SUMMARIZING DISCUSSION
Since the late 1960s and early 1970s, when (error-prone) post-replication repair was first described [1-3], much progress has been made in understanding the mechanism and regulation of TLS. In their landmark paper, Rupp and Howard-Flanders showed, using the newly developed alkaline sucrose gradient centrifugation technique, that UV-irradiated bacteria have similar DNA synthesis rates as mock treated ones. However, UV-irradiated bacteria synthesized their DNA in much smaller fragments, suggesting that gaps had been formed as a result of the UV-induced lesions [2]. Subsequent work showed that this was indeed correct [3]. Soon after, with the identification of the REV loci in Saccharomyces cerevisiae and the discovery of the variant form of Xeroderma pigmentosum, genes responsible for the bypass of UV-induced lesions were found that operated independently of excision or recombination-mediated repair [1,4]. It took more than twenty years to reveal the DNA polymerase activity of the proteins generated by these and other relevant loci. Rev3 turned out to be, together with Rev7, a DNA polymerase (ζ) that could inefficiently bypass CPDs in vitro, while Rev1 was shown to have dCMP transferase activity [5,6]. Three years later, the XPV locus was shown to encode Polη, which can efficiently bypass UV-induced thymidine dimers [7-9]. Two other TLS polymerases, ı and κ, were soon found [10-16]. At this point, most TLS polymerases and their biochemical activities had been discovered and characterized, but hardly anything was known about their regulation.

Besides TLS polymerases, genetic analysis in yeast revealed that the Rad6 epistasis group acts in some way in error-free or error-prone DNA damage tolerance (DDT). Nevertheless, based on the biochemical characterization of TLS polymerases, it was suspected that the error-prone branch of DDT is linked to translesion DNA synthesis (TLS) [17]. Previously, it had been established that the Rad6 protein contained E2 ubiquitin-conjugation activity, but its substrate within the Rad6 pathway remained elusive [17,18]. Later, PCNA was discovered as a substrate of Rad6 and Rad18-dependent ubiquitination, while successive work by the Ulrich laboratory revealed that PCNA monoubiquitination (PCNA-Ub) controls TLS polymerases η and ζ in yeast [19,20]. Hoege et al. also detected PCNA-Ub in HeLa cells after methyl methanesulphonate (MMS) treatment, which implied functional conservation in higher eukaryotes [19]. Similar to their yeast counterparts, higher eukaryotic Pcna<sup>K164R</sup> cells are also extremely sensitive to replication blocking lesions [21,22]. While in yeast PCNA-Ub is crucial in the activation of TLS polymerases η and ζ, the exact role in mammals remained unknown. On one hand it has been reported that TLS polymerases have higher affinity for PCNA-Ub than for unmodified PCNA [23-27], while on the other hand TLS can also be activated independently of RAD18 or ubiquitin binding domains (UBDs) [28-31].

**Relevance of PCNA modification in TLS regulation**

To define the exact function of PCNA-Ub during TLS activation in mammals, we made use of a mouse model previously generated in our laboratory: mutant mice that carry a non-modifiable Pcna<sup>K164R</sup> allele [32]. This mutant strain was crossed to the Polh-deficient mouse strain to generate the respective single mutants and the Pcna<sup>K164R</sup>;Polh<sup>−/−</sup> double mutant (Chapter 2). First, we showed that the Pcna<sup>K164R</sup> single mutant cell lines are sensitive to replication blocking lesions, which is consistent with previous findings in other model systems [19-22]. Likewise, Polh<sup>−/−</sup> single mutant cells are sensitive to UV- and cisplatin-induced lesions, as previously reported in
yeast and XPV cells [33-37]. These results corroborate the importance of DDT for cell survival after treatment with DNA damaging agents. Moreover, Polh-/- single mutant cells do not display sensitivity to MMS. In yeast, the role of Rad30 (Polη) is somewhat controversial as some groups report enhanced sensitivity to MMS in rad30Δ cells [34,38-40], while others do not [41-43]. This discrepancy might, at least in part, be explained by subtle genetic differences between the different yeast strains used in these studies. Xeroderma pigmentosum (XPV) cells, however, do not display sensitivity to methylating agents, consistent with our data [44]. Importantly, the 3-methyl adenine glycosylase Mag1 provides the primary defense against the replication blocking 3-methyl adenine lesion induced by methylating agents. Cells of S. cerevisiae lacking this protein are sensitive to MMS, but crossing the mag1Δ cells to a TLS-impaired background (i.e. mag1Δ rad30Δ rev3Δ triple mutant), further sensitizes these cells (with the respective double mutants being less sensitive than the mag1Δ rad30Δ rev3Δ triple mutant), suggesting that TLS plays a role in the bypass of persistent 3-methyl adenine [41]. The sensitivity of mag1Δ rad30Δ rev3Δ cells can be rescued to various degrees by introducing different human Y-family TLS polymerases. Introduction of either human Polκ or ι only moderately rescued sensitivity. Remarkably, only introduction of human Polκ rescued sensitivity to levels observed with reintroduced Rad30 (the S. cerevisiae genome does not contain a Polk gene) [41]. This suggests that mammalian TLS polymerases have evolved divergently from yeast TLS polymerases, as only Polκ can fully complement Rad30, but not its ortholog Polη.

When treated with chemical agents that induce replication blocking lesions, PcnaK164R;Polh-/- double mutant cells are more sensitive than the PcnaK164R cells, indicating that in mammals Polη can be activated independently of PCNA-Ub. Several other lines of evidence confirm the existence of a PCNA-Ub-independent activation of Polη in mammals. While replication block recovery was impaired in PcnaK164R cells, the alkaline DNA unwinding (ADU) assay alone was insufficient to distinguish between a defect in early or late DNA damage bypass (i.e. direct DNA damage bypass vs. post-replicative gap filling) [45,46]. To distinguish between these possibilities, we employed DNA fiber labeling experiments to investigate whether PCNA-Ub-dependent TLS occurs directly. As no defect in replication fork progression was detected in PcnaK164R cells, these data suggest that in mammals PCNA-Ub-dependent TLS occurs post-replicative.

In Chapter 3 we investigated whether apart from Polη, this principle of PCNA-Ub-independent activation also applied to other TLS polymerases. To this end we generated PcnaK164R;Polk-/- double mutant cell lines, to investigate if Polκ can be activated in the absence of PCNA-Ub. We observed that after MMS treatment PcnaK164R;Polk-/- double mutant cells displayed, compared to the respective single mutants: 1) decreased cell survival; 2) delayed S phase progression; and 3) increased replication stress. Apparently, after MMS treatment Polκ can also be activated independently of PCNA-Ub. Interestingly, while PcnaK164R and Polk-/- mutant cells were sensitive to N²-benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE), the PcnaK164R;Polk-/- double mutant cells were not more sensitive than the PcnaK164R mutants, indicating that damage bypass of BPDE-induced lesions by Polκ completely depends on PCNA-Ub. Additionally, DNA fiber analysis and ADU experiments revealed that PCNA modification and Polκ-dependent TLS past MMS-induced lesions probably occurs
post-replicative, a mechanism similar to Polη-dependent TLS after UV irradiation. Our data from Chapters 2 and 3 clearly demonstrate specificity among mammalian TLS polymerases for certain lesions, as well as different, PCNA-Ub-dependent and -independent, activation modes. It is evident that the increased collection of mammalian TLS polymerases has evolved distinct functions regarding lesion-specific DDT compared to their orthologues in lower eukaryotes such as yeast. However, how the polymerase selection at the site of the lesion is achieved remains elusive. One suggestion for polymerase selection is the lesion itself [47]. Future experiments should address this issue.

**Significance of mammalian PCNA polyubiquitination**

Besides monoubiquitination, PCNA can also be polyubiquitinated, thereby activating the error-free DDT pathway, TS [48]. In yeast K63-linked polyubiquitin chains on PCNA<sup>K164</sup> are generated by the E2 heterodimer Mms2/Ubc13 and the E3 Rad5 [19]. The mammalian genome contains two known Rad5 homologues, helicase-like transcription factor (HLTF) and SNF2 histone linker PHD RING helicase (SHPRH), both of which were shown to stimulate the formation of polyubiquitinated PCNA [49-52].

Current models of TLS regulation are based on genetic data from the *S. cerevisiae* model organism were monoubiquitination controls TLS. However, in another yeast model system, *S. pombe*, polyubiquitinated PCNA was shown to stimulate TLS as well [53]. In Chapter 4 we postulated that polyubiquitination of PCNA regulates TLS by potentially two mechanisms: 1) TLS might be suppressed by the formation of polyubiquitin chains on PCNA, as no monoubiquitination is present in this situation; or 2) PCNA polyubiquitination can stimulate TLS as was recently shown in *S. pombe* [53]. To investigate the role of PCNA polyubiquitination in regulating TLS in a genetically defined mammalian setting, we generated and analyzed cells lacking both functional HLTF and SHPRH. We showed that these cells do not display sensitivity to replication blocking lesions compared to their WT counterparts. Additionally, no difference was detected in the SHM spectra. Surprisingly, *Hltf* and *Shprh* double mutant cells were still able to generate PCNA polyubiquitination, albeit at lower levels. Collectively, our data suggest the existence of one or more additional E3s responsible for the generation of polyubiquitinated PCNA in mammals.

**Regulation of TLS by 9-1-1**

The PCNA-related DNA clamp RAD9-RAD1-HUS1 (9-1-1) has important functions in the efficient activation of the mammalian ATR/CHK1 DNA damage checkpoint, and is, similar to PCNA, essential for life [54]. Furthermore, several studies have implicated 9-1-1 in regulating yeast TLS [55-58]. Like PCNA, 9-1-1 in yeast can be monoubiquitinated site-specifically on the non-conserved K197 of the Rad17 (RAD1 in mammals) subunit by the E2/E3 heterodimer Rad6/Rad18 in a DNA damage-inducible manner [59]. Structural data of 9-1-1 revealed a conserved lysine residue to be a putative modification site of mouse RAD1 (K185) [60,61]. RAD1<sup>K185</sup> resides in a loop that is topologically equivalent to K164 of PCNA. Based on the facts that: 1) RAD1<sup>K185</sup> is a topological equivalent of PCNA<sup>K164</sup>; 2) PCNA-Ub by yeast Rad6/Rad18 exclusively takes place at PCNA<sup>K164</sup>; and 3) yeast Rad6/Rad18 ubiquitinate both PCNA and 9-1-1, we decided to mutate
RAD1<sup>K185</sup> in a mouse model system (Rad1<sup>K185R</sup>) to investigate the role of 9-1-1 modification in mammals (Chapter 5). As 9-1-1 ubiquitination at Rad17<sup>K197</sup> in yeast controls the phosphorylation of the Rad53 checkpoint protein after DNA damage, we wondered whether putative modifications on RAD1<sup>K185</sup> could also control checkpoint activation. Therefore, we analyzed the phosphorylation of CHK1 after UV irradiation, a 9-1-1-dependent event that is a requirement for efficient checkpoint activation. Our data showed that Rad1<sup>K185R</sup> cells display WT levels of CHK1 phosphorylation after UV irradiation, suggesting that putative modification of RAD1<sup>K185</sup> does not control checkpoint activation. Furthermore, Rad1<sup>K185R</sup> cells are as sensitive as WT cells to a wide variety of DNA damaging agents and have no defects in class switch recombination or SHM. Taken together, we conclude that RAD1<sup>K185</sup> is a topological equivalent of PCNA<sup>K164</sup>, but not a functional one. Our results, together with an independent study in yeast [62], indicate that the original research describing the role of DNA damage-inducible 9-1-1 ubiquitination might be flawed [59].

In addition to the amino acid substitution in exon 4, which contains RAD1<sup>K185</sup>, we also flanked this exon with LoxP sites to enable conditional inactivation of the Rad1 allele. This approach will allow us to perform detailed structure-function analyses of RAD1, as well as complete inactivation of Rad1 in various tissues in a defined mammalian system in future studies.

**Clinical relevance of TLS**

The DNA damage response (DDR) is a complicated, molecular network that is able to react to a multitude of DNA lesions. Defects in this network can lead to genomic instability, one of the hallmarks of cancer [63]. On the other hand, these defects can also be exploited to fight cancer [64–66]. Therapeutic targeting of the DDR, or defects therein, holds great promise in cancer treatment, as demonstrated by the specific killing of BRCA mutated tumors by treatment with poly(ADP-ribose) polymerase inhibitors [67,68]. TLS incompetent cells are to varying degrees sensitive to replication blocking lesions, suggesting that TLS inhibition might be a good strategy in chemosensitization. TLS inhibition can potentially be achieved in several ways: by inhibiting the dominant TLS polymerase (dependent of the lesion introduced by therapy), the generation of PCNA-Ub, or even both. Inhibition of both a TLS polymerase and the generation PCNA-Ub would have the greatest effect on chemosensitization, as our work has shown that cells that lack both are most sensitive to certain lesions (Chapters 2 and 3). So far, inhibitors for Y-family TLS polymerases in general or the TLS polymerase κ specifically, the interaction between PCNA and PCNA-interacting peptide (PIP)-box containing proteins, as well as RAD6 have been discovered [69–73]. RAD6, however, might be less favorable as a therapeutic target, since it also functions outside DDT [74].

XP-V is to date the only known human disease with a dysfunctional TLS polymerase. Nevertheless, altered expression of other TLS polymerases has been implicated in certain cancers, although no clear pattern of expression has emerged [75]. TLS overexpression has been observed [76–83], while downregulation has been observed as well [84,85]. For example, Polκ overexpression is observed in lung cancer and is associated with poorer prognosis of glioma patients, while downregulation of Polκ is observed in colorectal tumor biopsies [76,77,83,84]. It also remains to be investigated whether deregulation of TLS polymerases is
a cause or consequence of tumorigenesis or tumor progression. Regarding genome stability, TLS acts as a double-edged sword. Depending on the TLS polymerase and the lesion, TLS can be pro- or anti-mutagenic. Hence, the regulation of TLS is a matter of utmost importance to maintain genomic stability. One can imagine that overexpression of a particular TLS polymerase can increase the mutation rate of a cell, consequently increasing the chance of activating oncogenes or deactivating tumor suppressor genes. Alternatively, deactivation of TLS might also increase the mutation rate as potentially error-free bypass of particular lesions is disrupted and a redundant TLS polymerase(s) might bypass the lesion in an error-prone manner. This phenomenon is observed in the hypermutable XP-V cells, where in the absence of Polη, Polκ, ι and ζ are responsible for mutagenic TLS across cyclobutane thymine dimers (CPDs) [86]. Both scenarios would result in a higher mutation load, and thus could potentially drive tumorigenesis and tumor progression.

Cisplatin and its derivatives are successfully used in the clinic as anti-cancer drugs for almost 40 years [87]. However, many tumors become resistant to cisplatin treatment by a variety of mechanisms [88]. A growing body of evidence suggests that TLS polymerases, in particular Polζ, might also play a role in cisplatin resistance [89-91]. Cisplatin treatment can induce REV3L expression, the catalytic subunit of Polζ [91]. Interestingly, suppression of REV3L sensitizes otherwise drug-resistant glioma or lung tumor cells to cisplatin in vitro [89,90]. Of note, glioma cells have higher levels of REV3L compared to normal brain tissue, where the expression level of REV3L correlates with the tumor grade [89]. Transplantation experiments of REV3L-deficient lung tumors have shown that these tumors display an enhanced response to cisplatin compared to REV3L-proficient tumors [90]. These results provide the proof of principle that TLS inhibition might overcome cisplatin resistance observed in certain tumor types. Furthermore, one added benefit of Polζ suppression is reduced cisplatin-related mutagenesis that is observed in vitro, which might reduce the chance of secondary tumor formation and tumor progression due to cisplatin treatment [89-91].

**Concluding remarks**

In conclusion, we have shown that PCNA modification plays an important role in the efficient regulation of mammalian TLS, but, as opposed to lower eukaryotes such as yeast, TLS polymerases can also be activated without PCNA-Ub. Future experiments should investigate the molecular nature of PCNA-Ub-independent TLS activation. Furthermore, DDT by TLS is clinically relevant, as shown by deregulation in certain cancers and a suspected role in chemoresistance. Moreover, inhibition of DDT provides an attractive therapeutic drug target for chemosensitization.
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SUMMARIZING DISCUSSION


