This suggested that p21 downregulation and ubiquitin conjugation to PCNA could be coordinated. Hence, modulation of p21 levels could affect the extent of PCNA ubiquitination after UV irradiation.

So far two apparently conflicting results were obtained when this hypothesis was tested. Transient downregulation of p21 achieved by using specific siRNA oligonucleotides resulted in defects in PCNA ubiquitination after UV exposure.[39] Yet we have shown that the expression of stable p21 after UV impaired the accumulation of ubiquitinated PCNA.[3] If both results are biologically relevant this could imply that low levels of p21 promote PCNA ubiquitination but high levels of p21 downregulate this post-translational modification of PCNA. A similar bimodal effect was previously reported for the modulation of CDK4 activation by p21.[78] Interestingly, we have shown that the domain of p21 involved in the control of PCNA ubiquitination is the CDK binding region of p21. This suggests that independent functions linked to both domains of p21 could collaborate at the replicating forks to promote the polymerase switch. While much more work will be required to validate these speculations, this could suggest that p21 controls the phosphorylation (by CDKs) of PCNA or PCNA partners involved in the regulation of its ubiquitination after UV irradiation. In line with this, at least some PCNA functions involved in cell proliferation and chromatin recruitment were recently linked to PCNA phosphorylation.[79] Also, CDK and PCNA can interact and PCNA was shown to act as an adaptor for CDK phosphorylation of PCNA partners.[80,81]

While the above mentioned evidence points towards a role of p21 in the control of PCNA post-translational modifications, an inverse effect of PCNA on the stability of p21 has also been recently proposed. p21/PCNA interaction is required for p21 degradation triggered by the $CRL4^{Cdt2}$ E3 ligase complex after UV irradiation[82,83] and in S phase, to control replication licensing.[84] This suggests that p21 degradation could also take place in situ, on DNA, after UV irradiation. Interestingly, a detailed time course of p21 localization after UV irradiation indicated that residual p21 colocalizes with PCNA foci after UV irradiation (Soria G and Gottifredi V, unpublished). Moreover, the degradation of Xic1, the analog of p21 in Xenopus, takes place on DNA and requires PCNA loading and initiation of DNA synthesis.[85-87] The switch to a UV-driven mechanism for p21 proteolysis has been supported by the finding that UV-induced but not basal p21 degradation is controlled by ATR.[7] Moreover, the SCF^{Skp2} E3 ligase was previously reported to control p21 degradation after UV irradiation[7] which could at least partially contribute to increased p21 turnover after this insult. Whether p21 degradation is mainly controlled by PCNA before and after UV exposure or other treatments remains to be tested. However, it is interesting to speculate that that not only p21 degradation might be required for PCNA function/s after UV irradiation but also might be driven by PCNA and PCNA-associated molecules.

A Model for the Regulation of TLS by p21

A recent report by Livneh proposed a model based on the findings of his laboratory. In unstressed cells pol δ but not p21 is loaded onto DNA while after UV irradiation p53-dependent upregulation of p21 results in competition and displacement of pol δ from DNA. Since p21 is not a very bulky molecule, pol η and PCNA ubiquitinating enzymes could then be efficiently and specifically recruited to DNA lesions. Consequently, the reduction in mutagenic rate would directly depend on the more efficient/faster recruitment of pol η to DNA lesions.[88]

Three observations indicate that this model may need to be reevaluated. First, p21 is not upregulated but it is actively degraded after UV irradiation in many cellular systems[3-7] thus suggesting that pol δ displacement by p21 would not be easy to achieve. Second, we and others have shown that pol δ recruitment to PCNA is unaltered by UV irradiation[15,26,31,32] Third, pol η loading on DNA and pol η foci formation are inhibited by a stable p21 mutant that retains its capacity to bind PCNA.[89] Moreover, endogenous p21 expression delays pol η recruitment to foci structures or chromatin bound PCNA and this time-shift in pol η recruitment to DNA correlates with p21 degradation.[15]

Even under conditions of p21 overexpression, pol η is more sensitive to p21 inhibition than is pol δ. In fact, this was demonstrated using a stable mutant of p21 that fails to bind CDKs. The disruption of CDK binding in p21 was sufficient to allow normal rates of cell cycle progression and efficient PCNA focus formation in S phase cells. Due to its unaltered PCNA binding capacity, stable p21 (CDK^-) was recruited to those foci and colocalized with BrdU incorporation corresponding to replicative DNA synthesis.[15] This suggested that despite the recruitment of p21 to DNA synthesis factories, all necessary replicative polymerases were correctly localized at sites of DNA replication. By contrast, after UV irradiation p21 binding to replication foci prevented both pol η recruitment to those foci and chromatin-associated pol η/PCNA interaction.[15] Taken together these data suggest that pol η is more likely to be recruited to replication forks when p21 is degraded than when it is upregulated.

A model that takes the above mentioned findings into consideration is shown in Figure 1. Before UV irradiation, low levels of p21 compatible with S-phase would not prevent cell cycle progression even when p21 is recruited to replicating forks. However, they would prevent the accidental loading of pol η to undamaged template and therefore this would prevent mutagenesis as reported previously.[39] After UV exposure, p21 levels are reduced as a consequence of increased p21 proteolysis and PCNA ubiquitination is increased due to the specific activity of Rad6-Rad18. The local increment in PCNA ubiquitination and the reduced competitive effect of p21 for PCNA binding would then allow progressive recruitment of pol η to DNA lesions. Thus, appropriate p21 cellular levels might play a critical role

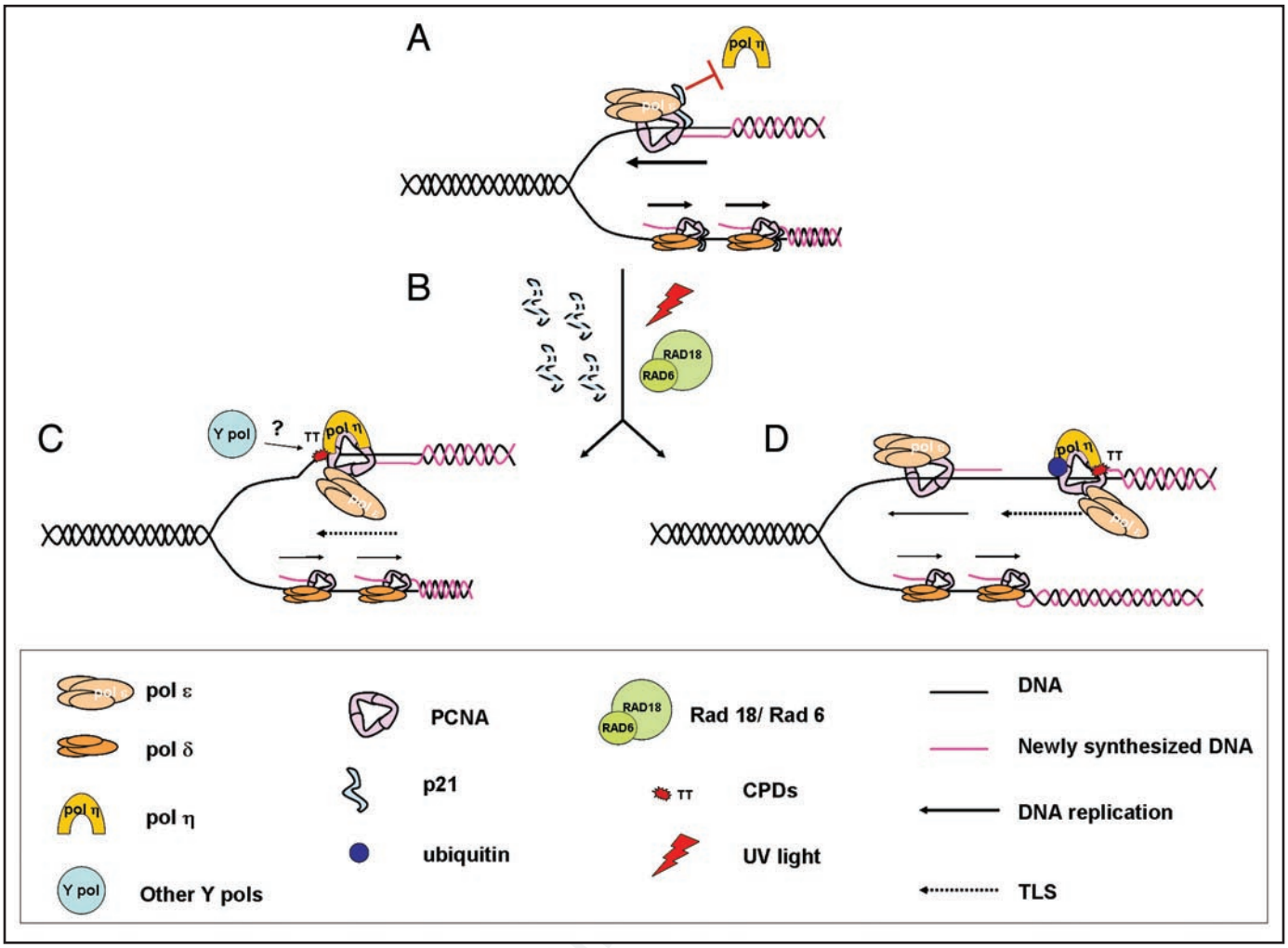

Figure 1. A model for the regulation of pol η recruitment to chromatin bound PCNA by p21. (A) During unstressed cell cycle progression p21 can associate to PCNA without impairing replicative DNA synthesis but avoiding the recruitment or the permanence of pol η at replication forks. (B) UV irradiation provokes a sustained accumulation of DNA lesions, mainly thymidine dimmers (TT) and triggers p21 proteolysis. (C) Consequentially, pol η is recruited to DNA lesions. The recruitment to stalled forks of other TLS-polymerases could also be favored when p21 levels are reduced. (D) PCNA ubiquitination is also triggered by the appearance of DNA lesions. The association of pol η to ubiquitinated PCNA could also promote lesion bypass at post-replicative gaps as well. For simplification purposes, only the TLS events on the leading strand are shown.

in the timing and loading management of pol η at stalled replication forks or post-replicative gaps.

Unraveling the mechanism of TLS regulation by p21 will require further investigation. It should also be borne in mind that the increase in p21 proteolysis after UV is very strong in many immortalized cell lines but is much more subtle in normal diploid human fibroblasts and sometimes is not evident at all when the UV dose is sufficiently low.[32,90] Also, under these conditions, while p21 is clearly degraded, the fraction of p21 that is bound to PCNA is much less sensitive to degradation.[32,39] The impact of these variables on TLS awaits to be determined. In addition, since it is not clear how TLS polymerases compete for their access to DNA lesions, exploring the effect of p21 on other B and Y-family polymerases will certainly help to identify the mechanism by which p21 controls the efficiency and accuracy of TLS. Since different signals might control the gap-filling and the restoration of blocked replication forks after UV irradiation,[65] it would be interesting to establish whether p21 interferes with pol η loading in both scenarios, particularly considering the indirect effect that p21 can exert on cell cycle regulation.

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