Regulation of DNA damage tolerance: Studies of the translesion synthesis DNA polymerase eta in *Saccharomyces cerevisiae*

by

Rachel Van Etten Woodruff

B.A. Molecular Biophysics and Biochemistry
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Signature of Author: _____
Department of Biology
October 31, 2007

Certified by: _____
Graham C. Walker
American Cancer Society Professor of Biology
Thesis Supervisor

Accepted by: _____
Stephen Bell
Professor of Biology
Chairman, Committee for Graduate Students

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ABSTRACT

All organisms must control the effects of DNA damage to protect the integrity of their genomes. In addition to DNA repair, this requires DNA damage tolerance pathways, which allow the continuation of essential processes in the presence of DNA damage. One such mechanism is translesion synthesis (TLS), in which a specialized polymerase replicates a damaged DNA template. These studies address the regulation of the eukaryotic TLS polymerase eta, which is particularly important for accurately bypassing UV-induced DNA damage.

Here I identify a regulatory domain of *Saccharomyces cerevisiae* polymerase eta and show that it is essential for pol eta's function. This domain interacts with ubiquitin, and has been named the UBZ (ubiquitin binding zinc finger) domain because its human homolog is a zinc finger. However, results presented here demonstrate that, in *S. cerevisiae*, neither the sequence nor the functional significance of the zinc finger within the UBZ domain is conserved. I present the first analysis of DNA-association of *S. cerevisiae* pol eta, demonstrating a UBZ-dependent increase in its chromatin-association in response to DNA damage. These results are consistent with the UBZ domain of *S. cerevisiae* pol eta forming a zincless finger, similar in structure and function to its human counterpart, and mediating a physical interaction with ubiquitinated PCNA to promote pol eta's association with damaged DNA.

Another eukaryotic TLS polymerase is Rev1, which is thought to play a structural role in TLS by binding the other TLS polymerases. In mammals, Rev1 and pol eta robustly interact. Here, I show that any physical interaction between Rev1 and pol eta in *S. cerevisiae* is too weak or conditional to observe by coimmunoprecipitation.

Finally, I describe a genetic study to elucidate the functional interactions of pol eta with Rev1 and another TLS polymerase, pol zeta. The results presented here are consistent with TLS during two distinct periods during the cell cycle, with pol eta contributing most to S-phase TLS. Pol eta's complex genetic relationships with the other polymerases show that pol eta generally acts independently, but may cooperate with the other polymerases in a damage-induced pathway. Alternative explanations of the data are also discussed.

Thesis Supervisor: Graham C. Walker
Title: American Cancer Society Professor of Biology

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Chapter 1: Introduction

The Role and Regulation of Polymerase eta in DNA Damage Tolerance

1. Overview

All organisms are equipped with an array of well-regulated DNA damage response mechanisms which allow them to survive and to maintain the continuity of genetic information. DNA damage is caused by a variety of agents, many of which are produced as byproducts of normal cellular metabolism. For example, reactive oxygen species, produced primarily by mitochondrial respiration, react with the DNA to cause adducts, strand breaks and base losses at a rate of several thousand per cell per day [1]. In addition, DNA is prone to spontaneous alterations such as demethylation, deamination, and even base loss (depyrimidation or depurination), any of which disrupts the structure of the DNA. As well as these endogenous sources, DNA is frequently subjected to damage by environmental agents such as ionizing radiation, reactive chemicals, and ultraviolet radiation (UV). These agents alter the structure of the DNA by chemical crosslinking, cleavage of the DNA strands, alkylation, or by other chemical alterations to the DNA bases. The net effect of spontaneous and exogenous DNA damage is that a human cell suffers over ten thousand DNA lesions per day [2], and therefore depends heavily on DNA damage responses to protect the genetic information. Their importance is highlighted by the many genetic diseases caused by deficiencies in the DNA damage response pathways, which are typically characterized by the early onset of cancer, premature aging, or both [3].

1a. DNA repair and DNA damage tolerance pathways. The DNA damage response mechanisms are placed into one of two broad categories: DNA repair and DNA damage tolerance. Mechanisms of the first class actually repair the lesions, by excision of the damaged base followed by re-synthesis, or direct reversal of damage, returning the DNA to a normal structure. DNA damage tolerance mechanisms, in contrast, only decrease the lethality of the lesions by allowing continuation of cellular processes, such as DNA replication and transcription, which otherwise would be disrupted by the presence of DNA damage. In this way, damage tolerance mechanisms essentially give the cell more time to repair DNA lesions. This study will focus on a damage tolerance

mechanism known as translesion synthesis (TLS), but begins with a brief introduction to DNA repair.

There are multiple DNA repair pathways, each of which promotes repair of a different subset of DNA lesions. though many specific lesions can be repaired by more than one mechanism. Base modifications can be repaired either by direct reversal, in which the damaged base is returned to its original structure, as in photoreactivation [4], or by excision repair, in which the damaged base is removed from the DNA and replaced by re-synthesis. The three major types of excision repair - mismatch repair (MMR), base excision repair (BER) and nucleotide excision repair (NER) - all involve recognition of damaged DNA by a specific damage recognition factor, followed by excision of the lesion (and some surrounding DNA, dependent on the pathway), to produce a gap which is refilled with newly-synthesized, undamaged DNA [3]. BER primarily repairs alkylated DNA and depurinations [3-5], while MMR repairs mismatches produced by DNA replication [3, 6]. NER repairs a great variety of large bulky or distorting lesions, including the (6-4) pyrimidine dimers produced by UV radiation [3, 7]. Defects in NER are the causes of several human diseases, including Xeroderma Pigmentosum (XP) [3], which will be discussed further in Section 3a-2. DNA breaks are repaired by another set of pathways, which include non-homologous end-joining (NHEJ), homologous recombination (HR) and single strand annealing (SSR).

DNA damage tolerance. Even with all these DNA repair pathways intact, DNA lesions are not repaired instantly, and are constantly generated, making DNA damage tolerance mechanisms important for cell survival and propagation. There are several DNA damage tolerance processes which allow DNA replication and transcription to continue in the presence of DNA lesions. Recombinational repair and replication fork regression (see Figure 1), use an undamaged homologous strand to complete DNA replication (reviewed in [3]). In contrast, translesion synthesis (TLS), which occurs in all domains of life, involves the direct replication, by a specialized DNA polymerase, of the damaged template DNA. The TLS polymerase inserts a base directly opposite an otherwise replication-blocking base modification.

TLS has inherent mutagenic potential because it uses a structurally altered DNA template. TLS therefore makes a significant contribution to damage-induced

mutagenesis (See Section 3c) [8], generally producing base substitutions or frameshifts. In addition to the potential for mutagenesis during bypass of damaged DNA, TLS polymerases are 100-1000 times less accurate than replicative polymerases when replicating undamaged DNA *in vitro* [9-11]. This loss of fidelity is offset by the gain in reliably quick replication, which helps to reduce genomic rearrangement and cell death. In spite of its mutagenic potential, TLS can also be an anti-mutagenic process. The presence of at least five distinct TLS polymerases in eukaryotes (see Section 2a), allows each polymerase to be specialized to bypass particular (cognate) DNA lesions with relatively high efficiency and often good accuracy, thus reducing the cumulative mutagenic effect of TLS.

This discussion will focus on the functions and regulation of one widely conserved eukaryotic TLS polymerase, polymerase eta (pol η) , whose cognate lesion is the cyclopyrimidine dimer (CPD), a major product of UV radiation of DNA [12, 13]. Pol η functions as part of the post-replicative repair pathway [14], which is discussed below.

Post-replicative repair: a DNA damage tolerance pathway. The historic observation of post-replicative repair (PRR) in yeast demonstrates the effect of DNA damage tolerance on replication. Even before molecular mechanisms of DNA damage tolerance were known, researchers measured the size distribution of nuclear DNA in alkaline sucrose density gradients after UV-induced DNA damage and pulse-labeling of the DNA. They observed that the nascent DNA in undamaged cells was longer than then nascent DNA from cells recently subjected to DNA damage. The nascent DNA from damaged cells grew longer the more recovery time was allowed after DNA damage, until it reached the full size observed in undamaged cells [15]. This process of recovery of full-length DNA was named post-replicative repair (PRR) [3], because it repairs the gaps or breaks created in the course of replication of damaged DNA, although unlike most "repair" pathways, does not repair the original DNA lesions.

In the budding yeast *Saccharomyces cerevisiae,* the PRR pathway is synonymous with the *RAD6* epistasis group (see also Sections 2c and 4c-1), which regulates several damage tolerance mechanisms. The *RAD6*-dependent damage tolerance pathway is initiated by the Rad6 and Rad18 proteins, which form a heterodimer [16]. The PRR subpathways include translesion synthesis as well as at least two additional "error-free"

sub-pathways which promote non-mutagenic DNA damage tolerance by poorly understood mechanisms [17] [18]. The PRR pathways which make up the *RAD6* epistasis group are not essential in yeast because other DNA damage responses, including Rad52-mediated homologous recombination, can substitute for the Rad6-dependent processes in their absence to prevent replication collapse. However, Rad18 becomes essential in yeast if other pathways, or their regulation, are disabled [19].

In yeast, persistent DNA damage leads to activation of the Mec1 (homolog of mammalian ATR) and Tel1 kinases, which play a central role in DNA damage or S phase checkpoint signal transduction [20]. Mec1 accumulates at stalled replication forks, in response to the accumulation of ssDNA, initiating a kinase cascade by controlling a web of secondary kinases including Rad53, Dun1, and Chk1 [21, 22]. These kinases in turn regulate various responses to DNA damage, including cell cycle arrest and the damage-induced transcription of specific target genes. In *Xenopus laevis,* the timing of activation of the ATR-dependent DNA damage checkpoint coincides with the activation of the PRR pathway [23]. Although the function of Rad6 and Rad18 does not depend upon the damage checkpoint response, this is consistent with the possibility of interactions between PRR and DNA damage checkpoint. Indeed, there is evidence of interaction between PRR and Mec1, as well as Mec1-target proteins including Rad9 and Ddc1 (discussed further in Section 4c-2) [24-26].

1b. Translesion synthesis in context: the replication of damaged DNA. DNA replication is carried out primarily by the replicative polymerases, pol delta and pol epsilon in eukaryotic nuclei, which are optimized for efficient, high fidelity replication of undamaged DNA, not for bypassing DNA damage. The replicative polymerases copy undamaged DNA quickly, efficiently, and with high fidelity, introducing a mismatch with a frequency of only 10^{-6} to 10^{-8} per nucleotide [10, 11]. The high fidelity of DNA replication by such enzymes is due both to highly stringent structural requirements for catalysis and to an intrinsic proofreading exonuclease domain [27]. These same features of the replicative polymerases can become liabilities when the replisome encounters a DNA lesion. Many DNA lesions distort the structure of the DNA such that it cannot be accommodated in the active site of the replicative polymerases. Even if the polymerase can insert a base opposite to a lesion, the improper structure can be recognized by the

polymerase's exonuclease domain, leading potentially to futile cycles of excision and extension [28]. In either case, the polymerase is stalled by a lesion in the template DNA.

It has recently been shown that damaged DNA can be replicated in a discontinuous fashion, even on the leading strand, where the nascent DNA is canonically thought of as one continuous strand. On both the leading and the lagging strand, repriming can occur downstream of an unrepaired blocking lesion, leaving behind a single strand gap in the DNA [29]. When the replicative polymerase on the leading strand encounters a blocking lesion, the leading strand polymerase and lagging strand polymerase temporarily uncouple, producing a region of single stranded DNA on the leading strand up to 3 kb long [29]. DNA damage tolerance mechanisms allow the completion of replication.

There are two distinct models of how a TLS polymerase gains access to a specific stretch of DNA: polymerase switching and gap filling. These two models were once seen as mutually exclusive, but now it is apparent that both are relevant. In the polymerase switching model, the TLS polymerase is recruited directly to the stalled replication fork just upstream of the lesion, where it replaces the replicative polymerase, incorporates a few nucleotides opposite and just past the lesion, and then is replaced by the replicative polymerase [30]. Polymerase-switching results in replication of continuous (not gapped) DNA, and thus implicitly occurs in S phase. In the gap-filling model, the TLS polymerase is recruited to the lesion-containing single strand gaps left behind in the nascent DNA by the replicative polymerase re-priming downstream of lesions as discussed above. Lesion-containing gaps can also be produced during NER, if two lesions are close together on opposite strands. Gap-filling TLS is thought to occur predominantly after S phase, during G2 [29]. Although both gap-filling and polymerase switching are now recognized as being relevant to TLS in general, specific TLS polymerases may contribute differentially to these two modes of damage tolerance. For example, in yeast, the TLS polymerase Rev1 (introduced in Section 2a below), which is most abundant during G2/M [31], is thought to act primarily through gap-filling, while pol η is thought to be act primarily during S phase [32]. It is unclear whether these two modes of TLS are regulated by the same or different pathways (this will be discussed further in Chapters 4 and 5).

2. TLS polymerases

2a. Kinds of TLS polymerases. The TLS polymerases are distinguished from the replicative polymerases by their ability to insert nucleotides opposite a lesion on the template. Based on sequence homology, the known DNA polymerases can be divided into five groups, or families, of which the most recently recognized is the Y family of DNA polymerases [33]. The majority of TLS polymerases are members of the recently discovered Y family, all of whose known members are involved in TLS [33]. Members of this family are found in all domains of life, and can be further categorized into five sub-families: UmuC/PolV, DinB/PolIV/Polymerase kappa (κ), Rev1, polymerase iota (ι), and pol η. Another TLS polymerase which will be discussed here, pol zeta (ζ), is a member of the B family of DNA polymerases [34]. It is common for an organism to have multiple TLS polymerases, including more than one member of the Y family. For example, *E. coli* have both Pol IV and Pol V, while humans have pol ζ and four Y family members: Rev1, and polymerases κ, ι, and η. The budding yeast *Saccharomyces cerevisiae*, the model organism used for this study, contains three TLS polymerases: polymerase ζ, Rev1, and pol η (also known as Rad30). The variety of TLS polymerases allows each to be specialized to bypass particular ("cognate") DNA lesions, and to play distinct roles in other processes, such as homologous recombination and somatic hypermutation (see Sections 3b and 3c) [35-39].

2b. Protein Structure and lesion-bypass mechanisms of the Y family TLS polymerases. The Y family polymerases, despite being unrelated to other polymerases by primary sequence, do have an overall structure similar to other DNA polymerases. The crystal structures of several Y family members including a truncated form of *S. cerevisiae* pol η have revealed that their structure resembles a right hand, including palm, thumb and finger domains [40-46]. Enzymatically, Y family polymerases function similarly to other known polymerases, in that catalysis of phosphodiester bond formation relies on a triad of magnesium-coordinating carboxylate groups. However, the active sites of Y family polymerases are characterized by other structural differences that enable them to accommodate unusual DNA structures.

The Y family polymerases make fewer contacts with the template DNA and have shorter fingers and thumb domains, resulting in looser, or more open, active sites [47] (Figure 2). They also notably lack intrinsic a 3' to 5' exonuclease proof-reading exonuclease activity. Some Y family polymerases, including pol η, compensate for the reduced DNA-interaction surface that results from this differences through an additional DNA-interaction domain, known as the little finger or palm-associated domain (PAD), which substantially strengthens the polymerase's interaction with DNA [41]. Additionally, Fingers domain of Y family polymerases such as pol η lacks the helices O and O1, which are critical for the high-fidelity mechanism used by replicative polymerases to enclose the active site and check the steric match between the incoming nucleotide and template base [48-50]. A more flexible interaction of the thumb domain with the DNA is thought to allow pol η to accommodate bulges in the template DNA and to reduce its processivity [51]. Finally, structural and mechanistic differences between the various TLS polymerases account for their distinct lesion bypass activities, the details of which are beyond the scope of this thesis (for a review on the subject, see [52].

Protein-protein interactions may activate catalysis by specific TLS polymerases, thereby influencing their functions. For example, there is evidence that factors such as RFC, Ctf18-RFC, PCNA and the Werner Syndrome protein WRN each can modulate the activity of TLS polymerases [53-55]. Some of these are discussed further in Chapter 5.

2c. Polymerase η. Polymerase (pol) η (eta), which is very broadly conserved among eukaryotes, was the first member of the Y family to be characterized as a TLS DNA polymerase [12]. It was found to be an effective replicator of damaged DNA templates, although it replicates undamaged DNA with the relatively low fidelity of 10^{-2} to 10^{-3} errors per base [12, 56]. The TLS activity will be explored in greater detail in section 3a-3.

Pol η was first studied in *S. cerevisiae*, in which it is one of three TLS polymerases. The story of their discovery gives some insights into their functional similarities and differences. *REV1*, *REV3* and *REV7* (the latter two of which encode the subunits of pol ζ) were identified in a screen for "reversionless" mutants, defective in UV-induced mutagenesis [57, 58]. *RAD30* was not found in any genetic screen. Instead,

RAD30 was discovered decades later when it was identified by its sequence similarity to *REV1* and to two bacterial genes, *dinB* (pol IV) and *umuC* (pol V) [14, 59], which were already known to contribute to DNA damage tolerance and mutagenesis [57, 60]. Knocking out the *RAD30* gene in *S. cerevisiae* resulted in hyper-sensitivity to killing by UV radiation, and it was therefore designated a RAD gene despite not having been identified in the screens that found most other RAD genes in *S, cerevisiae* [14]. This phenotype was consistent with a role in DNA damage tolerance or repair, and similar to the effects of both *rev1* and *umuC* mutants. However, functional differences were also immediately apparent: while the *REV* genes are required for the majority of damage-induced mutagenesis [61], loss of *RAD30* function has only subtle and complex effects on reversion frequencies (discussed further in Section 3c), which explains why it was not found in the screens that identified the *rev* mutants [59].

It was clear that *RAD30* is involved in DNA damage tolerance, rather than repair, when epistasis analysis revealed that, like *REV1, REV3* and *REV7, RAD30* belongs to the *RAD6/RAD18* epistasis group, defining the post-replicative repair (PRR) pathway (see sections 1b and 4c-1) [14]. There is a synergistic relationship, indicating partial redundancy, between *rad30* and the error-free pathway represented by *rad5* [14]. Epistasis analysis also revealed that *RAD30* can function independently of the error prone pathway involving *REV1, REV3* and *REV7* [14]; the complex functional relationships between pol η, Rev1 and pol ζ will be addressed further in Chapters 3 and 4. The importance of pol η's function was made clear by the discovery that a human disease, Xeroderma Pigmentosum Variant (XPV), results from loss of the human homolog (see Section 3a-2) [62, 63].

3. Physiological significance of pol η

3a-1. Pol η's role in the survival of DNA damage: CPDs/UV. Pol η's primary role in organisms from yeast to humans is thought to be in bypassing the lesions produced by ultraviolet radiation, a DNA damaging agent to which most organisms are frequently exposed in the form of sunlight. The major lesions produced by UV irradiation are covalent dimers of adjacent pyrimidines on the same DNA strand: the *cis-syn* cyclobutane pyrimidine dimer (CPD), and the somewhat less abundant (6-4)

photoproduct [3] (Figure 3). While some organisms also encode photolyase enzymes capable of direct reversal of CPDs, (6-4) photoproducts, or both, humans do not [3, 4]. The 6-4 photoproduct causes greater distortion of the DNA helix than the CPD, but is a substrate for efficient nucleotide excision repair (NER) [64]. The CPD, in contrast, is repaired so inefficiently that nearly 50% of CPDs remain unrepaired even 24 hours after UV irradiation [65]. As a result of their persistence, CPDs cause most of the UV-associated mutagenesis and cell death [66]. DNA damage tolerance is therefore particularly important for survival of CPDs, and is also responsible for the avoidance or creation of mutations at the sites of CPDs.

Although the CPD lesion does not severely distort the B-DNA helix structure, it does present major problems for bypass by most DNA polymerases. Because the adjacent bases of the CPD are fixed parallel to each other, they are resistant to the common polymerization mechanism of placing one templating base in the polymerase active site for each step of primer extension, while flipping out the base immediately 5' to the templating base [67]. Furthermore, although the 3' thymine of a T-T CPD is capable Watson-Crick base-pairing, the orientation of the 5' residue impairs its hydrogen bonding, impeding accurate replication [68]. However, pol η can accommodate and bypass CPDs [45], and does so both efficiently and accurately [12]. Mechanistic and structure studies suggest that pol η performs TLS by employing an induced-fit mechanism and uses the energy of hydrogen bonding, base-pairing, and base stacking in selection of a nucleotide to achieve this translesion synthesis [45, 69-72].

Pol η is the primary TLS polymerase responsible for error-free CPD bypass in many organisms, including yeast and human [66, 73, 74]. Without the CPD bypass activity of pol η, UV radiation results in the production of double strand DNA breaks when unrepaired lesions are encountered during DNA replication, which can ultimately cause cell death or genomic rearrangement [3, 75, 76]. Furthermore, in the absence of pol η, CPD bypass which does occur is significantly more mutagenic, resulting in the high frequency of UV-induced mutagenesis and skin cancer among XPV patients (see section 3a-3). More error-prone bypass of CPDs may be performed by other TLS polymerases such as pol ζ and pol ι [77, 78].

Pol η has only a limited, and relatively mutagenic, ability to bypass the other major UV photoproducts, (6-4) photoproducts or Dewar photoproducts [79, 80] and is less involved in bypassing them *in vivo* [74, 81]. Instead, these lesions are generally bypassed by polymerase ζ, acting together with Rev1 and polymerase delta [74]. To the extent that pol η does bypass (6-4) photoproducts, it can contribute to UV-induced mutagenesis (see Section 3c) [82].

3a-2. Cancer prevention: Xeroderma pigmentosum variant (XPV).

Xeroderma Pigmentosum is a rare autosomal recessive disease characterized by dramatically increased incidence of skin cancer and sensitivity to ultraviolet radiation (UV) [83]. XP is caused by mutations in any one of seven different genes, six of which are essential to the nucleotide excision repair (NER) pathway [84]. The seventh, which accounts for about 20% of XP patients, encodes pol η [62, 63], and is named *XPV* (for XP-Variant) because, unlike other XP cells, XPV cells have no defect in nucleotide excision repair [85]. Instead, cytological studies show that they are defective in post replication repair (PRR, also discussed in Section 1b) of UV-induced DNA damage, suggesting a defect in DNA damage tolerance [86]. Most of the mutations causing XPV are truncations of pol η which certainly result in severe loss of function, but there are also a few point mutations [87, 88].

TLS is not abolished in XPV cells, but without pol η, TLS past the major UV-induced DNA lesion is both less efficient and more error-prone (see section 3a-1), demonstrating that the fidelity of TLS depends on the pairing of the specific lesion and polymerase (see also Section 3c) [79]. For reasons discussed in Section 3a-1 above, XP-V cells are both more sensitive to UV and also more mutable by UV than regular human cell lines [89-95]. The lack of pol η results in a 25-fold increase in UV-induced mutation frequency in XPV cells [78], which contributes to the high cancer rates in XPV patients, since mutagenesis is frequently an early step in carcinogenesis. The accumulation of DNA strand breaks, which in turn produce increased genomic rearrangements [3], may also contribute to carcinogenesis. Pol η's dramatic role in mutation avoidance is particularly striking in contrast to the pro-mutagenic activities of Rev1, which contributes substantially to mutagenesis [61].

3a-3. Pol η's roles in the bypass of other DNA lesions. Pol η also contributes significantly to the survival of many other DNA lesions. *In vitro*, pol η is able to bypass a broad range of DNA lesions that are neither extraordinarily bulky nor dramatically distorting to the overall DNA structure [96-102], including byproducts of oxidation and alkylation damage, as well as base loss. Oxidative DNA damage, produced by the free radicals and reactive oxygen species that are continuously produced by the metabolic processes of living cells, as well as by ionizing radiation or UV [3], contributes to aging, carcinogenesis, and neurodegeneration. One common, and highly mutagenic, type of oxidative damage is 7,8-dihydro-8-oxoguanine (8-oxoG). This lesion is repaired chiefly by Ogg1, an 8-oxoG DNA glycosylase, to a lesser extent by NER [103]. The synergistic genetic relationship between *rad30* (*RAD30* encodes pol η in yeast) and *ogg1* for survival of oxidative damage in yeast demonstrates that pol η is also important to survival of 8-oxoG [98]. Like the CPD, 8-oxoG can be bypassed both efficiently and accurately by pol η [80, 98, 104, 105]. Pol η also bypasses other oxidative lesions, including thymine glycol [96]. Pol η is also able to successfully bypass alkylation lesions. For example, pol η is involved in bypass of damage caused by cisplatin and other platinum-based chemotherapeutic agents, which produce bulky alkyl adducts and crosslinks in the DNA [99, 106]. Because many alkylating agents are used in chemotherapy, this function of pol η also has clinical significance. Indeed, it has been suggested that pol η may reduce cisplatin's mutagenic effect in human cells [107]. Pol η also plays more minor roles in survival of other lesions such as abasic (AP) sites [96, 100, 108], and may cooperate with other polymerases to bypass other lesions, such as the bulky adducts produced by benzo[a]pyrene [109].

3b. Homologous recombination. Mammalian pol η promotes restart of stalled replication forks not only by TLS, but also through a role in homologous recombination. Other TLS polymerases have also been shown to be involved in recombinational repair [36, 110, 111]. [112], suggesting that this may be one of their general functional features. Recombination can rebuild a collapsed replication fork, but during this process an intermediate DNA structure, the D-loop, is formed. The D-loop structure is a poor substrate for the replicative polymerase, pol delta, and may instead be replicated by pol η. Pol η has recently been shown to efficiently interact with and replicate the D-loop

intermediate during homologous recombination *in vitro* [36]. Consistent with the *in vitro* findings, pol η is required *in vivo* for efficient Ig gene conversion and in double strand break (DSB)-induced homologous recombination in DT40 cells [111].

The price of using the error-prone TLS polymerases to fill gaps during homologous recombination could be a higher local mutation frequency at sites of recombination. Consistent with this prediction, DSB-induced recombination in mitotic cells causes a 100 to 1000 fold increase in mutagenesis in genes adjacent to DSBs [113]. This increased mutation frequency may be evolutionarily tolerated as a small cost relative to the loss of genomic stability which could result from replication fork collapse during recombination. Additionally, the use of TLS polymerases to replicate recombination intermediates could function to induced mutagenesis at specific, targeted sites.

3c. Mutagenesis. While XPV makes it clear that pol η has a beneficial role in preventing UV damage-induced mutagenesis in humans, these findings belie a much more complex role in mutagenesis. *RAD30,* encoding yeast pol η, has seemingly contradictory effects on reversion frequency, preventing mutations in some experiments, but promoting mutagenesis in others [14, 59]. This is probably due, at least in part, to differences in its fidelity when bypassing the many distinct lesions pol η encounters in the cell. As noted in the preceding discussion, (Section 3a-3) TLS by pol η is anti-mutagenic when employed to bypass certain DNA lesions (such as CPDs and 8-oxoG), but has a more mutagenic effect when bypassing others, such as (6-4) photoproducts. Additionally, pol η, like TLS polymerases in general, is highly error-prone when replicating undamaged DNA, producing errors at a frequency of 10^{-2} to 10^{-3} [12, 114]. [115]. Furthermore, human pol η can promote mutagenesis by incorporating oxidized nucleotides into the nascent DNA [116], and pol η has been shown to generate and extend from tandem base substitutions at an unusually high rate [117].

Additional data support a complex role for pol η in mutagenesis *in vivo*. Depletion of pol η by siRNA in human cells causes an increase in mutation frequency [118, 119], consistent with a role for pol η in avoidance of mutagenesis. Conflicting reports on whether pol η depletion affects the mutation spectrum, or only the frequency [119, 120], suggest that in limited circumstances, pol η probably does produce some mutations itself. Clearer evidence of the mutagenic potential of pol η is seen in yeast,

where over-expression of pol η can cause increased damage-induced mutagenesis [121, 122], although this effect has not been observed in human cells [115].

Mutagenesis in eukaryotes is not necessarily detrimental. A case in point is somatic hypermutation of immunoglobulin variable regions during antibody maturation, to which pol η contributes. After V(D)J recombination, somatic hypermutation (SHM) is a further step in many organisms, including humans, for fine-tuning Ig gene diversification in B lymphocytes in order to produce sufficiently high affinity antibodies during an infection. Transcription-coupled cytidine deamination within Ig genes creates dU lesions, which then act as targets for mutagenesis by error-prone DNA synthesis [39]. Pol η is the primary mutator of A:T base pairs during SHM [123-125]. Although XPV patients have altered SHM mutation spectra, they are normal with respect to SHM mutation frequency and immune response [126, 127], demonstrating that at least one other polymerase, with distinct mutagenic properties, can substitute for pol η in SHM. This is consistent with the observation that several other TLS polymerases also contribute to SHM [128-134].

Given the complex effects of mutagenesis on the survival of the cell or organism, it is not surprising that the mutagenic potential of pol η (and TLS in general) must be carefully regulated to prevent the excessive mutation frequencies that could result from its overuse or misuse. As pol η has the potential either to promote or to prevent mutagenesis, its global effect on mutagenesis is likely to be very dependent upon which specific DNA templates it is allowed access to: damaged or undamaged, and if damaged, with what specific lesion(s).

4. Regulation of polymerase η

Pol ηis a physiologically important enzyme, but one which presents a potential hazard if it is misused. The simple observation that pol η does not normally cause an increase in mutations, despite being an error-prone replicator of undamaged DNA, suggests the pol η does not usually replicate undamaged DNA. This implies that the polymerase is regulated in such a way as to allow it to be active only under specific conditions e.g., at sites of DNA damage. In this thesis project, I have undertaken to improve the understanding of the mechanisms by which pol η's activity is regulated,

especially in *S. cerevisiae*. What follows is a summary of the current literature regarding pol η regulation.

In general, proteins can be regulated at many levels. The most basic of these is the protein's abundance, which will be addressed in Section 4a. Protein abundance can be affected by regulation at the level of transcription, translation, or proteolysis. Proteins can also be regulated by post-translational modifications, which will be discussed in Section 4b. Post-translational modifications can be used to regulate a protein's physical interactions or their likelihood of proteolytic digest. A protein's physical interactions define another layer of regulation, the subject of Section 4c, resulting in the protein's sequestration into or away from protein complexes, or directly influencing its activity. Given the complex roles that pol η plays in the cell, it is not surprising that many of these mechanisms are implicated in its regulation.

4a. Regulation of protein abundance. Some regulation of pol η's activity at the level of protein abundance in yeast is suggested by the finding that overexpression of pol η in a repair-deficient strain can result in a mutator phenotype [121]. However, this observation does not address whether the abundance of pol η is changed, in response to conditions such as cell cycle or replication stress, or simply maintained at a constant level.

There have been very few studies on the protein abundance itself of pol η, but transcriptional regulation has been observed. In yeast, *RAD30* transcript (encoding pol η) is induced 3-4 fold in response to UV radiation [14, 59]. In mouse, however, expression of the XPV gene (encoding pol η) is not induced by UV radiation; instead, it has been found to increase about 4-fold during cell proliferation [135]. It is not yet clear how these species-specific changes in pol η transcript affect its abundance or function, as will be discussed further in Chapter 4.

A significant change in protein abundance has been observed in yeast for the other Y family member , Rev1. Rev1's abundance is responsive to the cell cycle: it is least abundant in G1, then gradually increases during S phase to become about 50-fold more abundant during G2/M [31], consistent with a model in which Rev1 is most active for post-replicative gap-filling. I found that, unlike Rev1, the abundance of pol η does

not change significantly in response to the cell cycle in yeast (my unpublished results and [31].

4b. Pol η and post-translational modifications. Pol η has been shown to be modified by covalent attachment of a mono-ubiquitin moiety [136, 137], although the functional significance of this modification is not yet understood. Ubiquitination of pol η is dependent on a ubiquitin-binding domain of pol η, which will be discussed further in Chapter 2. The monoubiquitination of pol η is not dependent on the post-replicative repair (PRR) proteins Rad6 and Rad18, and is not responsive to DNA damage [136], as might be expected for a regulatory modification of pol η. This has led some to argue that the modification is not regulatory [138]. However, because pol η is regulated by physical interactions with ubiquitin and with ubiquitinated proteins (See Section 4c) such as PCNA and Rad18, another hypothesis is that the covalently attached ubiquitin serves as a negative regulator of pol η by inhibiting these interactions. It has also been proposed that pol η monoubiquitination is a precursor for polyubiquitination, which targets pol η for proteolytic degradation [139]; this will be discussed in Chapter 4.

To date, pol η has not been shown to be phosphorylated, but various evidence points to a role for phosphorylation in translesion synthesis, mainly involving the Mec1 (ATR) damage checkpoint kinase (see Section 1b). The formation of Rad18 foci in CHO cells in response to DNA damage is presumed to be dependent on phosphorylation, as it can be prevented by kinase inhibitors, although it is not known whether Rad18 itself is phosphorylated [140]. There is more evidence for phosphorylation affecting Rev1 function. One study observed the apparent phosphorylation of Rev1 itself in yeast, in a Mec1-dependent fashion, although they did not convincingly demonstrate that the phosphorylated species is functionally significant [141]. In addition, Rev1's association with double strand breaks is also Mec1-dependent [142]. Rev1 may interact specifically with phosphorylated proteins through its BRCT domain, a type of phospho-protein binding domain [143]. The BRCT domain of Rev1 mediates an interaction with PCNA (the processivity clamp), and is required for Rev1 function and for its localization to nuclear foci in the absence of exogenous damage [144, 145]. The possibility that pol η is itself phosphorylated will be considered further in Chapter 2.

4c. Protein-protein interactions recruit pol η to damaged DNA. Pol η appears to be found in the nucleus constitutively [146], but it's access to the DNA is regulated by the post-replicative repair pathway (see also sections 1b and 2c). The post replication repair (PRR) pathway in *S. cerevisiae* is defined by the genes *RAD6* and *RAD18*, which are both epistatic to *RAD30* with respect to survival of UV-induced DNA damage [14]. Rad6 is a ubiquitin conjugator, and it forms a stable dimer with Rad18, a ubiquitin ligase and single stranded DNA binding protein [16, 147-150]. In HeLa cells, Rad18 localizes to sites of DNA damage independently of replication, cell cycle stage, or nucleotide excision repair [151], perhaps through its specific interaction with single stranded DNA.

In mammalian cells, pol η also forms damage-induced nuclear foci which co-localize with DNA lesions: they are seen in a small percentage of untreated cells and accumulate in the majority of cells that have been treated with DNA damaging agents such as UV or MMS [146]. Pol η foci also accumulate in cells subjected to hydroxyurea-induced replication stress [32]. The pol η foci co-localize with Rad18 foci [152]. Furthermore, the formation of pol η foci is largely dependent on Rad18: only a very small proportion of *rad18*$^{-/-}$ cells contain pol η foci, and, unlike the pol η foci in wildtype cells, their frequency does not increase in response to DNA damage [152]. Formation of pol η foci also depends on its C-terminal, non-catalytic region [146], suggesting that this region mediates the recruitment of pol η to sites of DNA damage (this is discussed further in Chapter 2). It is assumed that the nuclear pol η foci represent sites of TLS, although it should be kept in mind that focus formation does not necessarily imply activity. For example, a mutant form of Rad18 that is unable to form foci nonetheless activates DNA damage tolerance pathways [151].

Notably, while Rad18 functions similarly in yeast and mammals, its role can vary in other species, such as the chicken [153]. However, this discussion will focus on data from yeast and mammals. The Rad6/Rad18 complex recruits pol η to sites of DNA damage by two mechanisms: by ubiquitinating the replication factor PCNA (discussed further below), and also by a PCNA-independent effect on pol η function and localization, perhaps involving its direct physical interaction with pol η [152], which is discussed immediately below.

4c-1. Physical interaction of Rad18 and polymerase η. Mouse pol η forms a direct physical interaction with Rad18 involving C-terminal domains of both proteins [152]. This interaction can be observed in the presence or absence of DNA damage [152]. A (possibly indirect) physical interaction between Rad18 and pol η also occurs in human cells, from which a complex including Rad18, Rad6, Rev1 and pol η has been purified: the complex is enriched in the chromatin fraction in response to UV radiation or S phase arrest [154]. This is consistent with pol η's association with stalled replication forks, and therefore with the model that Rad18 is involved in the functionally significant recruitment of pol η to sites of damage.

A mutant form of Rad18 protein that is unable to physically interact with pol η scarcely supports formation of pol η foci in response to UV irradiation (about 15%, vs. 60% in the wildtype), despite retaining PCNA-ubiquitinating activity *in vivo* [152]. This suggests that the direct physical interaction with Rad18 plays an important role in pol η's recruitment to nuclear foci in response to DNA damage. However, the same mutant completely fails to rescue the UV sensitive phenotype of *rad18*$^{-/-}$ mouse cells [152], while the loss of polymerase η does not cause so severe a phenotype. Since the *rad18*$^{-/-}$ null is much more sensitive to DNA damage than a pol η deficient strain, the severity of the *rad18*$^{-/-}$ mutant's phenotype suggests that this region of Rad18 is needed to activate additional activities downstream of Rad18, not just pol η function. Thus, it is unclear whether the defect in pol η foci formation is due specifically to loss of the physical interaction between pol η and Rad18, or is instead caused by a larger disruption of Rad18 function (in which case the physical interaction between pol η and Rad18 may have a different, unknown function). Direct physical interaction between Rad18 and pol η in *S. cerevisiae* has not been demonstrated.

4c-2. Pol η's interaction with PCNA is regulated by Rad6/Rad18. The processivity clamp PCNA (Proliferating Cell Nuclear Antigen) is a homotrimeric ring protein which is loaded onto DNA at primer termini and localizes to replication forks where it interacts with many proteins involved in DNA replication and damage tolerance [155, 156]. Pol η has a direct physical interaction with PCNA, mediated by pol η's C-terminal PCNA-binding motif (PIP-box) [146]. The interaction between PCNA and pol

η plays an important role in pol η function, which is at least partially attributed to the stimulatory effect of PCNA on pol η's TLS activity *in vitro* [146, 157, 158].

In addition, pol η's interaction with PCNA can be enhanced by monoubiquitination of PCNA, a modification that is regulated (in yeast and mammals) by Rad6 and Rad18 [159]. The heterodimer made up of Rad6, a ubiquitin conjugator, and Rad18, a ubiquitin ligase, modifies PCNA by conjugating a mono-ubiquitin to PCNA's lysine-164 in response to a variety of DNA damaging agents including UV and MMS, as well as to replication stress induced by hydroxyurea [152, 159-162]. PCNA then remains mono-ubiquitinated and immobilized on the DNA for an extended time [161], where its modification state is thought to modulate its interactions with other proteins including pol η.

The ubiquitinated form of PCNA is created and maintained as a specific response to the presence of DNA damage. Monoubiquitination of PCNA in cell-free human extracts is efficient only in the presence of polymerase stalling or DNA secondary structures [163]. Single-stranded DNA, exposed at sites of stalled replication forks by the uncoupling of the replicative DNA polymerase and the MCM helicase, may be the signal that targets PCNA monoubiquitination to sites of damage, mediated by Rad18's single stranded DNA binding activity [23, 159]. While it persists, the ubiquitinated PCNA promotes DNA damage tolerance by TLS (see next section). Monoubiquitinated PCNA is eventually returned to the unmodified state by the deubiquitinating enzyme USP1 (ubiquitin specific protease) [118]. In the presence of DNA damage, USP1 is inactivated by self-cleavage, leading to protection of the ubi-PCNA while damage persists [118].

To complicate matters, there are two known alternative modifications of the same lysine on PCNA, which may compete with monoubiquitination (Figure 4). Lysine-164 of PCNA is conjugated to SUMO (small ubiquitin-like modifier) during S phase in yeast [159]. The SUMOylated form of PCNA acts to inhibit homologous recombination during DNA replication [164-166]. SUMOylated PCNA does not promote damage-induced mutagenesis, but does contribute to spontaneous mutagenesis (along with monoubiquitinated PCNA), suggesting that error-prone TLS by Rev1 and pol ζ can be activated by either SUMO or ubiquitin-modified PCNA [160].

PCNA is also modified at K164 by attachment of a K63-linked poly-ubiquitin chain, which requires members of a non-TLS "error-free" PRR subpathway: Mms2, Ubc13 and Rad5 [159, 167]. The K63-linked polyubiquitin chain does not target PCNA for proteolytic degradation (which typically involves a K48-linked poly-ubiquitin chain). Instead, polyubiquitinated PCNA initiates the "error-free" post replicative repair pathway, which promotes DNA damage tolerance by a recombination mechanism. It is interesting to note that in this system, the monoubiquitinated PCNA may be an intermediate in the production of the polyubiquitinated PCNA, suggesting that TLS may be activated prior to the "error-free" PRR pathway.

Monoubiquitinated PCNA. TLS by pol η is largely dependent upon monoubiquitinated PCNA, which is thought to recruit pol η to sites of DNA damage, as will be discussed in Chapter 2. Mammalian pol η foci co-localize with foci of mono-ubiquitinated PCNA in the nucleus [161], and accumulation of pol η foci in response to DNA damage is dependent upon mono-ubiquitinated PCNA [168], although a small proportion of cells (5-10%) do have pol η foci in *rad18*$^{-/-}$ or *pol30(K164R)* mutants, in which PCNA is not monoubiquitinated [152]. A similar proportion of cells contain pol η foci in the absence of DNA damage, consistent with a model in which PCNA monoubiquitination induces pol η's response to exogenous DNA damage, above a low level of uninduced DNA-association by pol η.

The dependence of pol η's damage-induced foci on mono-ubiquitinated PCNA is attributed to pol η's interactions with PCNA and ubiquitin [168], which give pol η a competitive advantage over the replicative polymerase delta for PCNA association [152, 154] Pol η's interaction with monoubiquitinated PCNA is mediated by both the PCNA interaction motif (PIP-box), and a novel ubiquitin interacting motif, the UBZ, which is a Ubiquitin Binding Zinc finger (Figure 5). The UBZ is the subject of Chapter 2. The UBZs of both yeast and mammalian pol η are required to complement the UV sensitivity of pol η deficient cells [136, 137]. Mono-ubiquitinated PCNA may also promote TLS by enhancing pol η's catalytic activity, but *in vitro* results have been inconsistent [169, 170]. Chapters 4 and 5 will address the extent to which pol η function can be independent of PCNA monoubiquitination.

4c-3. Pol η's interactions with other TLS Polymerases. There is mounting evidence that the TLS polymerases physically and functionally interact with one another. Mammalian Rev1 interacts not only with pol η, but also with pol ι, pol κ and pol ζ, all via its C-terminus [171-174]. The conservation of the physical interaction between Rev1 and the other TLS polymerases is the subject of Chapter 3. A complex including both pol η and Rev1 is enriched in the chromatin-associated fraction of HeLa cells after UV damage or arrest in S phase, consistent with its being an active complex [154, 175]. The interaction of pol η with Rev1 is not conserved in all species (see Chapter 4), but a novel interaction with pol ζ (Kosarek and Friedberg, personal communication) could functionally replace it in some organisms. As will be discussed further in Chapters 3 and 4, Rev1's interactions with so many other TLS polymerases may be involved in their recruitment or activation. The cell cycle specific changes in Rev1 protein abundance in yeast suggest it may specifically recruit other polymerases for post-replicative gap-filling [31, 171-173].

In addition to Rev1, pol η also interacts directly with pol ι [154, 171]. Foci of pol η colocalize, at least partially, with both Rev1 and pol ι foci [172, 176]. Furthermore, formation of pol ι (but not Rev1) foci is dependent on pol η [176], suggesting a role for pol η in recruitment of pol ι to sites of TLS. The colocalization of the TLS polymerases implies there is yet another layer of regulation required to select the appropriate polymerase for TLS past different lesions or in specific contexts.

5. Summary of this dissertation

In Chapter 2, I show that yeast pol η has a UBZ domain of degenerate sequence but conserved functional significance. I demonstrate that the association of pol η with chromatin increases in response to DNA damage in a manner that depends on the UBZ domain. By additional sequence and mutational analysis, I also identify potential modification sites and structural features of pol η. Chapter 3 is a comparison of the physical interactions of Rev1 with other polymerases in several model organisms. We show that the Rev1/pol η interaction, while conserved in flies and mammals, in absent from budding yeast, *S. pombe,* and *C. elegans.* I investigate the functional interactions of pol η with the other TLS polymerases of yeast, Rev1 and pol ζ, in Chapter 4 by genetic

analysis, including cell-cycle specific epistasis assays, and show that the DNA damage response of pol η is not due to an increase in protein abundance. Conclusions and discussion of all these studies are presented in Chapter 5.

Figure 1.

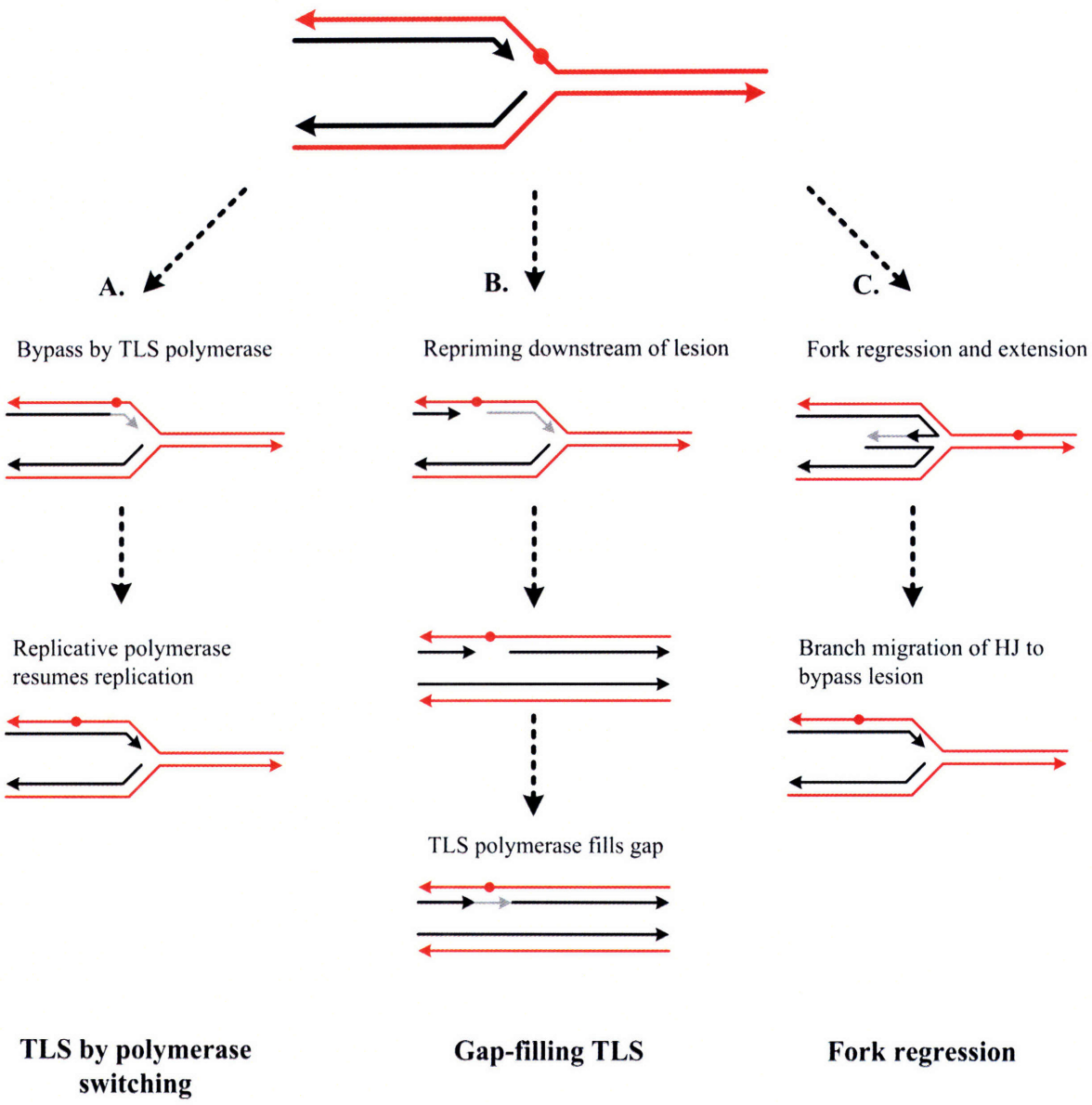


Figure 1. Some mechanisms of lesion bypass. The lesion is represented as a ball on the parental (red) DNA. A, Translesion synthesis by polymerase switching. The stalled replicative polymerase is replaced at the replication fork by a TLS polymerase, which bypasses the lesion, extends a few nucleotides, and is replaced by the replicative polymerase. B, Gap-filling translesion synthesis. The replicative polymerase reprimes downstream of the lesion, leaving behind a single stranded gap in the DNA which is later filled by a TLS polymerase. C, Fork regression allows use of the undamaged nascent DNA as template, thereby bypassing the lesion without directly replicating the damaged DNA.

Figure 2.

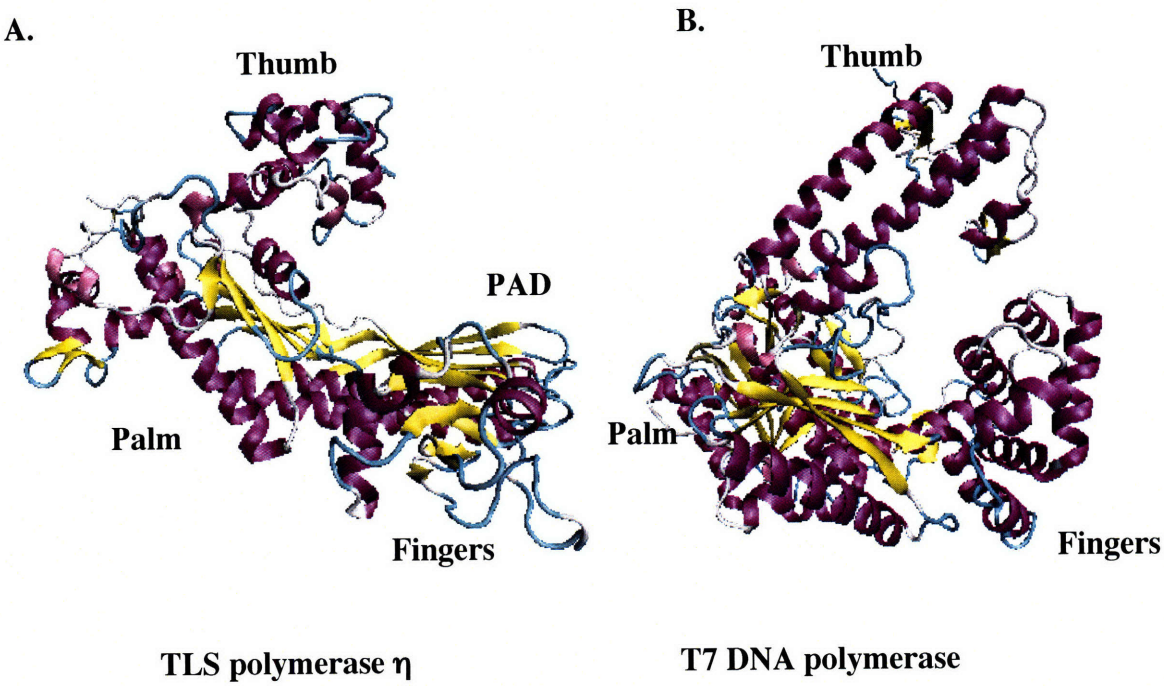
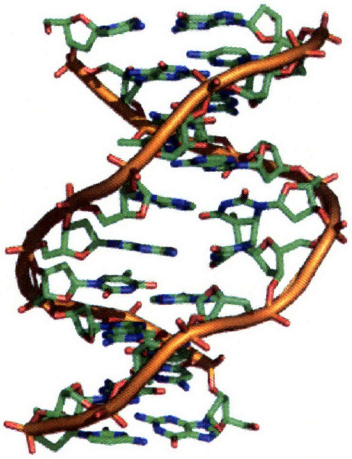


Figure 2. Structures of TLS polymerase and replicative polymerase. Both polymerases fold into a right-hand-shaped fold, which the active site at the palm. The TLS polymerase eta, shown in **A,** has smaller thumb and fingers domains, and has an additional PAD domain not present in T7 DNA polymerase, shown in **B.**

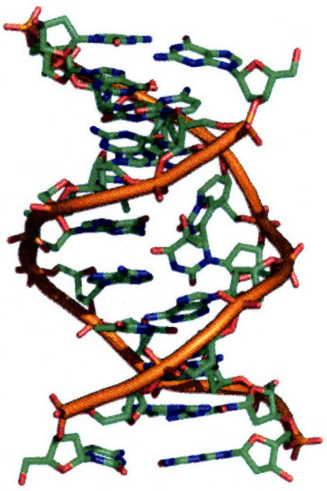
Figure 3.

A.



cyclopyrimidine dimer (CPD)

B.



(6-4) photoproduct

Figure 3. Common lesions produced by UV radiation. Structures of DNA helices containing UV-induced DNA lesions: **A,** cyclopyrimidine dimer or CPD; **B,** (6-4) photoproduct.

Figure 4.

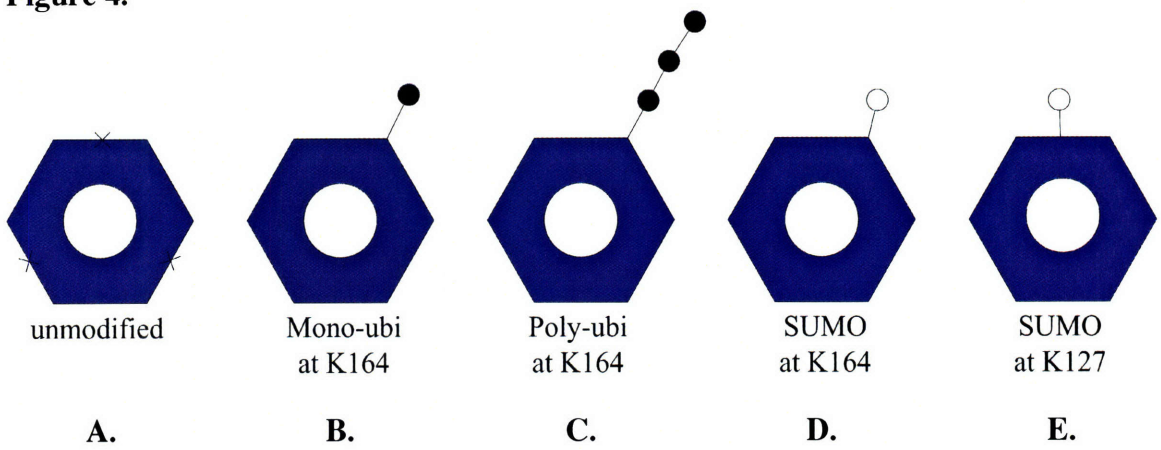


Figure 4. Modification states of PCNA. A, Unmodified. X marks the interaction site (on each of the three monomers) of the common PCNA-interaction motif known as the PIP-box. B, PCNA can be monoubiquitinated at the conserved residue K164. PCNA is monoubiquitinated by Rad6 and Rad18 in response to DNA damage, and promotes translesion synthesis. C, PCNA is polyubiquitinated at K164 by Mms2, Ubc13 and Rad5, as part of an error-free PRR pathway. D, E, PCNA is SUMOylated at K164 or K127. In *S. cerevisiae,* this modification is part of a regulatory pathway to prevent recombination during S phase. SUMOylated PCNA can also contribute to translesion synthesis in the absence of exogenous damage.

Figure 5

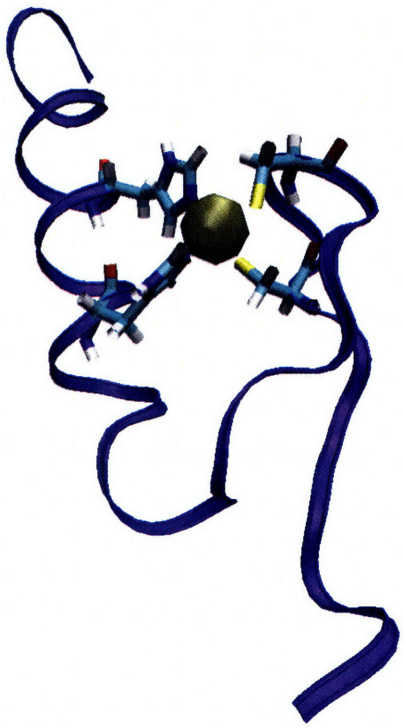


Figure 5. Solution structure of UBZ from human pol eta [23]. The zinc-coordinating cysteine and histidine side chains are shown. The outer face of the alpha-helix, on the left, interacts with ubiquitin [23].

References

1. Lindahl, T., and D.E. Barnes, *Repair of endogenous DNA damage*. Cold Spring Harb Symp Quant Biol, 2000(65): p. 127-133.
2. Lindahl, T., *Instability and decay of the primary structure of DNA*. Nature, 1993. **362**(6422): p. 709-15.
3. Friedberg, E.C., Walker, G.C., Siede, W., Wood, R. D., Schultz, R. A., Ellenberger, T., *DNA Repair and Mutagenesis*. Second Edition ed. 2006, Washington, DC: ASM Press.
4. Sancar, G.B., *Enzymatic photoreactivation: 50 years and counting*. Mutat Res, 2000. **451**(1-2): p. 25-37.
5. Barnes, D.E. and T. Lindahl, *Repair and genetic consequences of endogenous DNA base damage in mammalian cells*. Annu Rev Genet, 2004. **38**: p. 445-76.
6. Kunkel, T.A. and D.A. Erie, *DNA mismatch repair*. Annu Rev Biochem, 2005. **74**: p. 681-710.
7. Dip, R., U. Camenisch, and H. Naegeli, *Mechanisms of DNA damage recognition and strand discrimination in human nucleotide excision repair*. DNA Repair (Amst), 2004. **3**(11): p. 1409-23.
8. Becherel, O.J. and R.P. Fuchs, *SOS mutagenesis results from up-regulation of translesion synthesis*. J Mol Biol, 1999. **294**(2): p. 299-306.
9. Kunkel, T.A., Y.I. Pavlov, and K. Bebenek, *Functions of human DNA polymerases eta, kappa and iota suggested by their properties, including fidelity with undamaged DNA templates*. DNA Repair (Amst), 2003. **2**(2): p. 135-49.
10. Schaaper, R.M., *The mutational specificity of two Escherichia coli dnaE antimutator alleles as determined from lacI mutation spectra*. Genetics, 1993. **134**(4): p. 1031-8.
11. Kunkel, T.A., *DNA replication fidelity*. J Biol Chem, 2004. **279**(17): p. 16895-8.
12. Johnson, R.E., S. Prakash, and L. Prakash, *Efficient bypass of a thymine-thymine dimer by yeast DNA polymerase, Poleta*. Science, 1999. **283**(5404): p. 1001-4.
13. Washington, M.T., et al., *Fidelity and processivity of Saccharomyces cerevisiae DNA polymerase eta*. J Biol Chem, 1999. **274**(52): p. 36835-8.
14. McDonald, J.P., A.S. Levine, and R. Woodgate, *The Saccharomyces cerevisiae RAD30 gene, a homologue of Escherichia coli dinB and umuC, is DNA damage inducible and functions in a novel error-free postreplication repair mechanism*. Genetics, 1997. **147**(4): p. 1557-68.
15. Rupp, W.D. and P. Howard-Flanders, *Discontinuities in the DNA synthesized in an excision-defective strain of Escherichia coli following ultraviolet irradiation*. J Mol Biol, 1968. **31**(2): p. 291-304.
16. Bailly, V., et al., *Specific complex formation between yeast RAD6 and RAD18 proteins: a potential mechanism for targeting RAD6 ubiquitin-conjugating activity to DNA damage sites*. Genes Dev, 1994. **8**(7): p. 811-20.
17. Erlich, B., Danielle, Lahue, Samson, *Anc1, a protein associated with transcription, defines a novel branch of the postreplication repair pathway*.

18. Smith, S., et al., *Mutator genes for suppression of gross chromosomal rearrangements identified by a genome-wide screening in Saccharomyces cerevisiae.* Proc Natl Acad Sci U S A, 2004. **101**(24): p. 9039-44.
19. Hishida, T., et al., *Functional and physical interaction of yeast Mgs1 with PCNA: impact on RAD6-dependent DNA damage tolerance.* Mol Cell Biol, 2006. **26**(14): p. 5509-17.
20. Pasero, P., K. Shimada, and B.P. Duncker, *Multiple roles of replication forks in S phase checkpoints: sensors, effectors and targets.* Cell Cycle, 2003. **2**(6): p. 568-72.
21. Foiani, M., et al., *DNA damage checkpoints and DNA replication controls in Saccharomyces cerevisiae.* Mutat Res, 2000. **451**(1-2): p. 187-96.
22. Liberi, G., et al., *Srs2 DNA helicase is involved in checkpoint response and its regulation requires a functional Mec1-dependent pathway and Cdk1 activity.* Embo J, 2000. **19**(18): p. 5027-38.
23. Chang, D.J., P.J. Lupardus, and K.A. Cimprich, *Monoubiquitination of proliferating cell nuclear antigen induced by stalled replication requires uncoupling of DNA polymerase and mini-chromosome maintenance helicase activities.* J Biol Chem, 2006. **281**(43): p. 32081-8.
24. Barbour, L., et al., *DNA damage checkpoints are involved in postreplication repair.* Genetics, 2006. **174**(4): p. 1789-800.
25. Bi, X., et al., *Rad18 regulates DNA polymerase kappa and is required for recovery from S-phase checkpoint-mediated arrest.* Mol Cell Biol, 2006. **26**(9): p. 3527-40.
26. Kai, M. and T.S. Wang, *Checkpoint activation regulates mutagenic translesion synthesis.* Genes Dev, 2003. **17**(1): p. 64-76.
27. Kunkel, T.A. and K. Bebenek, *DNA replication fidelity.* Annu Rev Biochem, 2000. **69**: p. 497-529.
28. Pages, V., R. Janel-Bintz, and R.P. Fuchs, *Pol III proofreading activity prevents lesion bypass as evidenced by its molecular signature within E.coli cells.* J Mol Biol, 2005. **352**(3): p. 501-9.
29. Lopes, M., M. Foiani, and J.M. Sogo, *Multiple mechanisms control chromosome integrity after replication fork uncoupling and restart at irreparable UV lesions.* Mol Cell, 2006. **21**(1): p. 15-27.
30. Friedberg, E.C., A.R. Lehmann, and R.P. Fuchs, *Trading places: how do DNA polymerases switch during translesion DNA synthesis?* Mol Cell, 2005. **18**(5): p. 499-505.
31. Waters, L.S. and G.C. Walker, *The critical mutagenic translesion DNA polymerase Rev1 is highly expressed during G(2)/M phase rather than S phase.* Proc Natl Acad Sci U S A, 2006. **103**(24): p. 8971-6.
32. de Feraudy, S., et al., *Pol eta is required for DNA replication during nucleotide deprivation by hydroxyurea.* Oncogene, 2007.
33. Ohmori, H., et al., *The Y-family of DNA polymerases.* Mol Cell, 2001. **8**(1): p. 7-8.
34. Morrison, A., et al., *REV3, a Saccharomyces cerevisiae gene whose function is required for induced mutagenesis, is predicted to encode a nonessential DNA polymerase.* J Bacteriol, 1989. **171**(10): p. 5659-67.

35. Ogi, T. and A.R. Lehmann, *The Y-family DNA polymerase kappa (pol kappa) functions in mammalian nucleotide-excision repair.* Nat Cell Biol, 2006. **8**(6): p. 640-2.
36. McIlwraith, M.J., et al., *Human DNA polymerase eta promotes DNA synthesis from strand invasion intermediates of homologous recombination.* Mol Cell, 2005. **20**(5): p. 783-92.
37. Rattray, A.J., et al., *The roles of REV3 and RAD57 in double-strand-break-repair-induced mutagenesis of Saccharomyces cerevisiae.* Genetics, 2002. **162**(3): p. 1063-77.
38. Rattray, A.J. and J.N. Strathern, *Homologous recombination is promoted by translesion polymerase poleta.* Mol Cell, 2005. **20**(5): p. 658-9.
39. Casali, P., et al., *DNA repair in antibody somatic hypermutation.* Trends Immunol, 2006. **27**(7): p. 313-21.
40. Ling, H., et al., *Crystal structure of a Y-family DNA polymerase in action: a mechanism for error-prone and lesion-bypass replication.* Cell, 2001. **107**(1): p. 91-102.
41. Trincao, J., et al., *Structure of the catalytic core of S. cerevisiae DNA polymerase eta: implications for translesion DNA synthesis.* Mol Cell, 2001. **8**(2): p. 417-26.
42. Silvian, L.F., et al., *Crystal structure of a DinB family error-prone DNA polymerase from Sulfolobus solfataricus.* Nat Struct Biol, 2001. **8**(11): p. 984-9.
43. Zhou, B.L., J.D. Pata, and T.A. Steitz, *Crystal structure of a DinB lesion bypass DNA polymerase catalytic fragment reveals a classic polymerase catalytic domain.* Mol Cell, 2001. **8**(2): p. 427-37.
44. Uljon, S.N., et al., *Crystal structure of the catalytic core of human DNA polymerase kappa.* Structure, 2004. **12**(8): p. 1395-404.
45. Ling, H., et al., *Replication of a cis-syn thymine dimer at atomic resolution.* Nature, 2003. **424**(6952): p. 1083-7.
46. Nair, D.T., et al., *Hoogsteen base pair formation promotes synthesis opposite the 1,N6-ethenodeoxyadenosine lesion by human DNA polymerase iota.* Nat Struct Mol Biol, 2006. **13**(7): p. 619-25.
47. Yang, W., *Portraits of a Y-family DNA polymerase.* FEBS Lett, 2005. **579**(4): p. 868-72.
48. Doublie, S., et al., *Crystal structure of a bacteriophage T7 DNA replication complex at 2.2 A resolution.* Nature, 1998. **391**(6664): p. 251-8.
49. Li, Y., S. Korolev, and G. Waksman, *Crystal structures of open and closed forms of binary and ternary complexes of the large fragment of Thermus aquaticus DNA polymerase I: structural basis for nucleotide incorporation.* Embo J, 1998. **17**(24): p. 7514-25.
50. Suzuki, M., et al., *Thermus aquaticus DNA polymerase I mutants with altered fidelity. Interacting mutations in the O-helix.* J Biol Chem, 2000. **275**(42): p. 32728-35.
51. Cannistraro, V.J. and J.S. Taylor, *DNA-thumb interactions and processivity of T7 DNA polymerase in comparison to yeast polymerase eta.* J Biol Chem, 2004. **279**(18): p. 18288-95.

52. Prakash, S., R.E. Johnson, and L. Prakash, *Eukaryotic translesion synthesis DNA polymerases: specificity of structure and function.* Annu Rev Biochem, 2005. **74**: p. 317-53.
53. Kamath-Loeb, A.S., et al., *Werner syndrome protein interacts functionally with translesion DNA polymerases.* Proc Natl Acad Sci U S A, 2007. **104**(25): p. 10394-9.
54. Maga, G., et al., *Human DNA polymerase lambda functionally and physically interacts with proliferating cell nuclear antigen in normal and translesion DNA synthesis.* J Biol Chem, 2002. **277**(50): p. 48434-40.
55. Shiomi, N., et al., *Human RAD18 is involved in S phase-specific single-strand break repair without PCNA monoubiquitination.* Nucleic Acids Res, 2007. **35**(2): p. e9.
56. Masutani, C., et al., *Mechanisms of accurate translesion synthesis by human DNA polymerase eta.* Embo J, 2000. **19**(12): p. 3100-9.
57. Lemontt, J.F., *Mutants of yeast defective in mutation induced by ultraviolet light.* Genetics, 1971(68): p. 21-33.
58. Lawrence, C.W., B.R. Krauss, and R.B. Christensen, *New mutations affecting induced mutagenesis in yeast.* Mutat Res, 1985. **150**(1-2): p. 211-6.
59. Roush, A.A., et al., *Deletion of the Saccharomyces cerevisiae gene RAD30 encoding an Escherichia coli DinB homolog confers UV radiation sensitivity and altered mutability.* Mol Gen Genet, 1998. **257**(6): p. 686-92.
60. Kato, T. and Y. Shinoura, *Isolation and characterization of mutants of Escherichia coli deficient in induction of mutations by ultraviolet light.* Mol Gen Genet, 1977. **156**(2): p. 121-31.
61. Lawrence, C.W. and R. Christensen, *UV mutagenesis in radiation-sensitive strains of yeast.* Genetics, 1976. **82**(2): p. 207-32.
62. Masutani, C., et al., *The XPV (xeroderma pigmentosum variant) gene encodes human DNA polymerase eta.* Nature, 1999. **399**(6737): p. 700-4.
63. Johnson, R.E., et al., *hRAD30 mutations in the variant form of xeroderma pigmentosum.* Science, 1999. **285**(5425): p. 263-5.
64. Chandrasekhar, D. and B. Van Houten, *In vivo formation and repair of cyclobutane pyrimidine dimers and 6-4 photoproducts measured at the gene and nucleotide level in Escherichia coli.* Mutat Res, 2000. **450**(1-2): p. 19-40.
65. Mitchell DL, N.R., *The biology of the (6-4) photoproduct.* Photochem Photobiol, 1989. **49**: p. 805-819.
66. Abdulovic, A.L. and S. Jinks-Robertson, *The in vivo characterization of translesion synthesis across UV-induced lesions in Saccharomyces cerevisiae: insights into Pol zeta- and Pol eta-dependent frameshift mutagenesis.* Genetics, 2006. **172**(3): p. 1487-98.
67. Patel, P.H., et al., *Prokaryotic DNA polymerase I: evolution, structure, and "base flipping" mechanism for nucleotide selection.* J Mol Biol, 2001. **308**(5): p. 823-37.
68. Taylor, J.S., et al., *1H NMR assignment and melting temperature study of cis-syn and trans-syn thymine dimer containing duplexes of d(CGTATTATGC).d(GCATAATACG).* Biochemistry, 1990. **29**(37): p. 8858-66.

69. Washington, M.T., L. Prakash, and S. Prakash, *Yeast DNA polymerase eta utilizes an induced-fit mechanism of nucleotide incorporation.* Cell, 2001. **107**(7): p. 917-27.
70. Washington, M.T., et al., *The mechanism of nucleotide incorporation by human DNA polymerase eta differs from that of the yeast enzyme.* Mol Cell Biol, 2003. **23**(22): p. 8316-22.
71. Hwang, H. and J.S. Taylor, *Role of base stacking and sequence context in the inhibition of yeast DNA polymerase eta by pyrene nucleotide.* Biochemistry, 2004. **43**(46): p. 14612-23.
72. Hwang, H. and J.S. Taylor, *Evidence for Watson-Crick and not Hoogsteen or wobble base pairing in the selection of nucleotides for insertion opposite pyrimidines and a thymine dimer by yeast DNA pol eta.* Biochemistry, 2005. **44**(12): p. 4850-60.
73. Yagi, Y., et al., *DNA polymerases eta and kappa are responsible for error-free translesion DNA synthesis activity over a cis-syn thymine dimer in Xenopus laevis oocyte extracts.* DNA Repair (Amst), 2005. **4**(11): p. 1252-69.
74. Gibbs, P.E., et al., *The relative roles in vivo of Saccharomyces cerevisiae Pol eta, Pol zeta, Rev1 protein and Pol32 in the bypass and mutation induction of an abasic site, T-T (6-4) photoadduct and T-T cis-syn cyclobutane dimer.* Genetics, 2005. **169**(2): p. 575-82.
75. Garinis, G.A., et al., *Transcriptome analysis reveals cyclobutane pyrimidine dimers as a major source of UV-induced DNA breaks.* Embo J, 2005. **24**(22): p. 3952-62.
76. Limoli, C.L., et al., *Inaugural article: polymerase eta deficiency in the xeroderma pigmentosum variant uncovers an overlap between the S phase checkpoint and double-strand break repair.* Proc Natl Acad Sci U S A, 2000. **97**(14): p. 7939-46.
77. Lawrence, C.W. and D.C. Hinkle, *DNA polymerase zeta and the control of DNA damage induced mutagenesis in eukaryotes.* Cancer Surv, 1996. **28**: p. 21-31.
78. Wang, Y., et al., *Evidence that in xeroderma pigmentosum variant cells, which lack DNA polymerase eta, DNA polymerase iota causes the very high frequency and unique spectrum of UV-induced mutations.* Cancer Res, 2007. **67**(7): p. 3018-26.
79. Ishikawa, T., et al., *Mutagenic and nonmutagenic bypass of DNA lesions by Drosophila DNA polymerases dpoleta and dpoliota.* J Biol Chem, 2001. **276**(18): p. 15155-63.
80. Kozmin, S.G., et al., *Roles of Saccharomyces cerevisiae DNA polymerases Poleta and Polzeta in response to irradiation by simulated sunlight.* Nucleic Acids Res, 2003. **31**(15): p. 4541-52.
81. Yao, J., K. Dixon, and M.P. Carty, *A single (6-4) photoproduct inhibits plasmid DNA replication in xeroderma pigmentosum variant cell extracts.* Environ Mol Mutagen, 2001. **38**(1): p. 19-29.
82. Bresson, A. and R.P. Fuchs, *Lesion bypass in yeast cells: Pol eta participates in a multi-DNA polymerase process.* Embo J, 2002. **21**(14): p. 3881-7.
83. Leibeling, D., P. Laspe, and S. Emmert, *Nucleotide excision repair and cancer.* J Mol Histol, 2006. **37**(5-7): p. 225-38.

84. Lichon, V. and A. Khachemoune, *Xeroderma pigmentosum: beyond skin cancer.* J Drugs Dermatol, 2007. **6**(3): p. 281-8.
85. Robbins, J.H., K.H. Kraemer, and B.A. Flaxman, *DNA repair in tumor cells from the variant form of xeroderma pigmentosum.* J Invest Dermatol, 1975. **64**(3): p. 150-5.
86. Boyer, J.C., et al., *Defective postreplication repair in xeroderma pigmentosum variant fibroblasts.* Cancer Res, 1990. **50**(9): p. 2593-8.
87. Yuasa, M., et al., *Genomic structure, chromosomal localization and identification of mutations in the xeroderma pigmentosum variant (XPV) gene.* Oncogene, 2000. **19**(41): p. 4721-8.
88. Tanioka, M., et al., *Molecular Analysis of DNA Polymerase Eta Gene in Japanese Patients Diagnosed as Xeroderma Pigmentosum Variant Type.* J Invest Dermatol, 2007.
89. Lehman, A.R., et al., *Xeroderma pigmentosum cells with normal levels of excision repair have a defect in DNA synthesis after UV-irradiation.* Proc Natl Acad Sci U S A, 1975. **72**(1): p. 219-23.
90. Maher, V.M., et al., *Frequency of ultraviolet light-induced mutations is higher in xeroderma pigmentosum variant cells than in normal human cells.* Nature, 1976. **261**(5561): p. 593-5.
91. Wang, Y.C., V.M. Maher, and J.J. McCormick, *Xeroderma pigmentosum variant cells are less likely than normal cells to incorporate dAMP opposite photoproducts during replication of UV-irradiated plasmids.* Proc Natl Acad Sci U S A, 1991. **88**(17): p. 7810-4.
92. Wang, Y.C., et al., *Evidence from mutation spectra that the UV hypermutability of xeroderma pigmentosum variant cells reflects abnormal, error-prone replication on a template containing photoproducts.* Mol Cell Biol, 1993. **13**(7): p. 4276-83.
93. Misra, R.R. and J.M. Vos, *Defective replication of psoralen adducts detected at the gene-specific level in xeroderma pigmentosum variant cells.* Mol Cell Biol, 1993. **13**(2): p. 1002-12.
94. Waters, H.L., et al., *Ultraviolet hypermutability of a shuttle vector propagated in xeroderma pigmentosum variant cells.* J Invest Dermatol, 1993. **101**(5): p. 744-8.
95. Raha, M., et al., *Mutagenesis by third-strand-directed psoralen adducts in repair-deficient human cells: high frequency and altered spectrum in a xeroderma pigmentosum variant.* Proc Natl Acad Sci U S A, 1996. **93**(7): p. 2941-6.
96. Kusumoto, R., et al., *Translesion synthesis by human DNA polymerase eta across thymine glycol lesions.* Biochemistry, 2002. **41**(19): p. 6090-9.
97. Pollack, M., et al., *Translesion DNA Synthesis across the Heptanone-Etheno-2'-Deoxycytidine Adduct in Cells.* Chem Res Toxicol, 2006. **19**(8): p. 1074-9.
98. Haracska, L., et al., *Efficient and accurate replication in the presence of 7,8-dihydro-8-oxoguanine by DNA polymerase eta.* Nat Genet, 2000. **25**(4): p. 458-61.
99. Vaisman, A., et al., *Efficient translesion replication past oxaliplatin and cisplatin GpG adducts by human DNA polymerase eta.* Biochemistry, 2000. **39**(16): p. 4575-80.
100. Haracska, L., et al., *Inefficient bypass of an abasic site by DNA polymerase eta.* J Biol Chem, 2001. **276**(9): p. 6861-6.

101. Minko, I.G., et al., *Translesion DNA synthesis by yeast DNA polymerase eta on templates containing N2-guanine adducts of 1,3-butadiene metabolites.* J Biol Chem, 2001. **276**(4): p. 2517-22.
102. Choi, J.Y. and F.P. Guengerich, *Adduct size limits efficient and error-free bypass across bulky N2-guanine DNA lesions by human DNA polymerase eta.* J Mol Biol, 2005. **352**(1): p. 72-90.
103. Boiteux, S., L. Gellon, and N. Guibourt, *Repair of 8-oxoguanine in Saccharomyces cerevisiae: interplay of DNA repair and replication mechanisms.* Free Radic Biol Med, 2002. **32**(12): p. 1244-53.
104. de Padula, M., et al., *The post-replication repair RAD18 and RAD6 genes are involved in the prevention of spontaneous mutations caused by 7,8-dihydro-8-oxoguanine in Saccharomyces cerevisiae.* Nucleic Acids Res, 2004. **32**(17): p. 5003-10.
105. Avkin, S. and Z. Livneh, *Efficiency, specificity and DNA polymerase-dependence of translesion replication across the oxidative DNA lesion 8-oxoguanine in human cells.* Mutat Res, 2002. **510**(1-2): p. 81-90.
106. Albertella, M.R., et al., *A role for polymerase eta in the cellular tolerance to cisplatin-induced damage.* Cancer Res, 2005. **65**(21): p. 9799-806.
107. Bassett, E., et al., *The role of DNA polymerase eta in translesion synthesis past platinum-DNA adducts in human fibroblasts.* Cancer Res, 2004. **64**(18): p. 6469-75.
108. Kokoska, R.J., S.D. McCulloch, and T.A. Kunkel, *The efficiency and specificity of apurinic/apyrimidinic site bypass by human DNA polymerase eta and Sulfolobus solfataricus Dpo4.* J Biol Chem, 2003. **278**(50): p. 50537-45.
109. Zhang, Y., et al., *Two-step error-prone bypass of the (+)- and (-)-trans-anti-BPDE-N2-dG adducts by human DNA polymerases eta and kappa.* Mutat Res, 2002. **510**(1-2): p. 23-35.
110. Lovett, S.T., *Replication arrest-stimulated recombination: Dependence on the RecA paralog, RadA/Sms and translesion polymerase, DinB.* DNA Repair (Amst), 2006.
111. Kawamoto, T., et al., *Dual roles for DNA polymerase eta in homologous DNA recombination and translesion DNA synthesis.* Mol Cell, 2005. **20**(5): p. 793-9.
112. Chen, C.C., et al., *Genetic analysis of ionizing radiation-induced mutagenesis in Saccharomyces cerevisiae reveals TransLesion Synthesis (TLS) independent of PCNA K164 SUMOylation and ubiquitination.* DNA Repair (Amst), 2006. **5**(12): p. 1475-88.
113. Rattray, A.J. and J.N. Strathern, *Error-prone DNA polymerases: when making a mistake is the only way to get ahead.* Annu Rev Genet, 2003. **37**: p. 31-66.
114. Matsuda, T., et al., *Low fidelity DNA synthesis by human DNA polymerase-eta.* Nature, 2000. **404**(6781): p. 1011-3.
115. King, N.M., et al., *Overproduction of DNA polymerase eta does not raise the spontaneous mutation rate in diploid human fibroblasts.* DNA Repair (Amst), 2005. **4**(6): p. 714-24.
116. Shimizu, M., et al., *Efficient and erroneous incorporation of oxidized DNA precursors by human DNA polymerase eta.* Biochemistry, 2007. **46**(18): p. 5515-22.

117. Matsuda, T., et al., *Error rate and specificity of human and murine DNA polymerase eta.* J Mol Biol, 2001. **312**(2): p. 335-46.
118. Huang, T.T., et al., *Regulation of monoubiquitinated PCNA by DUB autocleavage.* Nat Cell Biol, 2006. **8**(4): p. 339-47.
119. Choi, J.H. and G.P. Pfeifer, *The role of DNA polymerase eta in UV mutational spectra.* DNA Repair (Amst), 2005. **4**(2): p. 211-20.
120. Vaisman, A., et al., *DNA polymerase iota-dependent translesion replication of uracil containing cyclobutane pyrimidine dimers.* DNA Repair (Amst), 2006. **5**(2): p. 210-8.
121. Pavlov, Y.I., D. Nguyen, and T.A. Kunkel, *Mutator effects of overproducing DNA polymerase eta (Rad30) and its catalytically inactive variant in yeast.* Mutat Res, 2001. **478**(1-2): p. 129-39.
122. Northam, M.R., et al., *A novel function of DNA polymerase zeta regulated by PCNA.* Embo J, 2006.
123. Mayorov, V.I., et al., *DNA polymerase eta contributes to strand bias of mutations of A versus T in immunoglobulin genes.* J Immunol, 2005. **174**(12): p. 7781-6.
124. Zeng, X., et al., *Absence of DNA polymerase eta reveals targeting of C mutations on the nontranscribed strand in immunoglobulin switch regions.* J Exp Med, 2004. **199**(7): p. 917-24.
125. Pavlov, Y.I., et al., *Correlation of somatic hypermutation specificity and A-T base pair substitution errors by DNA polymerase eta during copying of a mouse immunoglobulin kappa light chain transgene.* Proc Natl Acad Sci U S A, 2002. **99**(15): p. 9954-9.
126. Zeng, X., et al., *DNA polymerase eta is an A-T mutator in somatic hypermutation of immunoglobulin variable genes.* Nat Immunol, 2001. **2**(6): p. 537-41.
127. Yavuz, S., et al., *The role of polymerase eta in somatic hypermutation determined by analysis of mutations in a patient with xeroderma pigmentosum variant.* J Immunol, 2002. **169**(7): p. 3825-30.
128. Ukai, A., et al., *Role of DNA polymerase theta in tolerance of endogenous and exogenous DNA damage in mouse B cells.* Genes Cells, 2006. **11**(2): p. 111-21.
129. Masuda, K., et al., *Absence of DNA polymerase theta results in decreased somatic hypermutation frequency and altered mutation patterns in Ig genes.* DNA Repair (Amst), 2006.
130. McDonald, J.P., et al., *129-derived strains of mice are deficient in DNA polymerase iota and have normal immunoglobulin hypermutation.* J Exp Med, 2003. **198**(4): p. 635-43.
131. Martomo, S.A., et al., *Normal hypermutation in antibody genes from congenic mice defective for DNA polymerase iota.* DNA Repair (Amst), 2006. **5**(3): p. 392-8.
132. Faili, A., et al., *Induction of somatic hypermutation in immunoglobulin genes is dependent on DNA polymerase iota.* Nature, 2002. **419**(6910): p. 944-7.
133. Poltoratsky, V., et al., *Expression of error-prone polymerases in BL2 cells activated for Ig somatic hypermutation.* Proc Natl Acad Sci U S A, 2001. **98**(14): p. 7976-81.
134. Zan, H., et al., *The translesion DNA polymerase zeta plays a major role in Ig and bcl-6 somatic hypermutation.* Immunity, 2001. **14**(5): p. 643-53.

135. Yamada, A., et al., *Complementation of defective translesion synthesis and UV light sensitivity in xeroderma pigmentosum variant cells by human and mouse DNA polymerase eta.* Nucleic Acids Res, 2000. **28**(13): p. 2473-80.
136. Parker, J.L., et al., *Contributions of ubiquitin- and PCNA-binding domains to the activity of Polymerase eta in Saccharomyces cerevisiae.* Nucleic Acids Res, 2007. **35**(3): p. 881-9.
137. Bienko, M., et al., *Ubiquitin-binding domains in Y-family polymerases regulate translesion synthesis.* Science, 2005. **310**(5755): p. 1821-4.
138. Acharya, N., et al., *Mutations in the ubiquitin binding UBZ motif of DNA polymerase {eta} do not impair its function in translesion synthesis during replication.* Mol Cell Biol, 2007.
139. Skoneczna, A., et al., *Polymerase eta is a short-lived, proteasomally degraded protein that is temporarily stabilized following UV irradiation in Saccharomyces cerevisiae.* J Mol Biol, 2007. **366**(4): p. 1074-86.
140. Nikiforov, A., et al., *DNA damage-induced accumulation of Rad18 protein at stalled replication forks in mammalian cells involves upstream protein phosphorylation.* Biochem Biophys Res Commun, 2004. **323**(3): p. 831-7.
141. Sabbioneda, S., et al., *Yeast Rev1 is cell cycle regulated, phosphorylated in response to DNA damage and its binding to chromosomes is dependent upon MEC1.* DNA Repair (Amst), 2007. **6**(1): p. 121-7.
142. Hirano, Y. and K. Sugimoto, *ATR homolog Mec1 controls association of DNA polymerase zeta-Rev1 complex with regions near a double-strand break.* Curr Biol, 2006. **16**(6): p. 586-90.
143. Yu, X., et al., *The BRCT domain is a phospho-protein binding domain.* Science, 2003. **302**(5645): p. 639-42.
144. Nelson, J.R., et al., *Evidence for a second function for Saccharomyces cerevisiae Rev1p.* Mol Microbiol, 2000. **37**(3): p. 549-54.
145. Guo, C., et al., *REV1 protein interacts with PCNA: significance of the REV1 BRCT domain in vitro and in vivo.* Mol Cell, 2006. **23**(2): p. 265-71.
146. Kannouche, P., et al., *Domain structure, localization, and function of DNA polymerase eta, defective in xeroderma pigmentosum variant cells.* Genes Dev, 2001. **15**(2): p. 158-72.
147. Joazeiro, C.A. and A.M. Weissman, *RING finger proteins: mediators of ubiquitin ligase activity.* Cell, 2000. **102**(5): p. 549-52.
148. Wood, A., et al., *Bre1, an E3 ubiquitin ligase required for recruitment and substrate selection of Rad6 at a promoter.* Mol Cell, 2003. **11**(1): p. 267-74.
149. Dohmen, R.J., et al., *The N-end rule is mediated by the UBC2(RAD6) ubiquitin-conjugating enzyme.* Proc Natl Acad Sci U S A, 1991. **88**(16): p. 7351-5.
150. Sung, P., et al., *Yeast RAD6 encoded ubiquitin conjugating enzyme mediates protein degradation dependent on the N-end-recognizing E3 enzyme.* Embo J, 1991. **10**(8): p. 2187-93.
151. Nakajima, S., et al., *Replication-dependent and -independent responses of RAD18 to DNA damage in human cells.* J Biol Chem, 2006. **281**(45): p. 34687-95.
152. Watanabe, K., et al., *Rad18 guides poleta to replication stalling sites through physical interaction and PCNA monoubiquitination.* Embo J, 2004. **23**(19): p. 3886-96.

153. Simpson, L.J., et al., *RAD18-independent ubiquitination of proliferating-cell nuclear antigen in the avian cell line DT40.* EMBO Rep, 2006.
154. Yuasa, M.S., et al., *A human DNA polymerase eta complex containing Rad18, Rad6 and Rev1; proteomic analysis and targeting of the complex to the chromatin-bound fraction of cells undergoing replication fork arrest.* Genes Cells, 2006. **11**(7): p. 731-44.
155. Majka, J. and P.M. Burgers, *The PCNA-RFC families of DNA clamps and clamp loaders.* Prog Nucleic Acid Res Mol Biol, 2004. **78**: p. 227-60.
156. Moldovan, G.L., B. Pfander, and S. Jentsch, *PCNA, the maestro of the replication fork.* Cell, 2007. **129**(4): p. 665-79.
157. Haracska, L., et al., *Interaction with PCNA is essential for yeast DNA polymerase eta function.* Mol Cell, 2001. **8**(2): p. 407-15.
158. Haracska, L., et al., *Physical and functional interactions of human DNA polymerase eta with PCNA.* Mol Cell Biol, 2001. **21**(21): p. 7199-206.
159. Hoege, C., et al., *RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO.* Nature, 2002. **419**(6903): p. 135-41.
160. Stelter, P. and H.D. Ulrich, *Control of spontaneous and damage-induced mutagenesis by SUMO and ubiquitin conjugation.* Nature, 2003. **425**(6954): p. 188-91.
161. Kannouche, P.L., J. Wing, and A.R. Lehmann, *Interaction of human DNA polymerase eta with monoubiquitinated PCNA: a possible mechanism for the polymerase switch in response to DNA damage.* Mol Cell, 2004. **14**(4): p. 491-500.
162. Leach, C.A. and W.M. Michael, *Ubiquitin/SUMO modification of PCNA promotes replication fork progression in Xenopus laevis egg extracts.* J Cell Biol, 2005. **171**(6): p. 947-54.
163. Schmutz, V., et al., *Requirements for PCNA monoubiquitination in human cell-free extracts.* DNA Repair (Amst), 2007.
164. Pfander, B., et al., *SUMO-modified PCNA recruits Srs2 to prevent recombination during S phase.* Nature, 2005. **436**(7049): p. 428-33.
165. Haracska, L., et al., *Opposing effects of ubiquitin conjugation and SUMO modification of PCNA on replicational bypass of DNA lesions in Saccharomyces cerevisiae.* Mol Cell Biol, 2004. **24**(10): p. 4267-74.
166. Papouli, E., et al., *Crosstalk between SUMO and ubiquitin on PCNA is mediated by recruitment of the helicase Srs2p.* Mol Cell, 2005. **19**(1): p. 123-33.
167. Tsui, C., A. Raguraj, and C.M. Pickart, *Ubiquitin binding site of the ubiquitin E2 variant (UEV) protein Mms2 is required for DNA damage tolerance in the yeast RAD6 pathway.* J Biol Chem, 2005. **280**(20): p. 19829-35.
168. Plosky, B.S., et al., *Controlling the subcellular localization of DNA polymerases iota and eta via interactions with ubiquitin.* Embo J, 2006. **25**(12): p. 2847-55.
169. Garg, P. and P.M. Burgers, *Ubiquitinated proliferating cell nuclear antigen activates translesion DNA polymerases eta and REV1.* Proc Natl Acad Sci U S A, 2005. **102**(51): p. 18361-6.
170. Haracska, L., et al., *Ubiquitylation of yeast proliferating cell nuclear antigen and its implications for translesion DNA synthesis.* Proc Natl Acad Sci U S A, 2006. **103**(17): p. 6477-82.

171. Guo, C., et al., *Mouse Rev1 protein interacts with multiple DNA polymerases involved in translesion DNA synthesis.* Embo J, 2003. **22**(24): p. 6621-30.
172. Tissier, A., et al., *Co-localization in replication foci and interaction of human Y-family members, DNA polymerase pol eta and REVl protein.* DNA Repair (Amst), 2004. **3**(11): p. 1503-14.
173. Ohashi, E., et al., *Interaction of hREV1 with three human Y-family DNA polymerases.* Genes Cells, 2004. **9**(6): p. 523-31.
174. D'Souza, S. and G.C. Walker, *Novel Role for the C-terminus of Saccharomyces cerevisiae Rev1 in Mediating Protein-Protein Interactions.* Mol Cell Biol, 2006.
175. Hoeller, D., et al., *Regulation of ubiquitin-binding proteins by monoubiquitination.* Nat Cell Biol, 2006. **8**(2): p. 163-9.
176. Kannouche, P., et al., *Localization of DNA polymerases eta and iota to the replication machinery is tightly co-ordinated in human cells.* Embo J, 2003. **22**(5): p. 1223-33.

Chapter 2:

Variant UBZ Domain of *S. cerevisiae* Polymerase eta is required for its damage-induced chromatin-association

Rachel Woodruff and Graham C. Walker.

SUMMARY

I have constructed a set of substitution mutations to alter the sequence of the C-terminus of the translesion synthesis polymerase eta (η) from *Saccharomyces cerevisiae*. I find that the UBZ (ubiquitin binding zinc finger) domain of polymerase η is both highly conserved and functionally important. Intriguingly, although the UBZ domain of human polymerase η requires a zinc finger motif to stabilize its structure, my genetic data indicate that the functional contribution of the UBZ domain from *S. cerevisiae* polymerase η to survival of UV-induced DNA damage does not require zinc binding. I propose that the UBZ domain from *S. cerevisiae* polymerase η is a rare zincless UBZ domain. I have assayed the chromatin-association state of the translesion synthesis polymerase η in *S. cerevisiae*. Polymerase η is found in the chromatin-associated fraction in the absence of exogenous DNA damage, and the amount of chromatin-associated polymerase η increases in response to UV-induced DNA damage. Furthermore, a UBZ mutant which disrupts polymerase η function also precludes this damage-induced increase in chromatin-association, consistent with a role for the UBZ domain in recruitment of polymerase η to damaged DNA. In addition, I identify some potential phosphorylation sites in the sequence of *S. cerevisiae* polymerase η.

INTRODUCTION

All organisms are equipped with an array of well-regulated DNA damage response mechanisms which allow them to survive and to maintain the continuity of genetic information. DNA damage tolerance mechanisms are needed to allow cells to continue normal cellular processes, such as DNA replication and cell cycle progression, which otherwise would be disrupted by DNA lesions. One such DNA damage tolerance mechanism is translesion synthesis (TLS), in which specialized DNA polymerases directly replicate a damaged DNA template.

Polymerase (pol) eta (η), a TLS polymerase encoded by *RAD30* in *S. cerevisiae,* functions in the *RAD6* post-replicative repair (PRR) epistasis group [1], which includes both error-prone and error-free pathways that are activated by Rad6 and Rad18 [2-4]. The mechanism by which Rad6 and Rad18 activate the PRR pathways has recently been elucidated. Rad6, an E2 ubiquitin conjugator, and Rad18, an E3 ubiquitin ligase, form a heterodimer which associates with single-stranded DNA at stalled replication forks [5, 6]. The Rad6/Rad18 heterodimer ubiquitinates PCNA at lysine 164 in response to DNA damaging agents such as UV and MMS [7]. Mono-ubiquitinated PCNA localizes to sites of DNA damage. PCNA can also be polyubiquitinated or SUMOylated at the same site [7, 8]. Measurements of sensitivity to UV radiation indicate that a *pol30K164R* mutation, which prevents any modification of PCNA at lysine 164, is epistatic to *rad30* [8, 9]. Monoubiquitination of PCNA is thought to promote the recruitment of pol η to stalled replication forks, as it is required for the damage-induced increase in pol η foci which is observed in mammalian cells [10].

Pol η is a member of the Y family of DNA polymerases, which is made up of the pol η, pol κ/DinB/PolIV, pol ι, Rev1, and PolV sub-families [11]. The polymerases of the Y family share a conserved polymerase domain, composed of five motifs, which form a right hand structure including Fingers, Thumb, and the Palm domain where catalysis takes place. All the Y family DNA polymerases are involved in the bypass (translesion synthesis) of DNA damage.

Pol η is broadly conserved among eukaryotes, where it bypasses a variety of DNA lesions [12], but is particularly important for its ability to accurately and efficiently bypass UV radiation-induced cyclobutane pyrimidine dimers (CPDs) [13, 14]. Closest to

the N-terminus is the Y family polymerase domain, followed by the PAD (Palm Associated Domain), also known as the Little Finger, which binds DNA and is required for DNA polymerase activity *in vitro* [15].

In addition to these domains, most pol η homologs include another 100 to 200 amino acids at their C-termini. The C-terminal region of pol η is the region of the protein whose structure and functions are least characterized. It is not required for pol η's catalytic activity, as a truncated form of pol η is capable of TLS *in vitro* [16]. Instead, the C-terminus is known to mediate protein-protein interactions. One of these is the interaction between pol η and PCNA [17]. PCNA is a homotrimeric ring-shaped protein which is loaded onto DNA at primer termini and acts as a processivity factor for replicative polymerases, but also regulates access to DNA for many DNA damage repair and tolerance factors [18]. The very C-terminus of pol η harbors its PCNA interaction motif (PIP box), as well as a nuclear localization sequence (NLS). In mammals, pol η's C-terminus interacts with Rev1 and Rad18 [19-21] (See Chapter 3). The C-terminal region of mammalian pol η is also required for its re-localization into nuclear foci in response to DNA damage [17], pointing to an important role in the regulation of pol η recruitment to the DNA. Between the PAD and the NLS of *S. cerevisiae* pol η is a region of about 100 amino acids which has been the focus of this study.

In this chapter, I present the first study of chromatin-association of pol η in yeast, showing a DNA damage-induced increase in chromatin-associated pol η. As one approach to a better understanding of the regulation and function of pol η, I have performed a thorough analysis of the C-terminus of *S. cerevisiae* polymerase η using both bioinformatic and genetic approaches. My mutational analysis of the conserved motif surrounding the degenerate C2H2 zinc finger demonstrates that it is required for pol η's contribution to survival of UV-induced DNA damage and for pol η's damage-induced increase in chromatin-association. However, my genetic studies strongly suggest that these functions do not depend upon coordination of zinc. These findings are remarkable in light of other concurrent studies which found that this motif is in fact a conserved ubiquitin interaction domain, now called the UBZ (ubiquitin binding zinc finger) domain [22], whose structure and ubiquitin-interacting function in human pol η depend upon an intact zinc finger [10, 23]. UBZ domains have also been identified in

Rad18 and Mgs1 (WRNIP1) [24]. Furthermore, I compared all available pol η sequences, and found that the UBZ domain is broadly conserved and nearly always includes a C2H2 zinc finger consensus sequence that should be capable of binding zinc. I identify a new UBZ domain in the transcription termination factor Pcf11, and find that the UBZ domains outside of pol η all include an intact zinc finger motif, suggesting that the UBZ domain of *S. cerevisiae* pol η has a rare variant structure which can be stabilized without coordinating zinc. Structural and functional implications are discussed.

My analysis includes the rest of the C-terminal sequence of *S. cerevisiae* pol η as well as the UBZ domain. I also identify a generally conserved region of predicted protein disorder located between the polymerase domains and the UBZ domain, which may explain why crystallization of pol η has required its truncation [15]. By bioinformatic analyses, I have identified potential phosphorylation sites, and I present genetic evidence that they are needed for pol η function.

RESULTS

Conservation of the UBZ domain. Early characterizations of pol η identified a putative C2H2 zinc finger motif located near the C-terminus of the protein [25, 26]. However, in *S. cerevisiae* pol η, the motif is degenerate. The consensus sequence of a C2H2 zinc finger is CxxC.....HxxxH, whereas *S. cerevisiae* pol η's sequence is CCKY.....HADYH (Figure 1c-d). In spite of this degeneracy, and perhaps because of the presence of the two adjacent cysteines, this motif of *S. cerevisiae* pol η is often referred to as a C2H2 zinc finger motif, using the same terminology that is used for the conserved C2H2 motifs in other pol η homologs.

When I initially aligned the C-termini of pol η homologs, I identified a conserved region including, but notably not limited to, the C2H2-like motif, as the most highly conserved amino acid sequence between the PAD domain and the NLS (Figure 1c) [27], suggesting that this region has functional significance separate from, or in addition to, the zinc finger itself. In the course of my study, this region was identified as a ubiquitin interaction domain, the UBZ (ubiquitin binding zinc finger) domain [27]. Similar UBZ domains have since been identified in polymerases kappa (κ) as well as in human Rad18,

WRNIP1/Mgs1, and Mtmr15 [24]. I searched for additional UBZ domains (using PSI-BLAST), and identified one novel UBZ domain in the transcription termination factor Pcf11 (Figure 1d).

To further investigate how well the UBZ domain is conserved among pol η homologs, I used BLAST and protein alignments to identify known or putative proteins homologous to pol η from about sixty different species [27]. The great majority of these pol η homologs contain a readily identifiable UBZ domain, indicating a high level of conservation. Several of the predicted pol η homologs that do not have a UBZ domain lack the C-terminus entirely (Supplemental Table 1a). The existence of truncated pol η homologs raises the possibility that, in some species, functions normally performed by the C-terminus are instead carried out by a protein interaction partner of pol η.

The broad conservation of the UBZ domain among pol η homologs is consistent with it performing one or more functions important to pol η activity and/or regulation. The known function of the UBZ domains in pol κ, Rad18, and WRNIP1, as well as pol η, is to bind ubiquitin non-covalently [22, 24, 28]. I therefore searched the UBZ-free pol η homologs from *Arabidopsis thaliana, Caenorhabditis elegans, Oryza sativa,* and *Leishmania major* for an alternative ubiquitin interaction motif, the UBM (ubiquitin binding motif), which is found in the pol η orthologs Rev1 and pol ι [22]. Intriguingly, one pol η homolog, from *A. thaliana,* contains a UBM-like motif (Figure 1e) instead of a UBZ domain. These results imply that the interaction with ubiquitin is important for the function of pol η in most species.

Among the many pol η homologs with UBZ domains, I found that all but two include the canonical C2H2 zinc finger sequence, CxxC....HxxxH. The two degenerate zinc fingers are pol η from *S. cerevisiae*, as previously mentioned, and a predicted protein from the wasp *Nasonia vitripennis* (accession number XP_001607964). This suggests that, in most species, the domain is a true zinc finger. Indeed, a recent NMR study determined the structure of the UBZ domain from human pol η, revealing that it is indeed a zinc-coordinating beta-beta-alpha zinc finger (see Chapter 1) [23]. Also in human pol η, the UBZ domain's interaction with ubiquitin can be disrupted by substitution of alanine for one of the conserved, zinc-coordinating histidines or cysteines [10, 22, 23]. Based on these results, one might predict that the degenerate UBZ domain of

S. cerevisiae pol η would fold improperly, due to failure to coordinate zinc, and therefore would not be functional. However, my finding that the UBZ domain of *S. cerevisiae* pol η is required for its function suggests that it is a properly folded, functional motif.

Mutational analysis of the UBZ motif of *S. cerevisiae* pol η. My observations led me to conduct a mutational analysis to learn, first, whether the UBZ domain of *S. cerevisiae* pol η affects its function and, second, whether it might coordinate zinc in spite of its degenerate C2H2 sequence. I therefore mutated specific conserved residues within the UBZ domain, both singly and in groups, and mutants were assayed for their ability to rescue the UV sensitivity of a *rad30* null strain (Figure 2).

My results clearly demonstrated that the degenerate UBZ is required for pol η function. Substitution of leucines for both histidines (H568L+H572L) resulted in a dramatic loss of function, close in severity to the null or the catalytic dead mutant (in which alanines are substituted for two residues of the catalytic triad, D155 and E156) (Figure 2b; Figure 3c, e). Because this phenotype could have been caused by a failure to express the protein or by degradation of the mutant protein, I tagged both wildtype pol η and H568L+H572L double mutant pol η, and compared their expression levels by immunoblotting. To avoid any tag-specific effects on pol η, I used two different tagged forms of pol η: one with an N-terminal 6His tag, the other with a C-terminal –TEV-ProA-7His tag. Neither of these tags interferes with the function of the wildtype protein ([12] and Chapter 3, Figure 4a). In both cases, I observed, by immunoblotting, that the cellular levels of the mutant pol η was similar to that of the wildtype protein, while, regardless of the tag, the H568L+H572L double mutant exhibited a severe defect in UV survival (Figure 3a-d). These results demonstrated that the H568L+H572L double mutant pol η is expressed but inactive. These results demonstrate that the UBZ domain of *S. cerevisiae* pol η is functional and is required for pol η function, even though it has a degenerate sequence.

To gain greater insight into the function disrupted by the H568L+H572L double mutation, I tested the mutant for a dominant negative effect. I assayed the UV sensitivity of a strain in which wildtype pol η is expressed endogenously, while the H568L+H572L double mutant is expressed from a plasmid (Figure 3e). The presence of the H568L+H572L double mutant did not cause increased UV sensitivity in the wildtype

background (Figure 3e). Thus, like the catalytically dead mutant shown here as a control, the H568L+H572L double mutant results in a recessive phenotype, demonstrating that it does not interfere with the function of the wildtype protein.

These results demonstrate that the UBZ domain is essential to pol η's function in survival of UV-induced DNA damage. This conclusion was corroborated by another recent study, in which mutation of the conserved aspartate residue (D570) of *S. cerevisiae* pol η was shown to disrupt its function [29]. Additionally, the authors found that the UBZ domain of yeast pol η, like its zinc-coordinating human counterpart, physically interacts with ubiquitin [30]. A simple hypothesis is that the H568L+H572L double mutant disrupts the physical interaction between pol η and ubiquitin, and that this interaction is required for pol η function. Taken together, these results implied that, despite having a degenerate zinc finger sequence, the UBZ domain of *S. cerevisiae* pol η does have a functional structure. This raised the question of whether the variant UBZ is stabilized without binding zinc.

My analysis of other mutations disrupting the C2H2-like motif demonstrate that loss of any potential capacity to coordinate zinc is not sufficient to cause the severe phenotype that results from the H568L+H572L double mutant. If zinc coordination were necessary for the function of *S. cerevisiae* pol η's UBZ domain, then substitution mutations at the cysteine or either histidine individually should result in the same loss of function. I tested single site substitutions of either alanine or leucine for each of the histidines individually, and for both of the adjacent cysteines (since either of them could, in principle, be involved in zinc coordination). Notably, the C552R+C553R double mutant (Figures 2c and 3a), and the H572A (Figure 2b) and H572L (Figure 3c) single mutants, had no detrimental effect on the ability of pol η to rescue the UV sensitive phenotype of a *rad30* null strain, while H568A (Figure 2b) and H568L (Figure 3c) single mutations resulted in a much milder defect in UV sensitivity than is seen in the H568L+H572L double mutant (Figures 2 and 3). This result in *S. cerevisiae* contrasts dramatically with the effect of substituting alanine either for one of the conserved cysteines or for one of the conserved histidines in human pol η, which results in a severe (thought not complete) loss of function [10, 22]. Thus it appears that, unlike human pol η, yeast pol η's function does not depend on a zinc-coordinating UBZ.

Therefore, I conclude that this variant UBZ domain has a function which is independent of any structural requirement for zinc binding. This is consistent with a hypothesis suggested by its sequence: namely, that the UBZ domain of *S. cerevisiae* pol η forms a "zincless finger", in which the structure (and function) of the zinc finger are conserved without requiring zinc for stabilization. A zincless finger structure has been observed once before in a natural protein [31], and has also been successfully engineered [32, 33].

Another very recent study, by Acharya et al. [34], made a similar finding with respect to mutation of the cysteines of *S. cerevisiae* pol η's UBZ domain. Intriguingly, in the same study, the authors substituted alanines for both histidines (H568A+H572A), and observed no significant defect in UV survival of the resulting mutant pol η. This result shows that loss of the potentially zinc-binding histidines does not affect pol η function. This result confirms that the severe loss of function resulting from my H568L+H572L double mutation is not caused by a loss of zinc binding, but is the result of a structural perturbation which is caused by substitution of leucines, but not alanines, for the histidine residues.

This leaves open the question of what function is disrupted by the H568L+H572L mutation. The ability of the UBZ domain of human pol η to interact with ubiquitin is dependent on zinc coordination [10, 22, 23], presumably due to loss of structure of the UBZ domain in the absence of zinc. When interpreting the mutant phenotype, Acharya et al. [34] assume that the same is true in yeast, and therefore conclude that *S. cerevisiae* pol η's UBZ domain-mediated interaction with ubiquitin is not essential for pol η function distinct from ubiquitin binding. They therefore posit that the defect of the previously studied UBZ mutation, D570A[29, 34], results from disruption of an unknown zinc-finger-independent function. However, ubiquitin binding would not be disrupted by the mutations used in their study if the UBZ motif of *S. cerevisiae* pol η folds independently of zinc, either into a zincless zinc finger, or into a structure which differs more from the structures of other UBZ domains but is still capable of interacting with ubiquitin.

I constructed two more UBZ domain mutants in addition to the cysteine and histidine mutants described above. One of these is an alanine patch (patch-548) at positions 548 to 551, of the sequence PKLE. These amino acids immediately precede the

first cysteine of the C2H2-like motif (Figure 1c). The other, Y571A, is a substitution of alanine for the well-conserved tyrosine residue at position 571, which is located between the two histidines. Both of these mutations resulted in mild but significant defects in UV survival (Figure 2d-e).

It is possible to rationalize the effects of all of my mutants without positing a function of the UBZ domain beyond ubiquitin binding. The partial loss of function caused by the single mutations H568A, H568L, and Y571A, (Figures 2b, 2d, 3c) might result from a weakening of the UBZ domain's interaction with ubiquitin by each mutation. In the structural model of the human pol η UBZ domain's interaction with ubiquitin, the ubiquitin-interaction surface of the UBZ domain is one face of the alpha-helix in the β–β–α fold (See model in Chapter 1 Figure 5) [23]. If we assume a similar (though zincless) structure of the yeast pol η UBZ domain, it places both the histidines and tyrosine 571 on the helix close to, but not directly involved in, the ubiquitin interaction surface. This assumption is consistent with the finding that the conserved aspartate residue (D570 in *S. cerevisiae*), which was shown by NMR to be directly involved in the interaction with ubiquitin in the UBZ of human pol η [23], is also required to allow *S. cerevisiae* pol η to bind ubiquitin [29]. Thus, substitutions at the individual histidines, H568 and H572, or at Y571, could interfere with the ubiquitin binding surface by altering the structure of the helix. Similarly, the finding that the H568L+H572L double mutation causes a much greater defect than substitution (of either leucine or alanine) for either histidine alone (Figures 2b and 3c), or of alanines for both [34], cannot be due to loss of zinc-binding, but can be explained by a cumulative steric disruption of the structure of the ubiquitin-interacting face of the UBZ domain.

The cysteines, in contrast, are located away from the interaction interface in human pol η's UBZ domain, and would not be expected to have any direct effect on the interaction surface if zinc coordination is not required to stabilize the entire structure. Therefore, the finding that the C552R+C553R double mutant is fully functional in UV survival (Figure 2c and 3a) is not inconsistent with an important role for ubiquitin binding in pol η's function.

The patch-548 mutation, which causes a partial defect in UV survival, substitutes an alanine patch for the residues immediately N-terminal to the cysteines. Substitution of

these residues is unlikely to affect ubiquitin binding, based on the structural model, but might partially destabilize the interior structure of the UBZ domain. Another possibility is that the lysine at position 549 could be a site of modification by either SUMO or ubiquitin. This motif is similar to the SUMO conjugation consensus sequence (PK-E), but there is currently no evidence that pol η is SUMOylated. Pol η is known to be covalently modified by ubiquitination: a ubiquitin association distinct from, but apparently dependent on, the non-covalent ubiquitin interaction mediated by the UBZ domain [10, 22, 29, 35]. Although there is not a known consensus sequence for ubiquitination sites, one known feature of the sequence surrounding sites of ubiquitin conjugation is that they tend to be enriched for serine and threonine residues [36]. The presence of a threonine at position 547 (see Figure 1c) is therefore consistent with the possibility that K549 may be a site of ubiquitination.

DNA damage causes a UBZ domain-dependent change in chromatin-association of pol η in yeast. In mammalian cells, the accumulation of pol η foci in response to DNA damage is dependent on a functional UBZ domain [10] as well as on Rad18 and PCNA [19]. PCNA is ubiquitinated at K164 by Rad6/Rad18 in response to DNA damage [7]. One model of pol η foci formation is that the pol η molecules are recruited into foci by their physical interaction with PCNA at sites of DNA damage. The UBZ domain is thought to play a central role in this process by promoting a stronger physical interaction of pol η with mono-ubiquitinated PCNA than with unmodified PCNA, thereby mediating focus formation specifically in response to DNA damage.

To address whether the UBZ domain of *S. cerevisiae* pol η is involved in pol η's association with damaged DNA, I used a well-established chromatin association assay, the chromatin spindown [37 Diffley 1997], to characterize the wildtype pol η and compare it with the H568L+H572L double mutant (Figure 4). Orc1, a protein which is almost entirely chromatin-associated [38] was used as a control to compare the amounts of pol η in the chromatin-associated fraction in the presence or absence of DNA damage. This is the first report to address the DNA association of pol η in *S. cerevisiae*. Intriguingly, I found that a significant amount of pol η is DNA associated even in the absence of DNA damage. The amount of wildtype pol η (relative to Orc1) found in the chromatin-associated fraction is greater after exposure to UV than after mock-treatment

without exogenous DNA damage (Figure 4). The H568L+H572L double mutant is also found in the chromatin-associated fraction, but in contrast to the wildtype, the amount of chromatin-associated H568L+H572L pol η is decreased after UV irradiation (Figure 4). The D570A mutant, which has been shown to disrupt the ubiquitin interaction of pol η, behaved similarly to the H568L+H572L double mutant. These results are consistent with the UBZ domain functioning to promote chromatin-association of pol η in response to DNA damage.

Conservation of predicted disorder. Although I did not identify broadly conserved motifs outside of the UBZ domain, I observed that most pol η homologs include significant regions of predicted structural disorder on either side of the UBZ domain. I used the protein disorder prediction program PONDR [39, 40] to compare the regions of predicted disorder of several pol η homologs (Supplemental Figure 1). All the pol η homologs examined have regions of predicted disorder C-terminal to the UBZ domain. In most species analyzed, including human, *Xenopus*, mouse, chicken, *Drosophila*, and *S. pombe*, there is an additional region of predicted disorder N-terminal to the UBZ domain, such that the UBZ domain is sandwiched between two regions of predicted disorder. The prediction of a conserved region of protein disorder between the catalytic domain and the UBZ domain suggests the possibility of allosteric communication between the UBZ domain and the DNA-binding PAD domain. However, in the closely related *S. cerevisiae* and *Candida glabrata* pol η homologs, the sequence on the N-terminal side of the UBZ domain is not predicted to be disordered (Supplemental Figure 1). Because the region of predicted disorder between the PAD and UBZ is absent in *C. glabrata,* which has a normal UBZ sequence, as well as in *S. cerevisiae,* its absence seems to correlate with this particular lineage, rather than being an adjustment to an altered UBZ structure in *S. cerevisiae*. These yeast species may actually include disordered regions which are not predicted by PONDR [39, 40]. In addition, the C-terminal region of *C. elegans* pol η, which lacks a recognizable UBZ domain, is predicted to include several disordered regions, similar to the vertebrate pol η homologs. Given that *S. cerevisiae* pol η's UBZ is functionally conserved but of degenerate sequence, it should be noted that the pol η homologs, such as *C. elegans* pol η, in which I

have failed to identify a UBZ domain by sequence homology, may nevertheless contain a functionally similar domain of even more divergent sequence.

Additional mutations of the C-terminus of pol η. In the course of my analysis, I noted that some of the amino acids that flank the UBZ domain of *S. cerevisiae* pol η are conserved among ascomycetes species, although they are not more generally conserved (Figure 1b, c). To elucidate the functional significance such residues, I constructed *S. cerevisiae* pol η mutants in *RAD30* on a low-copy vector. *RAD30* expression was controlled by its own promoter, and fully rescued the UV sensitivity of a *rad30* null strain [1]. Mutant constructs were assayed for their ability to rescue the UV sensitive phenotype of the *rad30* null (Figure 5). Mutants which were unable to fully rescue the UV sensitive phenotype were shown to be expressed at a level similar to the wildtype pol η (data not shown).

The phenotypic effects of these mutants varied: some caused no defect, thereby demonstrating the specificity of the others, which resulted in partial loss of function. The most significant defect in UV survival was caused by the T514A mutation, which disrupts a potential phosphorylation site that is well conserved among fungi (Figure 5g). This is consistent with a model in which phosphorylation of the threonine at position 514 promotes pol η's activity. Two alanine patch mutations, patch-540 and patch-587, were both designed to disrupt a motif, SSK, which is not conserved at a specific location, but which recurs in pol η homolog sequences. Disruption of the SSK motif at positions 587 to 589, by alanine substitution for all three residues, resulted in a partial defect in UV survival (Figure 5c). Interestingly, substitution of aspartate for the first serine of this patch resulted in an increase in UV survival relative to the wildtype (Figure 5d), consistent with the possibility that phosphorylation at this site, in addition to T514, activates pol η for TLS.

Milder but reproducible defects in UV survival were also caused by replacing LLF with an alanine patch at positions 598-600 (Figure 5e) or by disruption of another predicted phosphorylation site [41] in the S592A mutation (Figure 5f). Substitution of an alanine patch for the sequence VDM, residues 516 to 518, did not impair pol η function at all (Figure 5a). Neither did substitution of alanines for two serines at positions 540-541, an SSK motif and potential phosphorylation site (Figure 5b). The partial loss of

function phenotypes resulting from mutations of potential phosphorylation sites, at T514, S587, and S592, suggest that phosphorylation at several partially redundant sites plays a role in activation of pol η,

In an effort to learn whether phosphorylation plays a role in pol η regulation or activity, I purified pol η from *S. cerevisiae* and analyzed it by mass spectrometry (by Forest White). No phosphorylation sites were detected on pol η from undamaged cells. A different assay was used to test a broader range of conditions. For this purpose, I immunoprecipitated pol η from undamaged cells and from cells which had been subjected to UV radiation, either when growing asynchronously or upon release from G1. Immunoprecipitated pol η was separated by polyacrylamide gel electrophoresis and the gel was stained specifically for phosphoproteins. Regardless of DNA damage or cell cycle stage, phosphorylated pol η was not observed (data not shown). However, both because pol η may have been de-phosphorylated in the extract, and because only a limited set of conditions was assayed, these experiments do not rule out the possibility that pol η is regulated by phosphorylation.

DISCUSSION

My analysis of the C-terminus of pol η has offered insights into the structure and function of the important TLS DNA polymerase η. I have shown that the degeneracy of the C2H2 motif within the UBZ domain of *S. cerevisiae* pol η is a very unusual variation in a well-conserved domain. My mutational analyses demonstrate that the UBZ domain of *S. cerevisiae* pol η is critically required for its function, but that the putative zinc-binding residues within the UBZ domain are dispensable for pol η function in *S. cerevisiae* pol η. Although I have not formally ruled out the possibility that the UBZ domain forms a highly unusual zinc finger in spite of its degenerate sequence and also has an additional zinc-independent function which is critical for TLS, my results strongly suggest that the UBZ domain of *S. cerevisiae* pol η does not bind zinc. This leads me to propose that this unusual UBZ domain folds into a zincless finger. A zincless finger has been previously described in NEIL1, a human DNA repair endonuclease [31]. The zincless finger domain of NEIL1 forms a structure similar to an antiparallel β-hairpin

zinc finger, although it cannot bind zinc [31]. NEIL1's zincless finger is required for its activity, and is therefore thought to be a functional DNA-binding domain, as is the structurally similar zinc finger of its homolog from *E. coli,* Nei [31, 42].

Furthermore, I have studied the chromatin-association of pol η in yeast, and showed an increase in chromatin-associated pol η in response to DNA damage. This damage-induced increase in chromatin-association is dependent on an intact UBZ domain, which accounts, at least partially, for the extreme UV sensitivity of some UBZ domain mutants. I hypothesize that the damage-dependent increase in chromatin association of yeast pol η requires the UBZ domain for its interaction with ubiquitin, most likely the ubiquitin modification of PCNA. Additionally, I have identified potential phosphorylation sites of *S. cerevisiae* pol η, as well as a region of predicted protein disorder separating the PAD and UBZ domains in most other species.

Zinc is not required for UBZ domain function. I have shown that mutation of the UBZ domain of *S. cerevisiae* pol η, specifically the H568L+H572L double mutation, can cause a severe loss of function, resulting in a UV sensitive phenotype similar to a *rad30* null. However, I also confirm that the cysteines and one histidine of the C2H2 motif, which in the human homolog coordinate a zinc ion [23], can be replaced in *S. cerevisiae* without phenotypic consequences, indicating that the loss of function caused by the H568L+H572L double mutant is not due to disruption of the putative zinc finger. These histidines, however, are located within the most conserved part of the UBZ domain's sequence, among the residues which form an alpha-helix in human pol η [23]. The surface of this alpha-helix, which is similar to previously recognized ubiquitin interaction motifs (the UIM/IUIM and reverse UIM), interacts directly with ubiquitin in the NMR structure of the human pol η UBZ domain [23]. As the histidines at positions 568 and 572 are likely to be located in this alpha helix (see Chapter 1, Figure 5), the H568L+H572L double mutant could cause loss of function by disrupting the UBZ domain's interaction with ubiquitin. The substitution of leucines for both histidines could either interfere directly with the interaction, or could disrupt it indirectly, by altering the structure of the UBZ domain.

This hypothesis assumes that the ability to interact with ubiquitin is independent of zinc coordination in *S. cerevisiae,* unlike human pol η, in which the interaction with

ubiquitin can be lost due to substitution at just one of the histidines (H654) [10]. My finding that neither the cysteine mutant nor either single histidine mutant in yeast causes a dramatic UV survival phenotype, could suggest either that the interaction with ubiquitin is independent of zinc binding in *S. cerevisiae* pol η, or alternatively that the interaction with ubiquitin is not actually required for pol η's function, as suggested by Acharya et al [34]. However, the latter possibility would require that there is an additional, unknown, function which accounts for the significance and conservation of the UBZ domain and is independent of zinc binding. The degeneracy of the UBZ domain of *S. cerevisiae* pol η, and the results of my genetic analysis, can be explained more simply by the hypothesis that *S. cerevisiae* pol η contains a zincless UBZ domain, in which both the structure and the ubiquitin-interacting function are stabilized without zinc coordination.

An ongoing project seeks to differentiate these two possibilities. To determine whether the UBZ domain of *S. cerevisiae* pol η folds into a stable structure in the absence of zinc, I will assay the structure and zinc content of the purified UBZ domain. In addition, I will compare the ubiquitin-binding activities of the mutant and wildtype UBZ domains. If the interaction with ubiquitin, rather than some unknown function, is the key activity of the UBZ domain whose disruption causes altered chromatin-association and loss of function in the H568L+H572L double mutant and the D570A mutant [29], then the UBZ domain mutants that did not affect UV survival, such as the C552R+C553R double mutant, should support a normal ubiquitin interaction, while H568L+H572L double mutant should not. If, as I hypothesize, *S. cerevisiae* pol η's UBZ domain does not coordinate zinc, further study will be needed to learn whether its structure is essentially conserved relative to the canonical UBZ domain structure, analogous to a zincless zinc finger [31-33], or whether it forms a substantially different structure than human pol η's UBZ domain.

As well as being structurally interesting, the possibility that *S. cerevisiae* pol η contains a zincless UBZ domain has broad implications for models of the regulation of pol η function, specifically with respect to the functional significance of the interaction with ubiquitin, and the probability of one or more additional UBZ domain functions.

UBZ domain is required for damage-induced increase in chromatin-association. Here I have shown that pol η is significantly chromatin-associated even in

the absence of exogenous DNA damage. Furthermore, I have found that in response to UV, more pol η becomes chromatin-associated in a UBZ domain-dependent fashion. This is consistent with a model in which pol η is recruited to sites of DNA damage by a UBZ domain-mediated interaction with ubiquitinated PCNA. Although the UBZ domain is not required for chromatin-association of pol η in the absence of DNA damage, disruption of the UBZ domain's function causes the pool of chromatin-associated pol η to decrease in response to UV. This suggests that the factors that promote pol η's association with chromatin are different in the presence and absence of exogenous DNA damage, and implies that the H568L+H572L double mutation disrupts a damage-specific interaction. The UBZ domain promotes pol η's interaction with its DNA damage-associated interaction partner, ubiquitinated PCNA, such that pol η lacking a functional UBZ domain cannot compete for this damage-associated interaction with other TLS polymerases or other PCNA- and ubiquitin-interacting proteins. Importantly, chromatin association alone must not be sufficient for pol η's function; if it were, the H568L+H572L double mutant, because it is detectably chromatin-associated even after damage, would be expected to have only a mild defect in UV survival, instead of the severe loss of function I have observed. This implies that pol η must be precisely located and/or oriented for activity, or may require a specific interaction to activate its TLS activity, and that the UBZ plays a role beyond general chromatin association.

Distribution and functions of the UBZ domain. A variety of ubiquitin binding domains have been characterized [43]. The presence of two different ubiquitin interaction motifs in different members of the Y family DNA polymerases, all of which derived from a common polymerase ancestor, suggests a functional difference between the two ubiquitin interaction motifs. Polymerases η and κ contain UBZ domains, but polymerase iota (ι) and Rev1 interact with ubiquitin via UBMs (Ubiquitin Binding Motifs) [22] [35, 44]. Like the UBZ domain, UBMs are also found outside of the Y family DNA polymerases; specifically, I have identified homologous motifs in Tom1 (Trigger of Mitosis or Temperature dependent Organization in Mitotic nucleus) and other HECT-domain E3 ubiquitin ligases (data not shown). It is particularly striking that pol ι, whose sequence is generally more similar to pol η than to Rev1 [11, 45], contains the UBM rather than a UBZ domain. The distribution of UBZ domains and UBMs among the Y

family polymerases may be the result of an evolutionarily conserved functional difference between the two ubiquitn-binding motifs. This difference might reside in their effects on the ubiquitin-interacting protein or the strength of the interaction with ubiquitin. Like the UBZ domain, the UBM can also mediate a functional interaction with ubiquitinated PCNA, as it does for Rev1 [35, 44].

The observation that UBZ domains are found predominantly in DNA damage response proteins [24] also suggests that the UBZ domain is not functionally interchangeable with other ubiquitin interaction domains. To gain insight into UBZ domain-specific functions, I have compared the functions of the proteins known to contain UBZ domains. Many of these proteins do have common interaction partners in addition to ubiquitin. Four of the UBZ domain proteins, pol κ, pol η, Rad18 and Mgs1, are known to physically interact with PCNA [7, 46, 47]. Although interaction with PCNA does not absolutely require the UBZ domain, the structure of the UBZ domain may enable it to uniquely influence the character of the interaction with ubiquitinated PCNA. Another potential interaction partner of several UBZ proteins is the Werner syndrome protein, WRN (Sgs1 in yeast), which interacts physically with Mgs1/WRNIP1 [48] and stimulates the extension activity of TLS polymerases *in vitro* [49].

The UBZ domain has been shown to play a role in self-association as well. Rad18 requires its UBZ domain for its self-interaction in both human and yeast [50, 51] and for the formation of replication-independent Rad18 foci [52 Usami 2006]. The UBZ domain of pol η is required for damage-induced accumulation of pol η foci [10], possibly mediated by a similar self-interaction. The formation of detectable nuclear foci by pol κ, pol η, Rad18 and Mgs1, may depend on self-association as well as on the interaction with ubiquitin/PCNA. However, it is doubtful that the UBZ domain mediates interactions among the different UBZ domain proteins because, although pol η and Rad18 do physically interact [19], and their interaction may involve pol η's UBZ domain, the accumulation of pol η foci after DNA damage depends on Rad18 but not on Rad18's UBZ domain or on Rad18 focus formation.

The UBZ domain is required for covalent ubiquitination of several UBZ domain-containing proteins, including Rad18 [50], WRNIP1 (at several sites) [24], and pol η [22, 29 Ulrich, 2006]. Monoubiquitination of yeast pol η does not require either Rad6 or

Rad18 [29, 53], but is diminished in their absence [53]. Because the UBZ domain is required for pol η ubiquitination, it has been suggested that pol η ubiquitination could involve autocatalytic ligation of an activated ubiquitin from an E1 or E2 onto pol η by the UBZ domain [54]. This would not be unusual, as many ubiquitin-interacting proteins are known to promote their own mono-ubiquitination [55-57].

The functional significance of pol η's ubiquitination is not yet understood, but it may play roles in self-association (through the UBZ domains), in regulation of interactions with ubiquitinated proteins such as PCNA, or in proteosomal degradation. The hypothesis that pol η ubiquitination targets it for proteosomal degradation has been suggested by the observation of poly-ubiquitinated pol η in a protease-deficient yeast strain [58], namely, that mono-ubiquitination of pol η may serve as the substrate for further polyubiquitination to promote protein degradation. However, my results, presented in Chapter 4 of this thesis, show that pol η protein levels do not change significantly in response to DNA damage, and others have found that pol η is not rapidly degraded ([53]; Mary Ellen Wiltrout and Graham Walker, unpublished results). These and other results presented in Chapter 4 suggest that the degradation observed by Skoneczna et al. [58] was due to their use of a destabilizing tag to detect pol η. I therefore propose that pol η ubiquitination is more likely to modulate pol η's interactions with itself, with ubiquitinated PCNA, or with other ubiquitin-binding proteins.

Mutational analysis of the UBZ domain of pol η does not yield insight into the hypothesis that pol η's ubiquitination negatively regulates its interaction with ubiquitinated PCNA, as mutating the UBZ domain simultaneously disrupts both the interaction with ubiquitinated PCNA and the ubiquitination of pol η. A direct assay of the effect of pol η ubiquitination would require identifying the ubiquitination site and mutating that alone, so as to disrupt ubiquitination without also disrupting the non-covalent interaction with ubiquitin.

The role of phosphorylation in TLS. Pol η is known to be ubiquitinated[29], and the recent finding that its paralog Rev1 is phosphorylated in yeast [59] led me to ask whether pol η is phosphorylated as well. Although I did not identify a phosphorylated species of pol η, my mutational analysis did identify a potential phosphorylation site which contributes to survival after UV-induced DNA damage. The UV sensitivity of the

T(514)A mutant, as well as the lesser defects of the S(592)A mutant and the alanine patch mutant at position 587, suggest that pol η may be activated by phosphorylation at multiple, partially-redundant sites. Recent evidence has pointed to a role for phosphorylation in the activity of pol ζ [60] as well as Rev1 [59, 60]. Therefore, one possible role of this putative phosphorylation of pol η may regulate a direct or indirect interaction with pol ζ and/or Rev1 (See Chapters 3 and 4). Further study is needed to determine whether pol η is, indeed, phosphorylated, and if so, under what conditions.

MATERIALS AND METHODS

Strains and plasmids

Yeast strains are listed in the table below. For the experiment shown in Figure 3a, I used a BY4741/BY4742 derivative strain constructed by mating of yeast deletion project strains 14255 and 6430. All other experiments use derivatives of W1588-4C (*MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5*), which is a W303 strain in which a *rad5* mutation is replaced by the wildtype *RAD5* sequence [61]. Deletion of *RAD30* was constructed by gene replacement using PCR amplified *rad30::KanMX* from the Saccharomyces Genome Deletion Project strain 4255. To produce the TEV-ProA-7His tagged Rad30 fusion protein, the tag cassette was amplified from pYM10 [62] and inserted by homologous recombination to replace the stop codon of *RAD30*. See Table 1 for additional information on strains. The plasmids pEGUh6 [63] and pEGUh6-RAD30 [12], of which the latter expresses 6His-Rad30 from Pgal, were the kind gifts of Zhigang Wang. Roger Woodgate and John McDonald generously provided the plasmid pJM96 (*RAD30* cloned into pRS415) [1]. See Table 2 for additional plasmid information. Mutants were constructed by site-directed mutagenesis using QuikChange. Mutants are listed in Table 3.

UV treatment

Cultures were grown to saturation for 3 days at 30 degrees C, diluted in water to approximately 6 CFUs per microliter, and equal amounts of the dilution were spread on

each of several selective minimal medium plates. Within 30 minutes, plates were irradiated using a G15T8 UV lamp (General Electric) at 254nm, 1 J/m^2 per second for varying amounts of time. After irradiation, plates were kept in the dark at 30 degrees C for 3 days before colonies were counted. The data shown are averages of at least three independent cultures, and error bars represent standard error.

Chromatin purification

For chromatin purification, exponentially growing cultures were collected by centrifugation and resuspended to OD600~0.5 in water. Cultures were then split, so that one half was not treated, while the other half was poured into a wide plate and irradiated with 50 J/m^2. Irradiated and untreated cells were separately collected by centrifugation immediately after UV treatment, resuspended in selective media, and incubated at 30 degrees C until 120 minutes after irradiation. The incubation time was based on previous work suggesting that both Rev1 and Rad30 appear to respond to DNA damage on this time scale [1, 58, 64, 65].

Chromatin-associated fractions were prepared as previously described [37 Diffley 1997] with modifications. Briefly, exponentially growing cells were pre-incubated in pre-spheroplasting buffer (100 mM Pipes-KOH, pH9.4), spheroplasted by treatment with Zymolyase in spheroplasting buffer (0.6 M Sorbitol, 10 mM DTT, 50 mM potassium phosphate, pH 7.5), then washed three times in lysis buffer (0.4 M Sorbitol, 150 mM potassium acetate, 2 mM magnesium acetate, 20 mM Pipes-KOH, pH 6.8). Cells were lysed by resuspending at no more than $8x10^8$ cells per mL in lysis buffer plus 1% Triton X-100 and protease inhibitors (Roche Complete cocktail). After 5 minutes on ice, the lysate was underlayed with 150 mL of 30% sucrose and the chromatin was pelleted by centrifugation at 15,800 RCF for 15 minutes. This first supernatant was carefully removed, and the pellet was washed once in lysis buffer, then re-collected by centrifugation at 15,800 RCF for 10 minutes. The soluble fraction was prepared by re-centrifugation of the first supernatant to ensure that it was free of all precipitate. The chromatin-associated fraction was solubilized from the washed pellet by resuspending it in lysis buffer plus 150 mM NaCl, 5 mM $CaCl_2$ and 0.5 mM $MgCl_2$ with MNase (Worthington) for 10 minutes at room temperature to digest the DNA. After

centrifugation at 15,800 RCF for 15 minutes, the chromatin associated fraction, separated as the supernatant, was carefully saved. Samples were then analyzed by immunoblot.

Immunoblotting

Whole cell extracts were prepared by trichloroacetic acid precipitation [62]. Protein samples were separated on 7.5% or 4-12% SDS-polyacrylamide gels, transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore), and probed with appropriate antibodies. ProA-tagged protein was detected using rabbit peroxidase anti-peroxidase (PAP) antibody diluted 1:5,000 (Sigma); the 6His tag, PGK, and Orc1 were detected using mouse anti-His (Qiagen), mouse anti-PGK (Molecular Probes) and rabbit anti-ORC (kind gift of Stephen Bell) antibodies, respectively, all diluted 1:1,000. Blots were visualized using HRP-conjugated goat anti-mouse or anti-rabbit secondary antibody (Pierce) and SuperSignal West Dura Extended Duration Substrate (Pierce) or SuperSignal West Femto Maximum Sensitivity Substrate (Pierce).

Phosphoprotein detection

For phosphoprotein detection assays, protein was separated on 4-12% polyacrylamide gels (Cambrex) and phosphorylated proteins were detected by Pro-Q Diamond Phosphoprotein stain (Invitrogen) according to manufacturer's protocol. The sensitivity of the stain is 1-16 ng/lane.

Sequence analysis

Alignments were made using T-Coffee [27]. BLAST and PSI-BLAST were used to identify homology in the database [66, 67]. Predictions of protein disorder were performed by PONDR [39, 40]. Access to PONDR® was provided by Molecular Kinetics (6201 La Pas Trail - Ste 160, Indianapolis, IN 46268; 317-280-8737; E-mail: main@molecularkinetics.com). VL-XT is copyright©1999 by the WSU Research Foundation, all rights reserved. PONDR® is copyright©2004 by Molecular Kinetics, all rights reserved. NetPhosYeast 1.0 was used to predict phosphorylation sites in *S. cerevisiae* Rad30 protein [41]. I used additional motif prediction tools including: Motif Scanner, NetPhos 2.0 [68], and NetPhosK 1.0 [69].

TABLES AND FIGURES

Table 1. Yeast strains used in this study

Strain	Genotype
RWY10	*MATα leu2Δ1 his3Δ1 met5Δ0 ura3Δ0 rad5::kanMX rad30::kanMX*
W1588-4C	*MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5*
RWY13	*MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5 RAD30-TEV-ProA-7His::HIS3MX*
RWY15	*MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5 rad30::KanMX*

Table 2. Plasmids used in this study

Plasmid	Relevant gene	Description/Comments
pJM96	*RAD30*	pRS415 derivative containing *RAD30*
pEGUh6Rad30	*6His-RAD30*	pEGUh6 derivative expressing His-tagged Rad30p from a galactose-inducible promoter
pRW1001	*6His-rad30(C552R+C553R)*	pEGUh6-Rad30 derivative
pRW1002	*6His-rad30(H568L+H572L)*	pEGUh6-Rad30 derivative
pRW1004	*rad30(H572A)*	pJM96 derivative
pRW1005	*rad30(Y571A)*	pJM96 derivative
pRW1006	*rad30(H568A)*	pJM96 derivative
pRW1007	*rad30(T514A)*	pJM96 derivative
pRW1018	*rad30(patch-516)*	pJM96 derivative
pRW1020	*rad30(patch-540)*	pJM96 derivative
pRW1021	*rad30(patch-548)*	pJM96 derivative
pRW1022	*rad30(patch-587)*	pJM96 derivative
pRW1024	*rad30(patch-598)*	pJM96 derivative
pRW1025	*rad30(H568L+H572L)*	pJM96 derivative
pRW1027	*rad30(C552R+C553R)*	pJM96 derivative
pRW1029	*rad30(D155A+E156A)*	pJM96 derivative containing catalytically inactive *rad30* mutant [70]
pRW1031	*RAD30-YM10*	pJM96 derivative with tag: -TEV-ProA-7His
pRW1045	*rad30(H568L+H572L)-YM10*	pJM96 derivative with tag: -TEV-ProA-7His
pRW1046	*rad30(H568L)-YM10*	pJM96 derivative with tag: -TEV-ProA-7His
pRW1047	*rad30(H572L)-YM10*	pJM96 derivative with tag: -TEV-ProA-7His
pRW1048	*rad30(H568A)-YM10*	pJM96 derivative with tag: -TEV-ProA-7His
pRW1049	*rad30(T514A)-YM10*	pJM96 derivative with tag: -TEV-ProA-7His
pRW1050	*rad30(patch-587)-YM10*	pJM96 derivative with tag: -TEV-ProA-7His
pRW1035	*rad30(S587D)-YM10*	pJM96 derivative with tag: -TEV-ProA-7His
pRW1051	*rad30patch-598)-YM10*	pJM96 derivative with tag: -TEV-ProA-7His
pRW1052	*rad30(D155A+E156A)-YM10*	pJM96 derivative with tag: -TEV-ProA-7His
pRW1054	*rad30(S592A)-YM10*	pJM96 derivative with tag: -TEV-ProA-7His
pRW1055	*rad30(patch-548)-YM10*	pJM96 derivative with tag: -TEV-ProA-7His

Table 3: Nomenclature for *RAD30* mutants used in this study

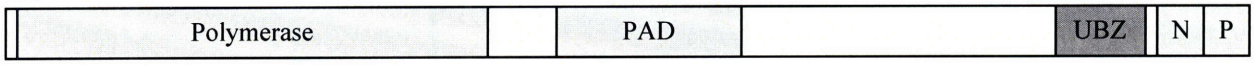
Mutant name	Mutant description
catalytic	Substitution of alanines for two residues of the catalytic triad, Asp155 and Glu156
H568L+H572L	Substitution of leucines for His568 and His572
C552R+C553R	Substitution of arginines for Cys552 and Cys553
H568L	Substitution of leucine for His568
H568A	Substitution of alanine for His568
H572L	Substitution of leucine for His572
H572A	Substitution of alanine for His572
Y571A	Substitution of alanine for Tyr571
D570A	Substitution of alanine for Asp570
Patch-548	Substitution of alanines for Pro548, Lys549, and Glu551
Patch-516	Substitution of alanine patch for Val516, Asp517 and Met518
Patch-598	Substitution of alanine patch for Leu598, Leu599 and Phe600
Patch-540	Substitution of alanines for Ser540 and Ser541
Patch-587	Substitution of alanine patch for Ser587, Ser588 and Lys589
T514A	Substitution of alanine for Thr514
S587D	Substitution of aspartate for Ser587
S592A	Substitution of alanine for Ser592

Figure 1. UBZ is broadly conserved in pol η, while flanking regions are not. A, domain architecture of human and yeast pol η. N represents the Nuclear Localization Sequence (NLS). P represents the PCNA interaction motif (PIP-box). **B,** Alignment of fungal pol η homologs for the region between the PAD and UBZ domains, positions 505 to 543 of *S. cerevisiae* pol η. Sites of substitution mutations are marked with asterisks (*). **C,** Alignment of pol η homologs from the start of the UBZ domain and to the NLS, positions 544 to 600 of *S. cerevisiae* pol η, showing broad conservation of the UBZ; the position of the conserved cysteine absent from *S. cerevisiae* pol η is highlighted in red. Sites of substitution mutations are marked with asterisks (*). **D,** Alignment of known and putative UBZ motifs found in *S. cerevisiae*. Alignments were made by TCOFFEE [27]. **E,** Alignment of residues 622 to 669 of *Arabidopsis thaliana* pol η with UBMs from pol ι and Rev1.

Figure 1

A.

H.



S. cerevisiae

B.

```
Ylip   GFVELPAGASIGHFFKTI--TTE-AREKAAVEKTKAQQVNTLWNK
Ncra   GFEDGVTGNMGIGAFLLK--GEE-AQASKTGSGTATVDSETEIRP
Pnod   GFEDGITNNKGIGSFLVR--GEE-AKAM----MSTGRSVSTGEPP
Cgla   NFDILDHQKTVLDMFGNQKQVFN-TKTI----SPKIEPVTEVEDK
Agos   NFDVLQPGKTIVDLLPRAPATPP-SP----------PARAASPAP
Klac   NFDIIDKGKTVLDMFGNRAISKS-SSTDNNVVKIEGEDIHSDSKG
Sbay   NFDILDLQKTVVDMFGNRANTFQ-SSIDK-----EREKKDSLGYT
Scas   NFEIIDQKKTVIDMFGQQAQIFQKNTANK-----EPELTVQGTDD
Sklu   NFEILDTGKSIVDMVGRQMHTIY-KRETS-----DGPTLPEEEND
Skud   NFDILDLQKTVVDMFGDQVHTFK-GSIGK-----GDEEKYPGLKD
Spar   NFDILDLQKTVVDMFGNQVHTFK-SSADK-----EDERKTTSSRD
Scer   NFDIIDLQKTVVDMFGNQVHTFK-SSAGK-----EDEEKTTSSKA
```

C.

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Hsap   LAAEDQVPCEKCG-SLVPVWDMPEHMDYHFALELQKSFLQPHSSN
Mmus   -ATEDQVLCEKCD-SLVPVWDMPEHTDYHFALELQKSFLQPCTSK
Cfam   MAAEDQVPCEKCG-SLVPVWEMPEHTDYHFALELQKSFLQPHSST
Btau   LAAEDQVPCEKCG-SLVPVWDMPEHSDYHFALELQNSFLQPHSSN
Ggal   VSPGDQQCCEKCG-GYVLAWELPEHMDYHFAVELQRSFQEPS---
Xlae   -SEEDIMCCEKCG--LKLVWEIPEHMDYHFAQELQDSFSAPSPSR
Aaeg   -TDDVTKACSECG-KLIPTANMPEHLDYHAARKLQIELNKLEMH-
Dmel   PSDPQMNQCPECK-AFIKCVDMPEHLDYHVAKNLQRELNQQDLRT
Spur   VPGEDEILCEKCQ-QIISVWDYPEHLDFHVALELQEQDRAEAKNT
Ncra   PQPIITLTCSRCNASLDSPEELQSHQDWHFAKELQEQERVSQTFV
Ylip   DEYIDTFTCPKCDKKYPI-DEEMEHSDWHVAVELSKA-NR----P
Pnod   QKTLDTYFCAQCN-IHLPTDEKAEHIDYHFALDLSKEMRHEERNP
Klac   PTMAENLTCKQCGETLQDKKLFQEHVDYHLSVQLSEQINGVSEN-TMLTHAERILLF
Skud   NRGTPKLECSKCQINFTDQKSFQEHGDYHLASKLSEGLNGAEET-KNLSFGEKRLLF
Spar   DEEIPKLECSKCQVTFTDQKAFQEHGDYHLALKLSEGLNGVEET-KNLSFGEKRLLF
Scer   DEKTPKLECCKYQVTFTDQKALQEHADYHLALKLSEGLNGAEESSKNLSFGEKRLLF
```

D.

```
Rad30  KTPKLECCKYQVTF---TDQKALQ-EHADYHLALKLS
Rad18  NEQMAQCPICQQFY----PLKALEKTHLDECLTLQSL
Mgs1   VEQLISCPICSRKVFF-S----LINSHLDICGKEKSK
Pcf11  RSKPNKCSVCGKRFGNSESEKLLQNEHLDWHFRINTR
```

E.

```
At pol eta     KTDEIDQSVFDELPVEIQRELRSFLRTNK
Hs Pol iota    LPEGVDQEVFKQLPVDIQEEILSGKSREK
               FPSDIDPQVFYELPEAVQKELLAEWKRTG
Hs Rev1        SPSQLDQSVLEALPPDLREQVEQVCAVQQ
               AFSQVDPEVFAALPAELQRELKAAYDQRQ
At Rev1        SLSQVDVSVLQELPEELRADVLGAFPSHR
```

Figure 2.

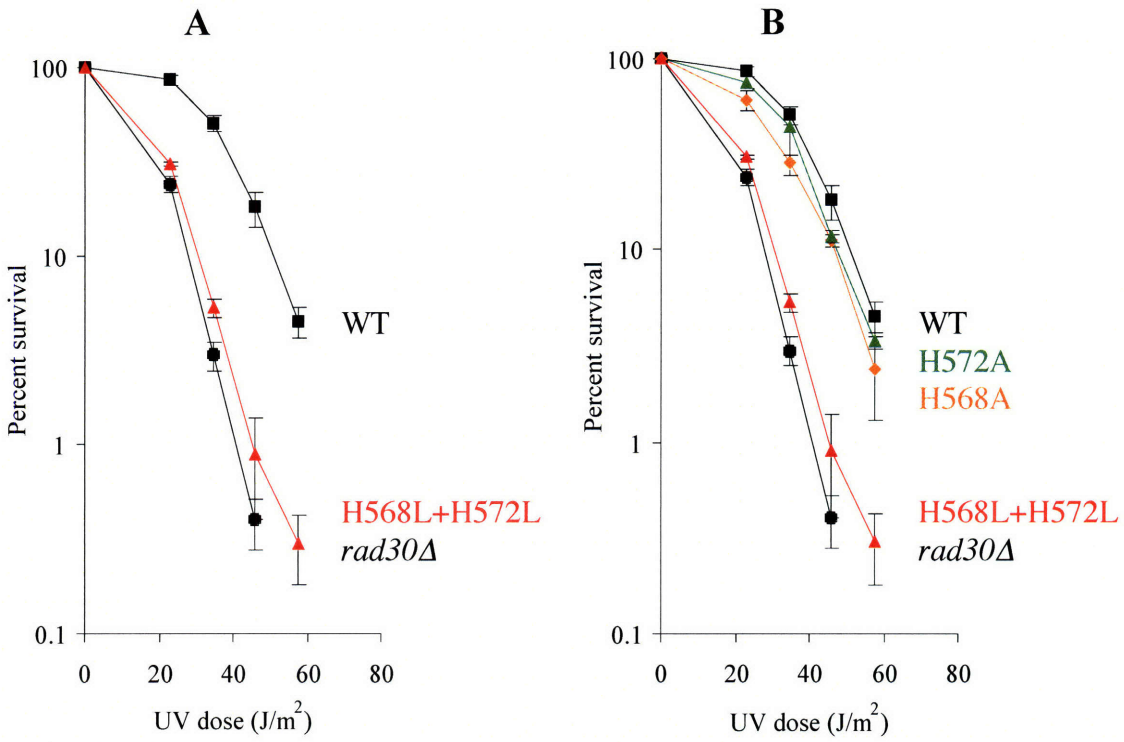


Figure 2. UV phenotypes of UBZ mutants. In a *rad30* null background (RWY15), effect on UV sensitivity of plasmids carrying *RAD30* (pJM96 or pRW1031), squares; specified mutants, triangles or empty vector (pRS415), circles. Error bars represent standard error. **A,** H568L+H572L double mutant (pRW1002), red triangles. **B,** H568L+H572L double mutant (pRW1025), red, causes greater loss of function than either H568A (pRW1006), orange, or H572A (pRW1004), green. **C,** C552R+C553R double mutant (pRW1027), blue triangles. **D,** Y571A (pRW1005), gray triangles. **E,** Alanine patch at PKLE 548 (pRW1021), open triangles.

Figure 2, continued.

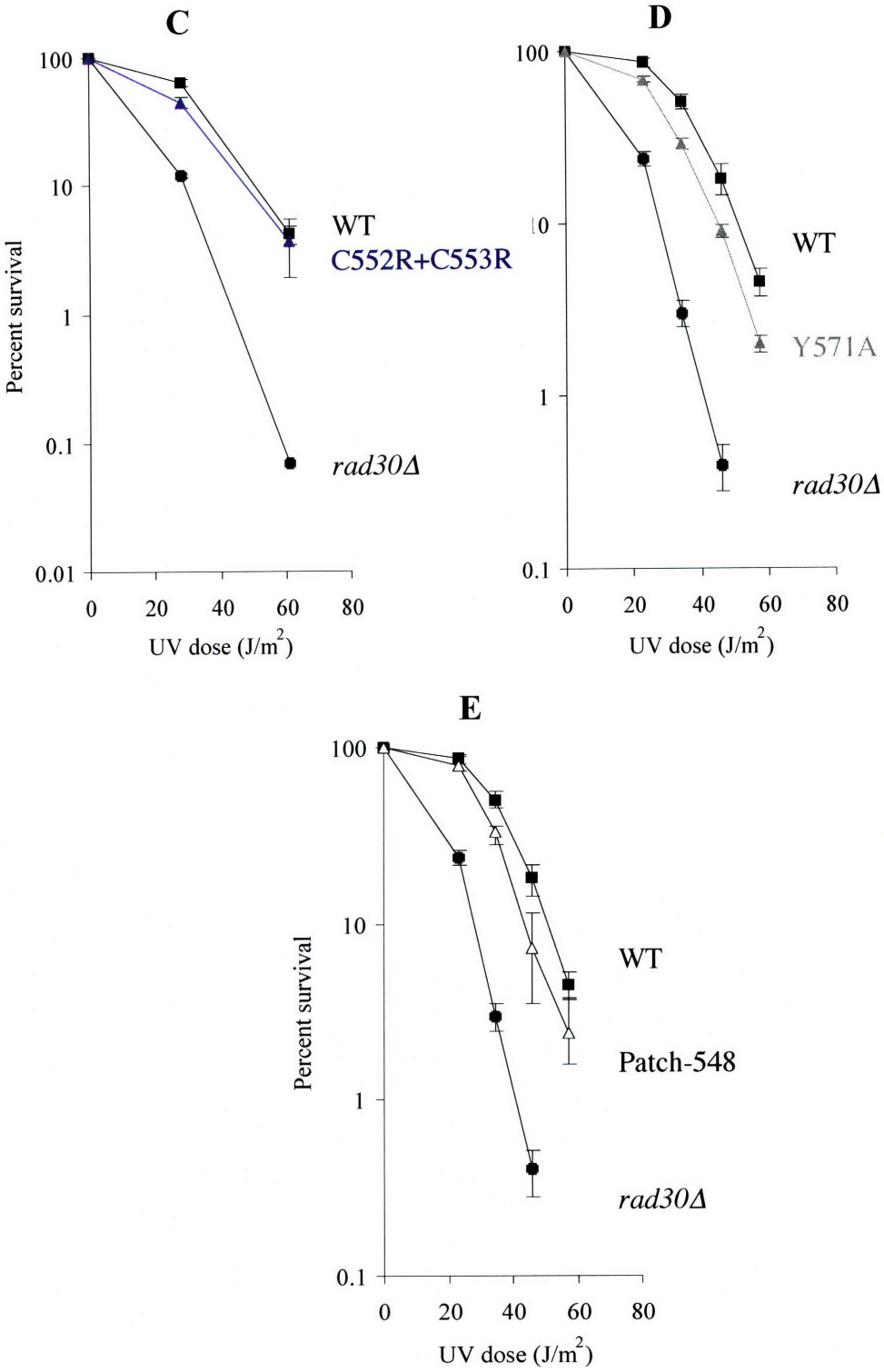


Figure 3.

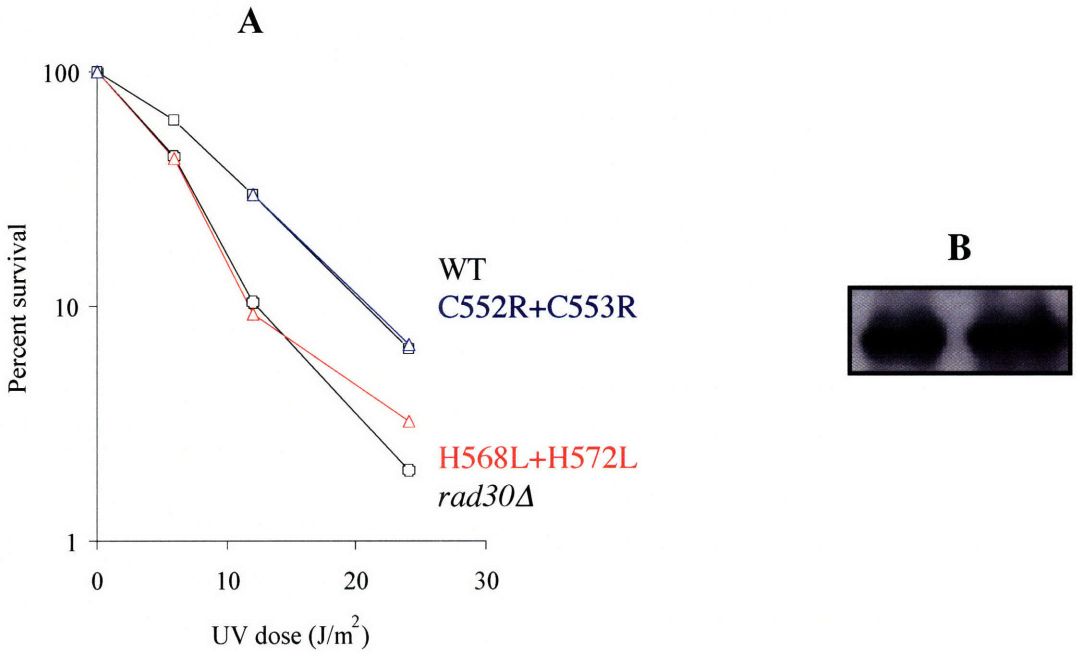
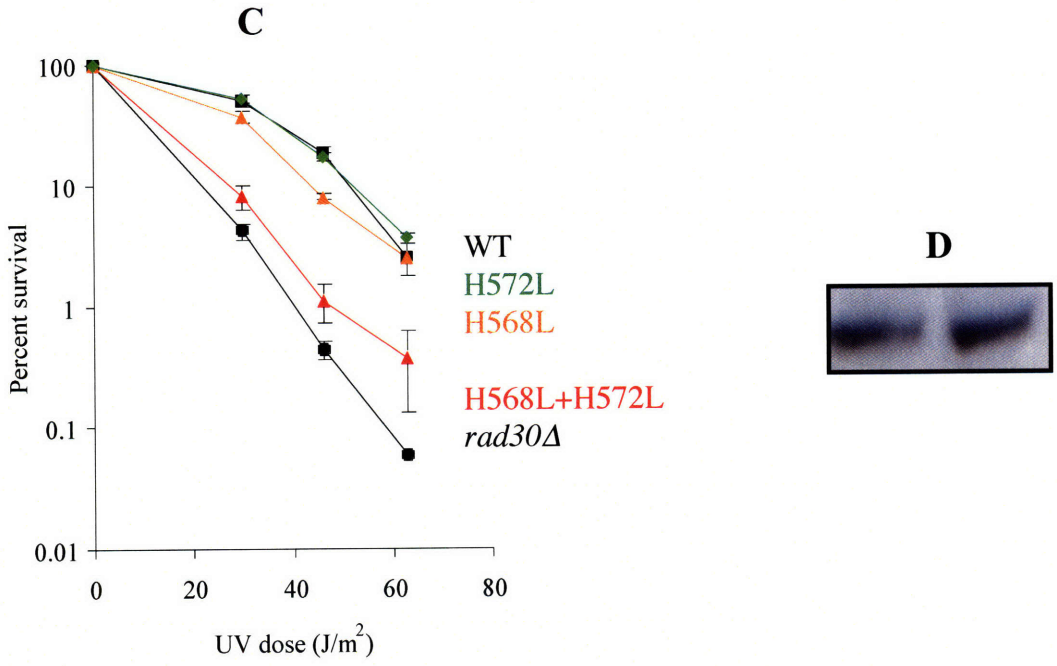


Figure 3. Mutation of histidines doubly, but not singly, causes recessive loss of function. A, Ability to rescue UV sensitivity in *rad5rad30* background of plasmid-born wildtype *RAD30* (pEGUh6-Rad30), black squares; H568L+H572L double mutant (pRW1002), red triangles; C552R+C553R double mutant (pRW1001), blue triangles; or empty vector (pEGUh6), circles. **B,** Anti-His immunoblot comparing expression of 6His-tagged mutant H568L+H572L Rad30p (pRW1002), left lane, with 6His-tagged wildtype Rad30p (pEGUh6-Rad30). **C,** In a *rad30* null background (RWY15), effect on UV sensitivity of Rad30p C-terminally tagged with -TEV-ProA-7His: Wildtype (pRW1031), black squares; H568L+H572L double mutant (pRW1045), red triangles; H568L mutant (pRW1046), orange; and H572L mutant (pRW1047), green; or empty vector (pRS415), black circles. Error bars represent standard error.
D, Immunoblot showing expression of the H568L+H572L double mutant (pRW1045), left lane, compared with the wildtype (pRW1031), right lane. **E,** H568L+H572L phenotype is recessive. Empty vector (pRS415) in *rad30* null (white) or wildtype background (light gray); plasmid-born wildtype Rad30p (pJM96) in wildtype background (dark gray); plasmid-born H568L+H572L double mutant (pRW1025) in *rad30* (yellow) or wildtype background (yellow striped); plasmid-born catalytically dead mutant Rad30 (pRW1029) in *rad30* (green) or wildtype background (green striped).

Figure 3, continued.



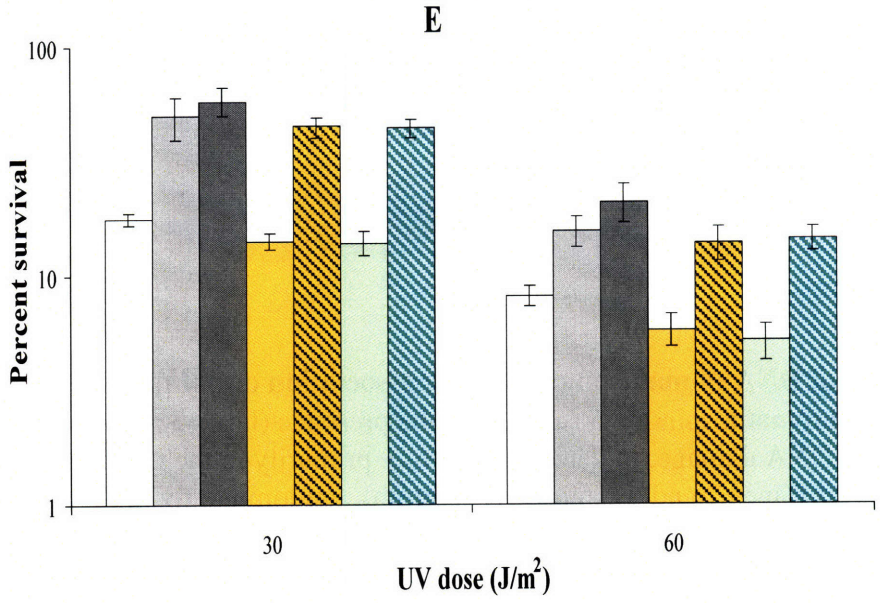


Figure 4.

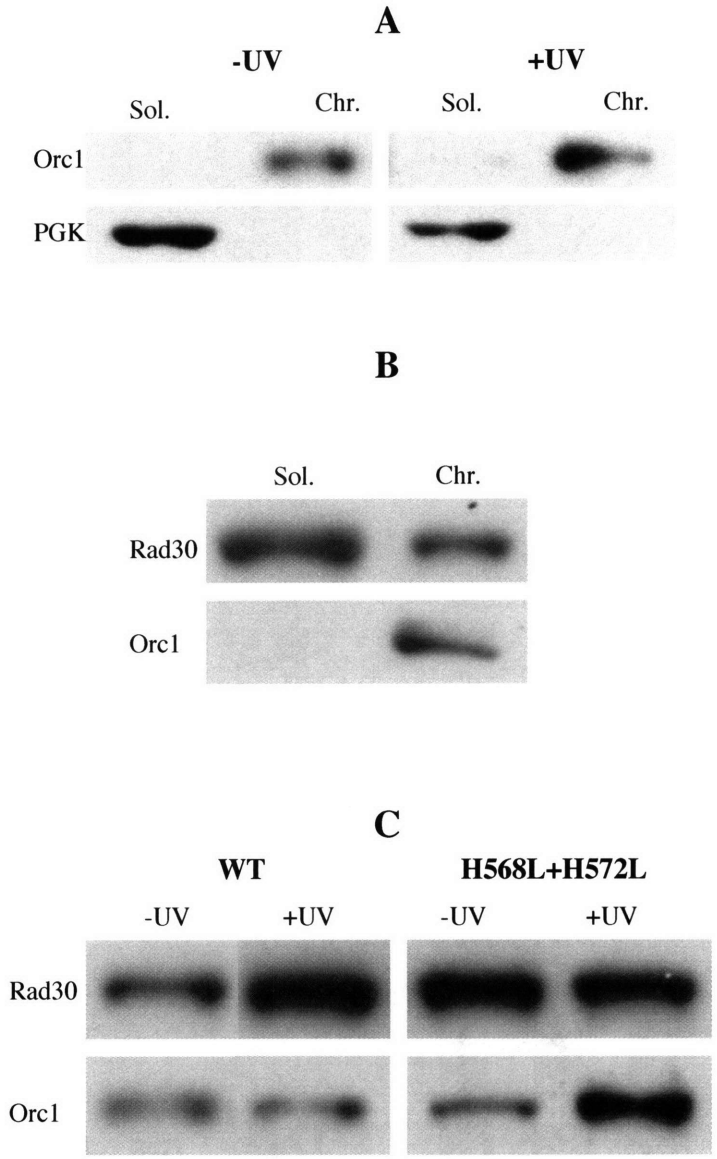


Figure 4. Effect of DNA damage on chromatin association of pol η. A, Controls: Immunoblots of chromatin spindown samples in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of DNA damage, to detect Orc1 (top). primarily in the chromatin associated fractions (lanes 2 and 4), and PGK (bottom), predominantly in the soluble fraction (lanes 1 and 3). **B,** Pol η is found in both the chromatin associated fraction (lane 2) and soluble fraction (lane1). Immunoblots detecting the ProA tag on Rad30-TEV-ProA-7His (top) and Orc1 (bottom) in chromatin spindown samples. **C,** UBZ domain mutant prevents damage-induced increase in chromatin-association of pol η. Chromatin associated fractions containing wildtype pol η (lanes 1 and 2) or H568L+H572L double mutant pol η (lanes 3 and 4). Lanes 1 and 3 are chromatin associated fractions of undamaged cultures; lanes 2 and 4 are the chromatin associated fractions from UV-irradiated cultures.

Figure 5.

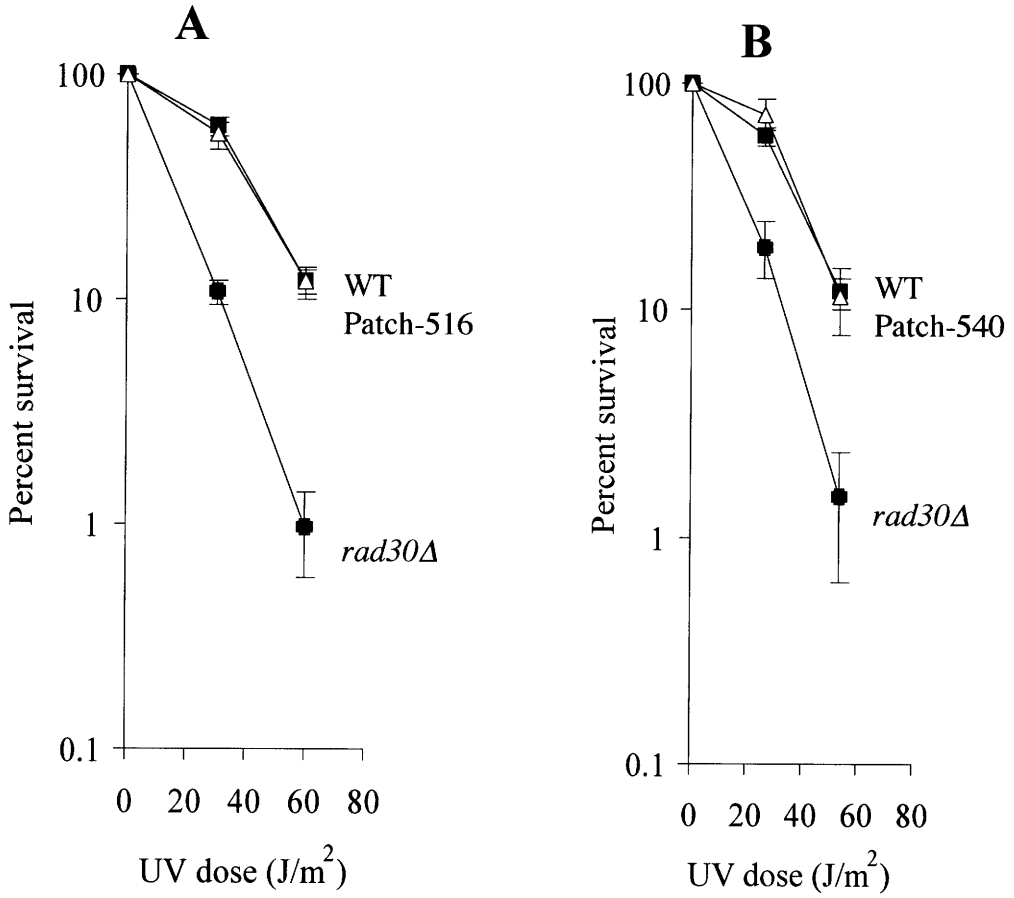


Figure 5. Effects on UV survival of pol η mutations outside the UBZ domain. In a *rad30* null background (RWY15), effect on UV sensitivity of plasmids carrying *RAD30* (pJM96 or pRW1031), squares; specified mutants, triangles or empty vector (pRS415), circles. Error bars (representing standard error) are included for all data points, but in many cases are smaller than the symbols marking the data points. **A,** Alanine patch at VDM 516-18 (pRW1018). **B,** Alanine patch at SS 540-41(pRW1020). **C,** Alanine patch at SSK 587-89 (pRW1022). **D,** S587D (pRW1035). **E,** Alanine patch at LLF 598-600 (pRW1024). **F,** S592A (pRW1054). **G,** T514A (pRW1007).

Figure 5, continued.

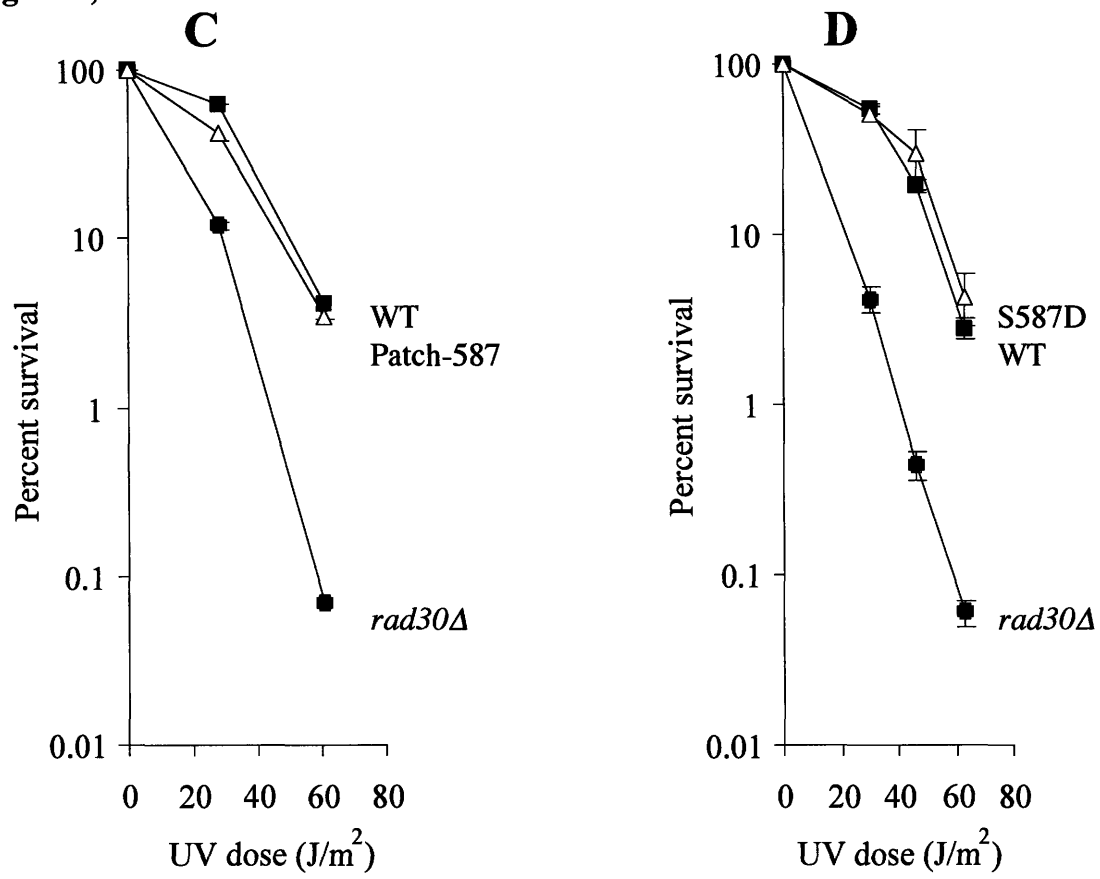


Figure 5. Effects on UV survival of pol η mutations outside the UBZ domain. In a *rad30* null background (RWY15), effect on UV sensitivity of plasmids carrying *RAD30* (pJM96 or pRW1031), squares; specified mutants, triangles or empty vector (pRS415), circles. Error bars (representing standard error) are included for all data points, but in many cases are smaller than the symbols marking the data points. **A,** Alanine patch at VDM 516-18 (pRW1018). **B,** Alanine patch at SS 540-41(pRW1020). **C,** Alanine patch at SSK 587-89 (pRW1022). **D,** S587D (pRW1035). **E,** Alanine patch at LLF 598-600 (pRW1024). **F,** S592A (pRW1054). **G,** T514A (pRW1007).

Figure 5, continued.

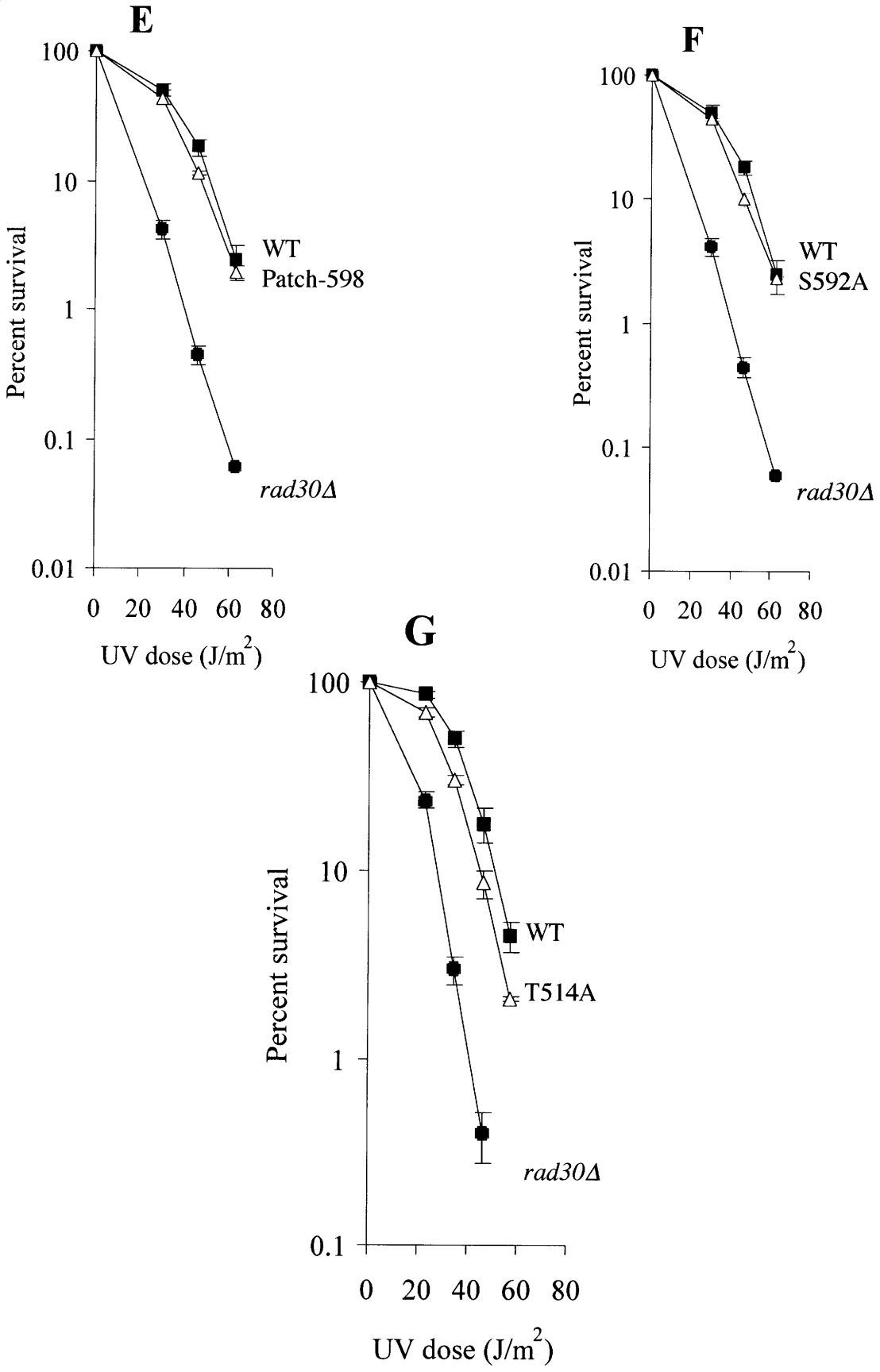
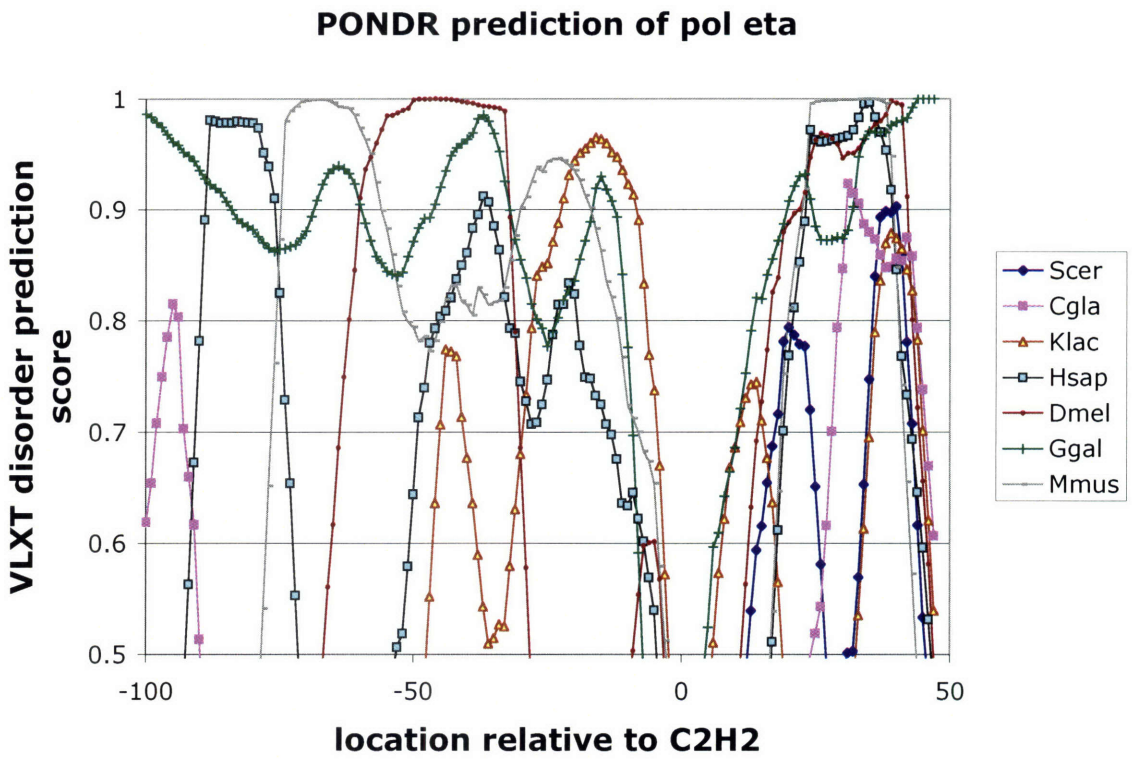


Figure S1

A.


PONDR prediction of pol eta
VLXT disorder prediction score
1
0.9
0.8
0.7
0.6
0.5
-100
-50
0
50
location relative to C2H2
Scer
Cgla
Klac
Hsap
Dmel
Ggal
Mmus

B.

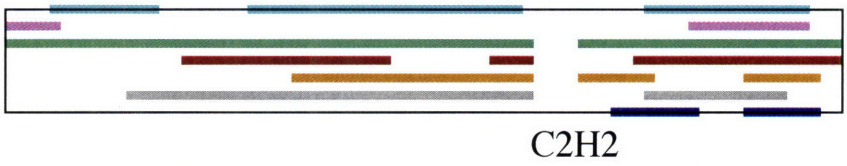

C2H2

Figure S1. **A,** Graphical representation of predicted probability of disorder for each residue of pol η homologs beginning 100 amino acids N-terminal to the first C of the C2H2. Predictions were performed by PONDR (see Materials and Methods). PONDR uses 0.5 as the cutoff below which disorder is not predicted. **B,** Another representation of the data shown in A. Box represents the C-terminus of polymerase η; colored lines represented regions of predicted disorder in each species.

Table S1. Lists species in which known or putative pol η homologs were identified, and whether I was able to recognize the UBZ motif in their sequences.

Classification	Organism	UBZ status
Ciliate	Paramecium tetraurelia	yes
Frog	Xenopus laevis	yes
Fungus: Ascomycetes	Saccharomyces cerevisiae	**Degenerate zinc finger:** cckyqvtftdqkalqehadyh
Fungus: Ascomycetes	Ashbya gossypii	yes
Fungus: Ascomycetes	Aspergillus nidulans	yes
Fungus: Ascomycetes	Aspergillus niger	yes
Fungus: Ascomycetes	Aspergillus terreus	yes
Fungus: Ascomycetes	Botryotinia fuckeliana	yes
Fungus: Ascomycetes	Candida albicans	yes
Fungus: Ascomycetes	Candida glabrata	yes
Fungus: Ascomycetes	Chaetomium globosum	yes
Fungus: Ascomycetes	Coccidioides immitis	yes
Fungus: Ascomycetes	Debaryomyces hansenii	yes
Fungus: Ascomycetes	Gibberella zeae	yes
Fungus: Ascomycetes	Kluyveromyces lactis	yes
Fungus: Ascomycetes	Lodderomyces elongisporus	yes
Fungus: Ascomycetes	Neosartorya fischeri	yes
Fungus: Ascomycetes	Neurospora crassa	yes
Fungus: Ascomycetes	Phaeosphaeria nodorum	yes
Fungus: Ascomycetes	Pichia guilliermondii	yes
Fungus: Ascomycetes	Pichia stipitis	yes
Fungus: Ascomycetes	Saccharomyces bayanus	yes
Fungus: Ascomycetes	Saccharomyces castellii	yes
Fungus: Ascomycetes	Saccharomyces kluyverii	yes
Fungus: Ascomycetes	Saccharomyces kudriavzevii	yes
Fungus: Ascomycetes	Saccharomyces paradoxus	yes
Fungus: Ascomycetes	Schizosaccharomyces pombe	yes
Fungus: Ascomycetes	Sclerotinia sclerotiorum	yes
Fungus: Ascomycetes	Yarrowia lipolytica	yes
Fungus: Basidiomyces	Coprinopsis cinerea	yes
Fungus: Basidiomyces	Cryptococcus neoformans	yes
Fungus: Basidiomyces	Ustilago maydis	yes
Green algae	Ostreococcus lucimarinus	**NO. Short**
Insect	Nasonia vitripennis	**Degenerate zinc finger:** pnicgqsilvekfdehndyh
Insect	Aedes aegypti	yes
Insect	Anopheles gambiae	yes
Insect	Tribolium castaneum	yes
Insect: fly	Drosophila melanogaster	yes
Insect: fly	Drosophila pseudoobscura	yes
Nematode	Caenorhabditis elegans	**NO. No UBM.**
Plant: eudicot	Arabidopsis thaliana	**NO. However, contains UBM**

Plant: monocot	Oryza sativa	**NO. No UBM.**
Sea urchin	Strongylocentrotus purpuratus	yes
Trypanosome	Leishmania braziliensis	**NO. No UBM.**
Trypanosome	Leishmania infantum	**NO. No UBM.**
Trypanosome	Trypanosoma brucei	**NO. short**
Trypanosome	Trypanosoma cruzi	**NO. Short**
Trypanosome	Leishmania major	**NO. No UBM.**
Vertebate: Mammal	Bos taurus	yes
Vertebate: Mammal	Canis familiaris	yes
Vertebate: Mammal	Equus caballus	yes
Vertebate: Mammal	Homo sapiens	yes
Vertebate: Mammal	Pan troglodytes	yes
Vertebate: Mammal: Opposum	Monodelphis domestica	yes
Vertebate: Mammal: platypus	Ornithorhynchus anatinus	yes
Vertebate: Mammal: Rodent	Mus musculus	yes
Vertebate: Mammal: Rodent	Rattus norvegicus	yes
Vertebrate	Danio rerio	yes
Vertebrate: bird	Gallus gallus	yes

REFERENCES

1. McDonald, J.P., A.S. Levine, and R. Woodgate, *The Saccharomyces cerevisiae RAD30 gene, a homologue of Escherichia coli dinB and umuC, is DNA damage inducible and functions in a novel error-free postreplication repair mechanism.* Genetics, 1997. **147**(4): p. 1557-68.
2. Xiao, W., et al., *The Saccharomyces cerevisiae RAD6 group is composed of an error-prone and two error-free postreplication repair pathways.* Genetics, 2000. **155**(4): p. 1633-41.
3. Darden, D.L., et al., *RNA differential display of scarless wound healing in fetal rabbit indicates downregulation of a CCT chaperonin subunit and upregulation of a glycophorin-like gene transcript.* J Pediatr Surg, 2000. **35**(3): p. 406-19.
4. Cejka, P., V. Vondrejs, and Z. Storchova, *Dissection of the functions of the Saccharomyces cerevisiae RAD6 postreplicative repair group in mutagenesis and UV sensitivity.* Genetics, 2001. **159**(3): p. 953-63.
5. Jentsch, S., J.P. McGrath, and A. Varshavsky, *The yeast DNA repair gene RAD6 encodes a ubiquitin-conjugating enzyme.* Nature, 1987. **329**(6135): p. 131-4.
6. Bailly, V., et al., *Yeast DNA repair proteins Rad6 and Rad18 form a heterodimer that has ubiquitin conjugating, DNA binding, and ATP hydrolytic activities.* J Biol Chem, 1997. **272**(37): p. 23360-5.
7. Hoege, C., et al., *RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO.* Nature, 2002. **419**(6903): p. 135-41.
8. Stelter, P. and H.D. Ulrich, *Control of spontaneous and damage-induced mutagenesis by SUMO and ubiquitin conjugation.* Nature, 2003. **425**(6954): p. 188-91.
9. Zhang, H., P.E. Gibbs, and C.W. Lawrence, *The Saccharomyces cerevisiae rev6-1 mutation, which inhibits both the lesion bypass and the recombination mode of DNA damage tolerance, is an allele of POL30, encoding proliferating cell nuclear antigen.* Genetics, 2006. **173**(4): p. 1983-9.
10. Plosky, B.S., et al., *Controlling the subcellular localization of DNA polymerases iota and eta via interactions with ubiquitin.* Embo J, 2006. **25**(12): p. 2847-55.
11. Ohmori, H., et al., *The Y-family of DNA polymerases.* Mol Cell, 2001. **8**(1): p. 7-8.
12. Yuan, F., et al., *Specificity of DNA lesion bypass by the yeast DNA polymerase eta.* J Biol Chem, 2000. **275**(11): p. 8233-9.
13. Johnson, R.E., S. Prakash, and L. Prakash, *Efficient bypass of a thymine-thymine dimer by yeast DNA polymerase, Poleta.* Science, 1999. **283**(5404): p. 1001-4.
14. Carty, M.P., et al., *The RAD30 cancer susceptibility gene.* Biochem Soc Trans, 2003. **31**(Pt 1): p. 252-6.
15. Trincao, J., et al., *Structure of the catalytic core of S. cerevisiae DNA polymerase eta: implications for translesion DNA synthesis.* Mol Cell, 2001. **8**(2): p. 417-26.
16. Masutani, C., et al., *Xeroderma pigmentosum variant (XP-V) correcting protein from HeLa cells has a thymine dimer bypass DNA polymerase activity.* Embo J, 1999. **18**(12): p. 3491-501.

17. Kannouche, P., et al., *Domain structure, localization, and function of DNA polymerase eta, defective in xeroderma pigmentosum variant cells.* Genes Dev, 2001. **15**(2): p. 158-72.
18. Moldovan, G.L., B. Pfander, and S. Jentsch, *PCNA, the maestro of the replication fork.* Cell, 2007. **129**(4): p. 665-79.
19. Watanabe, K., et al., *Rad18 guides poleta to replication stalling sites through physical interaction and PCNA monoubiquitination.* Embo J, 2004. **23**(19): p. 3886-96.
20. Guo, C., et al., *Mouse Rev1 protein interacts with multiple DNA polymerases involved in translesion DNA synthesis.* Embo J, 2003. **22**(24): p. 6621-30.
21. Tissier, A., et al., *Co-localization in replication foci and interaction of human Y-family members, DNA polymerase pol eta and REVl protein.* DNA Repair (Amst), 2004. **3**(11): p. 1503-14.
22. Bienko, M., et al., *Ubiquitin-binding domains in Y-family polymerases regulate translesion synthesis.* Science, 2005. **310**(5755): p. 1821-4.
23. Bomar, M.G., et al., *Structure of the ubiquitin-binding zinc finger domain of human DNA Y-polymerase eta.* EMBO Rep, 2007. **8**(3): p. 247-51.
24. Bish, R.A. and M.P. Myers, *Werner helicase-interacting protein 1 binds polyubiquitin via its zinc finger domain.* J Biol Chem, 2007. **282**(32): p. 23184-93.
25. Johnson, R.E., et al., *hRAD30 mutations in the variant form of xeroderma pigmentosum.* Science, 1999. **285**(5425): p. 263-5.
26. Gerlach, V.L., et al., *Human and mouse homologs of Escherichia coli DinB (DNA polymerase IV), members of the UmuC/DinB superfamily.* Proc Natl Acad Sci U S A, 1999. **96**(21): p. 11922-7.
27. Notredame, C., D.G. Higgins, and J. Heringa, *T-Coffee: A novel method for fast and accurate multiple sequence alignment.* J Mol Biol, 2000. **302**(1): p. 205-17.
28. Notenboom, V., et al., *Functional characterization of Rad18 domains for Rad6, ubiquitin, DNA binding and PCNA modification.* Nucleic Acids Res, 2007.
29. Parker, J.L., et al., *Contributions of ubiquitin- and PCNA-binding domains to the activity of Polymerase eta in Saccharomyces cerevisiae.* Nucleic Acids Res, 2007. **35**(3): p. 881-9.
30. Parker, V.P. and M.W. Lieberman, *Levels of DNA polymerases alpha, beta, and gamma in control and repair-deficient human diploid fibroblasts 1.* Nucleic Acids Res, 1977. **4**(6): p. 2029-37.
31. Doublie, S., et al., *The crystal structure of human endonuclease VIII-like 1 (NEIL1) reveals a zincless finger motif required for glycosylase activity.* Proc Natl Acad Sci U S A, 2004. **101**(28): p. 10284-9.
32. Mayorov, V.I., et al., *DNA polymerase eta contributes to strand bias of mutations of A versus T in immunoglobulin genes.* J Immunol, 2005. **174**(12): p. 7781-6.
33. Struthers, M.D., R.P. Cheng, and B. Imperiali, *Design of a monomeric 23-residue polypeptide with defined tertiary structure.* Science, 1996. **271**(5247): p. 342-5.
34. Acharya, N., et al., *Mutations in the ubiquitin binding UBZ motif of DNA polymerase {eta} do not impair its function in translesion synthesis during replication.* Mol Cell Biol, 2007.

35. Guo, C., et al., *Ubiquitin-binding motifs in REV1 protein are required for its role in the tolerance of DNA damage.* Mol Cell Biol, 2006. **26**(23): p. 8892-900.
36. Catic, A., et al., *Preferred in vivo ubiquitination sites.* Bioinformatics, 2004. **20**(18): p. 3302-7.
37. Donovan, S., et al., *Cdc6p-dependent loading of Mcm proteins onto pre-replicative chromatin in budding yeast.* Proc Natl Acad Sci U S A, 1997. **94**(11): p. 5611-6.
38. Ricke, R.M. and A.K. Bielinsky, *Easy detection of chromatin binding proteins by the Histone Association Assay.* Biol Proced Online, 2005. **7**: p. 60-9.
39. Li, X., et al., *Predicting Protein Disorder for N-, C-, and Internal Regions.* Genome Inform Ser Workshop Genome Inform, 1999. **10**: p. 30-40.
40. Romero, P., et al., *Sequence complexity of disordered protein.* Proteins, 2001. **42**(1): p. 38-48.
41. Ingrell, C.R., et al., *NetPhosYeast: prediction of protein phosphorylation sites in yeast.* Bioinformatics, 2007. **23**(7): p. 895-7.
42. Zharkov, D.O., et al., *Structural analysis of an Escherichia coli endonuclease VIII covalent reaction intermediate.* Embo J, 2002. **21**(4): p. 789-800.
43. Andersen, K.M., K. Hofmann, and R. Hartmann-Petersen, *Ubiquitin-binding proteins: similar, but different.* Essays Biochem, 2005. **41**: p. 49-67.
44. Wood, A., P. Garg, and P.M. Burgers, *A ubiquitin-binding motif in the translesion DNA polymerase Rev1 mediates its essential functional interaction with ubiquitinated proliferating cell nuclear antigen in response to DNA damage.* J Biol Chem, 2007. **282**(28): p. 20256-63.
45. McDonald, J.P., et al., *Novel human and mouse homologs of Saccharomyces cerevisiae DNA polymerase eta.* Genomics, 1999. **60**(1): p. 20-30.
46. Hishida, T., et al., *Functional and physical interaction of yeast Mgs1 with PCNA: impact on RAD6-dependent DNA damage tolerance.* Mol Cell Biol, 2006. **26**(14): p. 5509-17.
47. Haracska, L., et al., *Ubiquitylation of yeast proliferating cell nuclear antigen and its implications for translesion DNA synthesis.* Proc Natl Acad Sci U S A, 2006. **103**(17): p. 6477-82.
48. Kawabe, Y., et al., *A novel protein interacts with the Werner's syndrome gene product physically and functionally.* J Biol Chem, 2001. **276**(23): p. 20364-9.
49. Kamath-Loeb, A.S., et al., *Werner syndrome protein interacts functionally with translesion DNA polymerases.* Proc Natl Acad Sci U S A, 2007. **104**(25): p. 10394-9.
50. Miyase, S., et al., *Differential regulation of Rad18 through Rad6-dependent mono- and polyubiquitination.* J Biol Chem, 2005. **280**(1): p. 515-24.
51. Ulrich, H.D. and S. Jentsch, *Two RING finger proteins mediate cooperation between ubiquitin-conjugating enzymes in DNA repair.* Embo J, 2000. **19**(13): p. 3388-97.
52. Nakajima, S., et al., *Replication-dependent and -independent responses of RAD18 to DNA damage in human cells.* J Biol Chem, 2006. **281**(45): p. 34687-95.
53. Pabla, R., D. Rozario, and W. Siede, *Regulation of Saccharomyces cerevisiae DNA polymerase eta transcript and protein.* Radiat Environ Biophys, 2007.

54. Guo, C., et al., *REV1 protein interacts with PCNA: significance of the REV1 BRCT domain in vitro and in vivo.* Mol Cell, 2006. **23**(2): p. 265-71.
55. Di Fiore, P.P., S. Polo, and K. Hofmann, *When ubiquitin meets ubiquitin receptors: a signalling connection.* Nat Rev Mol Cell Biol, 2003. **4**(6): p. 491-7.
56. Hicke, L., H.L. Schubert, and C.P. Hill, *Ubiquitin-binding domains.* Nat Rev Mol Cell Biol, 2005. **6**(8): p. 610-21.
57. Katz, M., et al., *Ligand-independent degradation of epidermal growth factor receptor involves receptor ubiquitylation and Hgs, an adaptor whose ubiquitin-interacting motif targets ubiquitylation by Nedd4.* Traffic, 2002. **3**(10): p. 740-51.
58. Skoneczna, A., et al., *Polymerase eta is a short-lived, proteasomally degraded protein that is temporarily stabilized following UV irradiation in Saccharomyces cerevisiae.* J Mol Biol, 2007. **366**(4): p. 1074-86.
59. Sabbioneda, S., et al., *Yeast Rev1 is cell cycle regulated, phosphorylated in response to DNA damage and its binding to chromosomes is dependent upon MEC1.* DNA Repair (Amst), 2007. **6**(1): p. 121-7.
60. Hirano, Y. and K. Sugimoto, *ATR homolog Mec1 controls association of DNA polymerase zeta-Rev1 complex with regions near a double-strand break.* Curr Biol, 2006. **16**(6): p. 586-90.
61. Zhao, X., E.G. Muller, and R. Rothstein, *A suppressor of two essential checkpoint genes identifies a novel protein that negatively affects dNTP pools.* Mol Cell, 1998. **2**(3): p. 329-40.
62. Knop, M., et al., *Epitope tagging of yeast genes using a PCR-based strategy: more tags and improved practical routines.* Yeast, 1999. **15**(10B): p. 963-72.
63. Wu, X., E. Braithwaite, and Z. Wang, *DNA ligation during excision repair in yeast cell-free extracts is specifically catalyzed by the CDC9 gene product.* Biochemistry, 1999. **38**(9): p. 2628-35.
64. Waters, L.S. and G.C. Walker, *The critical mutagenic translesion DNA polymerase Rev1 is highly expressed during G(2)/M phase rather than S phase.* Proc Natl Acad Sci U S A, 2006. **103**(24): p. 8971-6.
65. Roush, A.A., et al., *Deletion of the Saccharomyces cerevisiae gene RAD30 encoding an Escherichia coli DinB homolog confers UV radiation sensitivity and altered mutability.* Mol Gen Genet, 1998. **257**(6): p. 686-92.
66. Altschul, S.F., et al., *Gapped BLAST and PSI-BLAST: a new generation of protein database search programs.* Nucleic Acids Res, 1997. **25**(17): p. 3389-402.
67. Schaffer, A.A., et al., *Improving the accuracy of PSI-BLAST protein database searches with composition-based statistics and other refinements.* Nucleic Acids Res, 2001. **29**(14): p. 2994-3005.
68. Blom, N., S. Gammeltoft, and S. Brunak, *Sequence and structure-based prediction of eukaryotic protein phosphorylation sites.* J Mol Biol, 1999. **294**(5): p. 1351-62.
69. Blom, N., et al., *Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence.* Proteomics, 2004. **4**(6): p. 1633-49.
70. Johnson, R.E., S. Prakash, and L. Prakash, *Requirement of DNA polymerase activity of yeast Rad30 protein for its biological function.* J Biol Chem, 1999. **274**(23): p. 15975-7.

Chapter 3:
Interactions between Rev1 protein and other Y-family polymerases in lower eukaryotes

J. Nicole Kosarek, Rachel V. Woodruff, Amanda Rivera-Begeman, Caixia Guo, Sanjay D'Souza, Eugene V. Koonin, Graham C. Walker and Errol C. Friedberg

I performed the coIPs and UV survival assay shown in Figure 4, and contributed significantly to the writing of the Discussion section.

Summary

Eukaryotes are endowed with multiple specialized DNA polymerases, some (if not all) of which are believed to play important roles in the tolerance of base damage in DNA. Among these DNA polymerases, Rev1 protein (a dCMP transferase) from vertebrates interacts with multiple other specialized polymerases via a highly conserved C-terminal region. The present studies sought to determine whether these interactions are retained in experimentally tractable yeasts and other invertebrates. Using well-documented technologies that identify *in vivo* interactions we observed an interaction between Rev1 protein and various Y-family DNA polymerases in Drosophila. However, regardless of the fact that the extent of amino acid identity and similarity in the C-terminal region of Drosophila Rev1 protein is no less than that in other invertebrates, such interactions were not observed in the yeasts *S. cerevisiae* or *S. pombe*. Futhermore, Rev1 protein from Drosophila and both yeasts readily interacts with Rev7 protein (the catalytic subunit of the B-family DNA polymerase Polζ). These studies advocate special consideration when making mechanistic extrapolations between diverse eukaryotes.

1. Introduction

The rescue of arrested DNA replication at sites of template base damage is critical for cell survival. Not surprisingly, prokaryotic and eukaryotic cells have evolved multiple strategies for mitigating the lethal effects of arrested DNA replication without prior removal of the offending DNA damage; so-called DNA damage tolerance [1]. The replicative bypass of base damage by DNA translesion synthesis (TLS) represents a specific mode of damage tolerance that utilizes specialized low-fidelity DNA polymerases to overcome arrested DNA replication, often at the expense of introducing errors and hence generating mutations [1] . To date ten such specialized DNA polymerases have been identified in vertebrates. A newly-discovered subset of these proteins (Rev1, Polη, Polι, and Polκ) is designated the Y-family of DNA polymerases [1-3] .

Among the Y-family of DNA polymerases Rev1 protein is highly conserved in eukaryotes, but no archaeal or bacterial Rev1 orthologs have been detected. Structural orthologs of Polη and Polι are also apparently absent in prokaryotes. In contrast, a readily identifiable ortholog of Polκ (DinB protein in *E. coli*) is present in bacteria. Rev1 is unique among the Y-family in that its DNA polymerase activity is restricted to the incorporation of one or two molecules of dCMP regardless of the nature of the template nucleotide. It is thus often referred to as a dCMP transferase [4]. Remarkably, while the catalytic domain of Rev1 protein is required for the replicative bypass of sites of base loss (AP sites), Rev1's catalytic function is not required for UV-induced mutagenesis in yeast or mammals. [5, 6].

Rev1 protein also possesses a conserved N-terminal BRCT domain that is required for TLS in yeast and mammalian cells exposed to UV radiation [7, 8] and presumably other types of base damage. Indeed, a single amino acid substitution in the BRCT domain of otherwise catalytically active yeast Rev1 abolishes the bypass of [6-4] photoproducts, suggesting a non-catalytic role(s) for Rev1 protein during UV radiation-induced mutagenesis [7]. Additional support for the notion that Rev1 has a TLS function(s) independent of its dCMP transferase activity is implicit in the observation that the protein interacts with the Y-family polymerases Polκ, Polη and Polι, and with Rev7 protein [a subunit of a heterodimeric specialized DNA polymerase called Polζ] through a C-terminal 100 amino acid region that is highly conserved among vertebrates [9-11]. The functional significance of these interactions is not understood. However, the additional observations that PCNA also interacts with these DNA polymerases and with Rev1 protein [8, 12-14], and that PCNA and some Y-family members (including Rev1 protein) undergo monoubiquitination, has prompted the hypothesis that Rev1 plays a key role in the process of TLS [3, 15, 16].

Several non-vertebrate eukaryotic organisms, such as the yeasts *S. cerevisiae* and *S. pombe*, the fruit fly *Drosophila melanogaster* and the nematode *C. elegans*, have proven to be informative model systems for various mechanistic studies in vertebrates. In view of the fact that these model organisms are endowed with Rev1 protein as well as one or more other Y-family DNA polymerases, they offer the potential for gaining fundamental insights into the molecular biology of TLS in eukaryotes. In the present

studies we have compared interactions between Rev1 protein and other members of the Y-family of DNA polymerases from animals and fungi.

Here we report that Y-family DNA polymerases from the fruit fly *Drosophila melanogaster* interact with Rev1 protein from this organism as well as that from the mouse. In contrast to the corresponding mouse proteins, *Drosophila* Y-family polymerases Polι and Polη each use two independent regions to interact with Rev1. However, comparison of a similarly located Rev1-interacting domain in Polη, Polι or Polκfrom mouse or Drosophila reveals little sequence conservation and does not obviously predict conserved structures. Indeed, in contrast to the extensive conservation of the C-terminal 100 amino acids of Rev1 protein in vertebrates, this region of Rev1 is also less conserved in the yeasts *S. cerevisiae* and *S. pombe*, and is not conserved in the nematode *C. elegans*. We also document a failure to observe interactions between Y-family DNA polymerases and Rev1 protein from the yeasts *S. cerevisiae* and *S. pombe* by the yeast two-hybrid or co-immunoprecipitation technologies, although Rev1 protein from both yeasts readily interacts with the Rev7 subunit of the specialized DNA polymerase ζ (Polζ). Thus, notwithstanding the presence of Rev1 protein and some specialized DNA polymerases in invertebrates and fungi, interactions between these proteins differ qualitatively among themselves and from the Rev1-DNA polymerase interactions observed in vertebrates.

We conclude that no single model eukaryote thus far examined can be considered a prototypic model system for generalizing the molecular mechanism of TLS in eukaryotes, and suggest that care must be exercised in making mechanistic extrapolations from one eukaryotic system to another.

2. Materials and Methods

2.1. Pair-wise yeast two-hybrid assays and interaction domain mapping

S. cerevisiae constructs. Rev1 was PCR amplified from Rev1p-GST-pJN60 (Christopher Lawrence) and cloned into the AD vector pACT2 (Clontech) or BD vector pGBKT7 (Clontech). Rad30 was PCR amplified from pEGUh6b-Rad30 (Zhigang Wang) and

cloned into BD vectors pGBKT7 or pGBT9 (Clontech). Rev7 was PCR amplified by colony PCR and cloned into AD vector pGADT7 (Clontech).

C. elegans constructs. Rev-1 was PCR amplified by RT-PCR of total RNA (prepared by bead disruption and RNAeasy prep of N2 hermaphrodite worms) and cloned into pGADT7. Polη-1 was PCR amplified by RT-PCR and cloned into pGBKT7. Two spliced products were detected, one with a 57 bp deletion in exon 7, as previously reported [17]. Both products were assayed. Polκ-1 was amplified by RT-PCR and cloned into pGBKT7.

S. pombe constructs. Rev1 (SPBC1347.01c) was PCR amplified by RT-PCR of total RNA and cloned into pGADT7 or pGBKT7. Eso1+(Polη), Polκ(SPCC553.07c), and Rev7 were amplified by RT-PCR and cloned into pGBKT7 or pGADT7. Exon boundaries for Rev7 were redefined and annotated accordingly on online databases.

Drosophila constructs. Rev1 was amplified by RT-PCR of total RNA prepared by Trizol extraction of Kc cells and cloned into pACT2. Polη and Polι were amplified from pGEX-dPolη and pGEX-dPolι [18] and cloned into pGBKT7. Rev7 was PCR amplified by RT-PCR and cloned into pGBKT7. Truncation experiments were performed using fragmented cDNAs cloned by PCR.

Mouse constructs. As previously described [9]. Truncation experiments were performed using fragmented cDNAs cloned by PCR.

2.2. Yeast transformation and growth selection

Pair-wise combinations of yeast two hybrid constructs and corresponding negative controls containing an empty vector were transformed into freshly prepared AH109 competent cells (Clontech) and plated on DDO media (-Trp/-Leu). After 4 days of growth at 30°C, 2-3 colonies were picked, suspended in sterile water, and plated on QDO media (-Trp/-Leu/-Ade/-His) and grown for up to 10 days at 30°C to select for positive interactions. Side by side plating on DDO was performed as a control.

2.3. β-galactosidase assays

Pair-wise combinations of full-length or truncated yeast two-hybrid constructs and corresponding negative controls containing an empty vector were transformed into

freshly prepared Y187 competent cells (Clontech) and plated on DDO media (-Trp/-Leu) to grow for 3-4 days at 30°C. Overnight cultures grown in selective media were used to prepare log phase cultures and 3 aliquots per culture in Z-buffer were flash frozen. Each sample was subjected to the addition of Z-buffer+β-mercaptoethanol and ONPG substrate (Sigma) and subsequently measured (<24 hours) for their spectrophotometric values with respect to time.

2.4. Immunoprecipitation and immunoblot analysis of dRev1 and dPolη

The full-length ORFs for dRev1 and dPolη were cloned into expression vectors using the Drosophila Gateway system. Kc *Drosophila* cells (40-80% confluency) were co-transfected with dRev1-pAMW(N-terminal Myc) and dPolη-pAWV(C-terminal YFP) or empty pAMW with dPolη-pAWV using Effectene reagent (Quiagen). Transfected cells selected in puromycin (20mg/mL)/CCM-3 reached confluency and were split after 24 hours. Transiently transfected cells were harvested after 48 hours and extracted in lysis buffer (50mM Tris pH 7.5, 150mM NaCl, 1mM EDTA, 0.5% NP-40) spiked with protease inhibitor (Sigma). The lysate was incubated with rabbit anti-GFP serum (Molecular Probes) and added to washed Protein A Sepharose (Amersham), followed by incubation for 3h at 4°C. The beads were washed and the contents bound to the beads were analyzed by Western blot using anti-Myc or anti-GFP.

2.4. Yeast strains

Strains are listed in Table 2. Yeast strains used for the Rev1/Rad30 coIP are derivatives of W1588-4C (*MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5*), which is a W303 strain corrected for *RAD5[19]*. Deletion of *REV1* and *RAD30* were constructed by gene replacement by PCR amplification of *rev1::KanMX* and *rad30::KanMX*, respectively, from the Saccharomyces Genome Deletion Project strains 1643 and 4255, respectively. To produce the tagged Rad30 fusion protein, the TEV-ProA-7His tag was PCR amplified from pYM10 [20] and inserted to replace the stop codon of *RAD30*. Rev1-HA was expressed from pAS311-REV1-HAC, which has been described previously along with YSD5, YLW20 YLW70 [21].

2.5. UV radiation survival of yeast

At least three independent cultures of each strain (RWY13, RWY15, and W1588-4C) were used. Cultures were grown to saturation for 3 days at 30°C, diluted in water, plated on SC-H, and immediately irradiated using a G15T8 UV lamp (General Electric) at 254nm, 1 J/m^2 per second for varying amounts of time. After irradiation, plates were kept in the dark at 30°C for 3 days before colonies were counted.

2.6. Survival after exposure to methyl methanesulfonate (MMS)

As described previously [21]. In short, after induction in galactose, appropriate dilutions of yeast cells (W1588-4C plus pAS311; YLW20 with pAS311 or with pAS311-REV1-HAC) were plated on SC-W plates with 2% galactose and the indicated amount of MMS.

2.7. Immunoprecipitation and immunoblot analysis of *S. cerevisiae* Rev1 and pol η

Yeast cultures were grown in selective media with raffinose for 2 days, then subcultured into selective media with galactose to induce protein expression overnight. For UV treatment, cells were spun down and resuspended in water to OD_{600} ~ 0.5, poured into large dishes to form a thin layer, then exposed to 50 J/m2 of UV (resulting in approximately 50% killing of WT). Irradiated cells were then resuspended in selective media with galactose and incubated at 30°C for 110-120 minutes after irradiation before harvesting, because previous work suggests that both Rev1 and Rad30 respond to DNA damage on this time scale [22-25]. Immunoprecipitations were performed essentially as described previously [21, 26]. Cell pellets were washed once in water, then resuspended in ice-cold lysis buffer (50 mM HEPES, pH7.6, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM DTT and Roche complete protease inhibitor cocktail). Cells were lysed either by bead-beating or by French Press. The lysate was centrifuged 13,500 RCF for 7 minutes, and PMSF was added to 1 mM. For the precipitation of ProA tagged proteins, the supernatant was bound to 50 ml IgG Sepharose (Amersham) for 1-2 hours. For Myc or HA tags, the supernatant was mixed with 2 μg of anti-Myc (mouse monoclonal 4A6; Upstate) or anti-HA (mouse monoclonal HA.11 clone 16B12; Covance) antibody and incubated for one hour on ice. 20 μl of ProG-agarose (Sigma) was then added and the whole was incubated for 1-2 hours at 4° C mixing

gently. The resin was washed 3 times in 500 ml of lysis buffer, and bound proteins were eluted by boiling the resin in SDS sample buffer.

Several alternate coIP procotols were performed, all yielding similar results. One alternate technique is represented in figure 4C. Yeast cultures were grown as above, but resuspended in alternate lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 5 mM $MgSO_4$, 10% glycerol, 0.05%NP40, 1 mM DTT, Roche complete protease inhibitor cocktail). Cell suspension was frozen drop-wise in liquid nitrogen, then lysed by grinding the frozen cells with dry ice in a coffee grinder. Thawed lysates were centrifuged 10,000 RCF for 15 minutes. The supernatant was then incubated with IgG-coupled magnetic beads (Dynabeads M270-Epoxy, Dynal) for 4 hours at 4°C. The beads were collected and washed three times in alternate lysis buffer lacking glycerol. Bound proteins were eluted by boiling the beads in SDS sample buffer.

For immunoblotting, protein samples were separated on SDS-polyacrylamide gels (Cambrex), transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore), and probed with appropriate antibodies. ProA-tagged proteins were detected using rabbit peroxidase anti-peroxidase (PAP) antibody (Sigma); Myc and HA tags were detected using mouse monoclonal antibody clone 4A6 (Upstate) and mouse monoclonal HA.11 clone 16B12 (Covance), respectively, followed by HRP-conjugated goat anti-mouse secondary antibody (Pierce).

2.8. Protein sequences analysis

Iterative searches of the non-redundant protein sequence database (National center for Biotechnology Information, NIH, Bethesda) were performed using the PSI-BLAST program [27] with standard parameters and the composition-based statistics applied to eliminate spurious hits emerging as a result of amino acid compositional biases [28]. Multiple alignments of protein sequences were generated using the Clustal W program [29]. Protein secondary structure prediction was performed using the JPred program [30].

3. Results

3.1. Interactions between Rev1 protein and Y-family DNA polymerases in animals and yeast

As already mentioned, interactions between the Y-family of DNA polymerases and Rev1 protein from humans and mice transpire via the C-terminal 100 amino acids of Rev1, a region of the protein that is highly conserved in vertebrates (Fig. 1). An iterative search of the NCBI's non-redundant protein sequence database demonstrated that this region of Rev1 is also conserved in a number of invertebrates, and fungi also reveal homologous sequences (Fig. 1). However, the extent of the amino acid conservation is considerably reduced compared to that in vertebrates (Fig. 1). Furthermore, the C-terminal 100 amino acids are not conserved in nematodes. Exhaustive sequence searches failed to reveal any sequences homologous to the nematode Rev1 C-terminus in other eukaryotes or in prokaryotes. Thus, it appears that the C-terminal domain of Rev1 is an innovation of the animal-fungal lineage that was lost in nematodes.

To examine physical interactions between Rev1 and specialized DNA polymerases from various non-vertebrate eukaryotes, cells were co-transformed with vectors that expressed the cloned *REV1* gene and a DNA polymerase of interest, and interactions were examined using the yeast two-hybrid system and in some cases by co-immunoprecipitation. In confirmation of previous studies, mouse Rev1 protein interacted with the mouse Y-family DNA polymerases Polη, Polι and Polκ (Fig. 2 and Table 1). Similar results were obtained when cells were transformed with vectors that express Rev1 and either Polη or Polι from *D. melanogaster* (Fig. 3A and Table 1), an organism which is not endowed with a *POLK* gene. This result was confirmed by immunoprecipitating YFP-tagged Polη from Drosophila cell lysates and detecting Myc-tagged Rev1 on YFP-Polη-bound beads (Fig. 3B).

No interactions were observed between Rev1 and Polη (Rad30/*eso1*+) from the yeasts *S. cerevisiae* and *S. pombe* using the two-hybrid assay (Fig. 3C). To confirm this result, an *S. cerevisiae* strain chromosomally modified to express endogenously tagged Rad30(Polη)-ProA was transformed with a vector expressing yeast Rev1 tagged with HA. Both tagged proteins were functional as evidenced by their ability to complement the sensitivity of yeast *rev1Δ* and *rad30Δ* (*polη*) mutants to killing by UV radiation or MMS

(Fig. 4A and 4B). However, in contrast to the control co-IP between Rev7-Myc and Rev1-HA (Fig. 4E and 4F), when Rad30 (Polη)-ProA was immunoprecipitated from cell extracts (either in the absence or the presence of DNA damage) Rev1-HA failed to co-precipitate (Fig. 4C, 4D). Like Drosophila, the yeast *S. cerevisiae* does not harbor a *POLK* gene. However, Rev1 protein from *S. pombe* failed to interact with Polκ protein from this organism (Table 1). Rev1 protein from the nematode *C. elegans* failed to demonstrably interact with either Polη or Polκ from this organism, an observation consistent with the divergent C-terminal domain of Rev1 in nematodes (Table 1).

3.2 Drosophila Polη and Polι have different requirements for an interaction with Rev1

The interaction between Drosophila Rev1 and Drosophila Polη or Polι was further examined to determine a requirement for the Rev1 C-terminal region, as previously demonstrated in mice and humans. As shown in Fig. 5A, the C-terminal 117 amino acids of Drosophila Rev1 are necessary and sufficient for an interaction with Polη. However, a region adjacent to the C-terminus of Rev1 is required for its interaction with Polι (Fig. 5B). Unlike the C-terminal domain, this region of Drosophila Rev1 is poorly conserved, even in the orthologs from mosquitoes (data not shown). Additional experiments demonstrated a robust interaction between mouse Rev1 C-terminus and Drosophila Polη, but not between the mouse Rev1 C-terminus and Drosophila Polι (Fig. 5C).

In summary, interactions between Rev1 protein and specialized DNA polymerases from the Y-family (Polη, Polι or Polκ) from mouse or humans are apparently conserved in the fruit fly *D. melanogaster*, but not in the worm *C. elegans* or the yeasts *S. cerevisiae* or *S. pombe*. Furthermore, whereas Drosophila Polη interacts with the conserved C-terminus of Rev1, Drosophila Polι exhibits a different requirement for interaction with Rev1.

3.3 Mapping Rev1-interaction domains in Y-family DNA polymerases

Having identified a requirement for the C-terminal region of mouse and Drosophila Rev1 protein for their interaction with some Y-family DNA polymerases, we sought to identify

and compare the Rev1-binding domains in these proteins. Truncated cDNAs for mouse Polη, Polι, and Polκ were constructed and tested for their ability to interact with full-length Rev1 in the yeast two-hybrid assay. With respect to the mouse polymerases, regions spanning ~50 amino acids in the C-terminal half of Polη, Polι, and Polκ supported an interaction with Rev1 (Fig. 6). Similar experiments were performed with truncations of Drosophila Polη and Polι. Once again, regions in the C-terminal half of both proteins supported an interaction with Drosophila Rev1 (Fig. 7). Surprisingly, interactions with Drosophila Rev1 were also observed in the presence of an N-terminal 280 amino acid peptide from Drosophila Polη and an N-terminal 300 amino acid peptide from Drosophila Polι(Fig. 7). These observations were confirmed using a β-galactosidase reporter assay (Fig. 8).

The amino acid sequences of the Rev1-interacting regions of Polη, Polι or Polκ from mouse and Drosophila are shown in Fig. 9. The Rev1-interacting regions that are located closer to the C-termini in these polymerases are poorly conserved. In contrast, the N-terminal regions of Polη and Polι comprise the polymerase domain proper and are highly conserved in most eukaryotes. These findings reveal a paradox. The ~50-amino acid regions interacting with Rev1 are similarly located in various Y-family polymerases. They represent the hinge between the N-terminal polymerase domain and the C-terminal Zn-finger and, as noted above, are poorly conserved, with no reliable alignment observed outside groups of closely related species. For instance, the Rev1-binding regions of mouse Polκ, Polη, and Polι show significant sequence conservation only within the respective sets of mammalian orthologous proteins: neither orthologs from more distant species nor paralogs could be reliably aligned within these regions. In the absence of sequence conservation, we compared the predicted secondary structures of these regions, and found that they are all predicted to be disordered (data not shown) [29, 30]. In contrast, the N-terminal regions of Polη and Polι, which also interact with Rev1 in Drosophila, belong to the polymerase domain proper that is extremely highly conserved in most eukaryotes.

3.4 Interactions between Rev1 protein and Rev7 protein, the catalytic subunit of the B-family DNA polymerase Polζ

In addition to its ability to interact with various Y-family DNA polymerases, the highly conserved C-terminal region of mouse Rev1 protein interacts with the Rev7 subunit of Polζ, a specialized DNA polymerase from the B-family, which is also implicated in TLS in eukaryotes. In the present studies, we demonstrated that Rev1 protein from Drosophila and the yeasts *S. pombe* and *S. cerevisiae* also interact with homologous Rev7 protein (Table1, Fig. 4, and Fig.10). Additionally, we observed by 2-hybrid that mouse Rev1 maintains an interaction with Rev7 from both yeasts and fly (data not shown), suggesting that the region of Rev7 responsible for binding Rev1 is structurally conserved.

4. Discussion

Previous studies indicate that Rev1 protein in eukaryotic organisms maintains one or more functions in DNA damage tolerance independent of its dCMP transferase activity [6, 7]. In light of the observation that human and mouse Rev1 interact with multiple Y-family DNA polymerases via a highly conserved C-terminal domain [9-11], we inquired whether similar if not identical interactions are conserved in invertebrates and fungi that also possess Y-family homologues. Surprisingly, given that *S. cerevisiae* Rev1 and Polη (Rad30) are both required for the replicative bypass (translesion DNA synthesis) of lesions in DNA generated by exposure of cells to UV radiation, we find no evidence of interaction between *S. cerevisiae* Rev1 and Polη (Rad30 protein), regardless of whether cells were exposed to UV radiation or not. Remarkably, the amino acid sequence of the Rev1 C-terminus shows considerable sequence similarity to the corresponding region of Drosophila Rev1, which interacts with both Polη and Polι (Fig. 3A, 3B). These findings are consistent with the observation that, unlike Rev1 and the Polζ complex (Rev3/Rev7) in *S. cerevisiae*, Rev1 and Rad30 do not exhibit an epistatic interaction with respect to UV radiation sensitivity [31].

An interaction between the polymerase accessory domain (PAD) of purified Rev1 and Polη (Rad30 protein) *in vitro* was recently reported [32]. This interaction was not

documented *in vivo*. However, the authors reported that purified yeast Rev1/Rev7 complex precludes interaction between Rev1 with Polη *in vitro* [32]. Conceivably, in the native cellular environment of *S. cerevisiae* where Rev7 (the regulatory subunit of DNA polymerase ζ) is abundant, this protein sequesters most, if not all Rev1, thus preventing complex formation between Rev1 and Polη (Rad30 protein).

Recent studies from one of our laboratories (GCW) have documented that Rev1 protein levels are cell cycle regulated in *S. cerevisiae* [33]. To further explore a possible functional relationship between Rev1 and Y-family polymerases in *S. cerevisiae* we performed epistasis analysis between Rev1 and Polη (Rad30 protein) in G1 or G2 arrested cells with respect UV radiation exposure and observed no cell-cycle dependent genetic relationship at low dosage. Similar results have been reported for asynchronous cells [23 and R. Woodruff and G. Walker, unpublished results]. The absence of physical and genetic interactions may explain the observation that *S. cerevisiae* Rev1 and Rad30 are not required for the replicative bypass of the same UV radiation-induced cognate lesions [7, 34]. Indeed, since Rad30 is apparently not required for UV radiation-induced mutagenesis (unlike Rev1 or Polζ), it has been speculated that Polη (Rad30) protein participates in an error-free repair pathway independent of Rev1 protein [31, 35]. In summary, it seems reasonable to suggest that the C-terminus of Rev1 acquired novel functions in more complex eukaryotes. Alternatively, different sets of interactions may execute similar functions, as suggested by the observation that *S. pombe* Rev7 protein interacts with Rev1, Polκ and Polη (*eso1+*) [J. N. Kosarek and E. C. Friedberg, unpublished results].

The observation that Drosophila Rev1 protein interacts with both Polη and Polι is intriguing. Drosophila is not endowed with an adaptive immune system [36] suggesting that these interactions did not evolve to support somatic hypermutation, a process in which several Y-family polymerases in higher eukaryotes are implicated [37, 38]. Remarkably, Drosophila Polη interacts with the C-terminal 117 amino acids of Drosophila Rev1, just as in mouse and humans. Drosophila Polη also maintains an interaction with the C-terminus of mouse Rev1, suggesting functional homology between the C-terminal domains of mouse and *Drosophila*, despite reduced sequence conservation. In contrast, Drosophila Polι does not interact with the C-terminus of Rev1,

but rather with a distinct domain that does not appear to be conserved in Rev1 protein from the other species examined, nor does it show any sequence similarity to closely related species (data not shown). The minimal conservation of this Polι-binding domain in Drosophila Rev1 suggests that Drosophila Rev1 may have a unique mechanism for switching between Polι and Polη.

Our experiments demonstrate that Drosophila Polη and Polι each utilize two independent domains for interacting with Rev1. In addition to the domain in the C-terminal half of these proteins, (similarly located to the Rev1-interaction domains identified in the mouse homologues of Polη, Polι, and Polκ) we identified a second Rev1-interaction domain located at the N-terminus of Drosophila Polη and Polι. The N-terminal motifs of Drosophila Polι and Polη that bind Rev1 contain the five characteristic Y-family motifs, including the catalytic domains of the polymerases, which are well conserved among all species. The N-terminal fragment of Drosophila Polη can also support an interaction with mouse Rev1 protein (data not shown), suggesting there are functional differences between the N-terminus of mouse Polη and Drosophila Polη, notwithstanding the high degree of amino acid conservation. The additional observation that Drosophila Rev1 interacts with the catalytic domains of Polη and Polι raises the possibility that these interactions may affect the catalytic properties of these proteins, as has been shown for the interaction between yeast Rev1 and Rev3 (Polζ) [39].

The Rev1-interacting regions in the similarly located mouse and Drosophila Y-family polymerases examined in our studies are predicted to share disordered structures. Disordered interaction domains have been observed among transcription factors [40] and a variety of other regulatory proteins. A structured protein that interacts with multiple unstructured partners has also been observed [41]. Furthermore, functionally analogous domains have been observed which have little sequence similarity but ~~to~~ share intrinsic disorder [42], which is predicted to be the case for Rev1-binding partners. UmuD and UmuD' proteins from *E. coli*, which are also involved in DNA damage tolerance, have also been shown to be intrinsically disordered (S.M. Simon, F.J.R. Sousa, R.S. Mohana-Borges and G. C. Walker, manuscript in preparation). UmuD and UmuD' are the products of the *umuD* gene; they stably interact with and functionally regulate the activity

of the prokaryotic Y-family member UmuC, and interact with many other proteins, including RecA, DinB, and polymerase subunits α, β, and ε [43-48].

Interaction between Rev1 and Rev7 (the catalytic subunit of Polζ) is maintained in all organisms studied, suggesting that these proteins co-evolved to maintain an essential function for TLS. Studies in yeast have shown that Polζ is indispensable for DNA damage-induced mutagenesis and that Rev1 is required for the function of Polζ [4, 49]. Furthermore, kinetic analyses have shown that Rev1 enhances Polζ function during mismatch extension as well as extension past abasic sites and [6-4] photoproducts [39]. While the specific role of the Rev1/Rev7 interaction remains to be determined, our results provide evidence that this interaction may underlie a distinctly conserved TLS function.

In conclusion, in our efforts to expand studies of the Rev1/Y-family polymerase interactions to a more tractable model organism, we conclude that no single eukaryote thus far examined can be considered a prototypic model system for generalizing the molecular mechanism of TLS in eukaryotes, and that particular domains of these proteins and their functions are more divergent than originally thought. These studies should advocate special consideration when making mechanistic extrapolations from lower to higher eukaryotes and vice versa.

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References

1. Friedberg, E., *Suffering in silence: the tolerance of DNA damage*. Nat Rev Mol Cell Biol, 2005. **6**(12): p. 943-953.
2. Friedberg, E., *DNA Repair and Mutagenesis*. 2 ed. 2006, Washington DC: ASM Press.
3. Friedberg EC, L.A., Fuchs RP, *Trading Places: how do DNA polymerases switch during tranlesion DNA synthesis?* Mol Cell, 2005. **18**(5): p. 499-505.
4. Lawrence, C., *Cellular functions of DNA polymerase zeta and Rev1 protein*. Adv Protein Chem., 2004. **69**: p. 167-203.
5. Otsuka C, L.D., Negishi K, *The role of deoxycytidly transferase activity of yeast Rev1 protein in the bypass of abasic sites*. Nucleic Acis Res Suppl., 2002. **2**: p. 87-88.
6. Ross AL, S.L., Sale JE, *Vertebrate DNA damage tolerance requires the C-terminus but not BRCT or transferase domains of Rev1*. Nucleic Acids Res., 2005. **33**(4): p. 1280-1289.
7. Nelson JR, G.P., Nowicka AM, Hinkle DC, Lawrence CW, *Evidence for a second function for Saccharomyces cerevisiae Rev1p*. Mol. Microbiol., 2000. **37**(3): p. 549-54.
8. Guo C, S.E., Tang TS, Parker JL, Bielen AB, Takeda S, Ulrich HD, Friedberg EC, *REV1 protein interacts with PCNA: significance of the REV1 BRCT domain in vitro and in vivo*. Mol Cell, 2006. **23**(2): p. 265-271.
9. Guo C, F.P., Luk-Paszyc MJ, Masuda , Zhou J, Kamiya K, Kisker C, Friedberg EC, *Mouse Rev1 protein interacts with multiple DNA polymerases involved in translesion DNA synthesis*. EMBO J, 2003. **22**(24): p. 6621-6630.
10. Tissier A, K.P., Reck MP, Lehmann AR, Fuchs RP, Cordonnier A, *Co-localization in replication foci and interaction of human Y-family members, DNA polyserase pol eta and REV1 protein*. DNA Repair, 2004. **3**(11): p. 1503-1514.
11. Ohashi E, M.Y., Kanjo N, Akagi J, Masutani C, Hanaoka F, Ohmori H, *Interaction of hREV1 with three human Y-family DNA polymerases*. Genes Cells, 2004. **9**(6): p. 523-531.
12. Haracska L, K.C., Unk I, Prakash S, Prakash L, *Interaction with PCNA is essential for yeast DNA polymerase eta function*. Mol Cell, 2001. **8**(2): p. 407-415.
13. Haracska L, J.R., Unk I, Phillips BB, Hurwitz J, Prakash L, Prakash S, *Targeting of human DNA polymerase iota to the replication machinery via interaction with PCNA*. Proc Natl Acad Sci USA, 2001. **98**(25): p. 14256-14261.
14. Haracska L, U.I., Johnson RE, Phillips BB, Hurwitz J, Prakash L, Prakash S, *Stimulation of DNA synthesis activity of human DNA polymerase kappa by PCNA*. Mol Cell Biol, 2002. **22**(3): p. 784-791.
15. Bienko M, G.C., Crosetto N, Rudolf F, Zapart G, Coull B, Kannouche P, Wider G, Peter M, Lehmann AR, Hofmann K, Dikic I, *Ubiquitin-binding domains in Y-family polymerases regulate translesion synthesis*. Science, 2005. **310**(5755): p. 1821-1824.

16. Guo C, T.T., Bienko M, Parker JL, Bielen AB, Sonoda E, Takeda S, Ulrich HD, Dikic I, Friedberg EC, *Ubiquitin-binding motifs in REV1 protein are required for its role in the tolerance of DNA damage*. Mol Cell Biol, 2006. **26**(23): p. 8892-8900.
17. Ohkumo T, M.C., Eki T, Hanaoka F, *Deficiency of the Caenorhabditis elegans DNA polymerase eta homologue increases sensitivity to UV radiation during germ-line development*. Cell Struct Funct, 2006. **31**(1): p. 29-37.
18. Ishikawa T, U.N., Mizukoshi T, Iwai S, Iwasaki H, Musutani C, Hanaoka F, Ueda R, Ohmori H, Todo T, *Mutagenic and nonmutagenic bypass of DNA lesions by Drosophila DNA polymerases dpoleta and dpoliota*. J Biol Chem, 2001. **276**(18): p. 15155-15163.
19. Zhao X, M.E., Rothstein R, *A supressor of two essential checkpoint genes identifies a novel protein that negatively affects dNTP pools*. Mol Cell, 1998. **2**(3): p. 329-340.
20. Knop, M., et al., *Epitope tagging of yeast genes using a PCR-based strategy: more tags and improved practical routines*. Yeast, 1999. **15**(10B): p. 963-72.
21. D'Souza, S. and G.C. Walker, *Novel Role for the C-terminus of Saccharomyces cerevisiae Rev1 in Mediating Protein-Protein Interactions*. Mol Cell Biol, 2006.
22. Waters, L.S. and G.C. Walker, *The critical mutagenic translesion DNA polymerase Rev1 is highly expressed during G(2)/M phase rather than S phase*. Proc Natl Acad Sci U S A, 2006. **103**(24): p. 8971-6.
23. Skoneczna, A., et al., *Polymerase eta is a short-lived, proteasomally degraded protein that is temporarily stabilized following UV irradiation in Saccharomyces cerevisiae*. J Mol Biol, 2007. **366**(4): p. 1074-86.
24. McDonald, J.P., A.S. Levine, and R. Woodgate, *The Saccharomyces cerevisiae RAD30 gene, a homologue of Escherichia coli dinB and umuC, is DNA damage inducible and functions in a novel error-free postreplication repair mechanism*. Genetics, 1997. **147**(4): p. 1557-68.
25. Roush, A.A., et al., *Deletion of the Saccharomyces cerevisiae gene RAD30 encoding an Escherichia coli DinB homolog confers UV radiation sensitivity and altered mutability*. Mol Gen Genet, 1998. **257**(6): p. 686-92.
26. Duncker, B.P., et al., *An N-terminal domain of Dbf4p mediates interaction with both origin recognition complex (ORC) and Rad53p and can deregulate late origin firing*. Proc Natl Acad Sci U S A, 2002. **99**(25): p. 16087-92.
27. Altschul SF, M.T., Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ, *Gapped BLAST and PSI-BLAST: a new generation of protein database search programs*. Nucleic Acids Res., 1997. **25**(17): p. 3389-3402.
28. Schäffer AA, A.L., Madden TL, Shavirin S, Spouge JL, Wolf YI, Koonin EV, Altschul SF, *Improving the accuracy of PSI-BLAST protein database searches with composition-based statistics and other refinements*. Nucleic Acids Res., 2001. **29**(14): p. 2994-3005.
29. Thompson JD, H.D.a.G.T., *CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice*. Nucleic Acids Res., 1994. **22**(22): p. 4673-4680.

30. Cuff JA, C.M., Siddiqui AS, Finlay M, Barton GJ, *JPred: a consensus secondary structure prediction server*. Bioinformatics, 1998. **14**: p. 892-893.
31. McDonald JP, L.A., Woodgate R, *The Saccharomyces cerevisiae RAD30 gene, a homologue of Escherichia coli dinB and umuC, is DNA damage inducible and functions in a novel error-free postreplication repair mechanism*. Genetics, 1997. **147**(4): p. 1557-1558.
32. Acharya N, H.L., Prakash S, Prakash L, *Complex formation of yeast Rev1 with DNA polymerase eta*. Mol Cell Biol, 2007. **IN PRESS**.
33. Waters LS, W.G., *The critical mutagenic translesion DNA polymerase Rev1 is highly expressed during G(2)/M phase rather than S phase*. Proc Natl Acad Sci USA, 2006. **103**(24): p. 8971-8976.
34. Gibbs PE, M.J., Woodgate R, Lawrence CW, *The relative roles in vivo of Saccharomyces cerevisiae Pol eta, Pol zeta, Rev1 protein and Pol32 in the bypass and mutation induction of an abasic site, T-T (6-4) photoadduct and T-T cis-syn cyclobutane dimer*. Genetics, 2005. **169**(2): p. 575-582.
35. Roush AA, S.M., Friedberg EC, Radman M, Siede W, *Deletion of the Saccharomyces cerevisiae gene RAD30 encoding an Escherichia coli DinB homolog confers UV radiation sensitivity and altered mutability*. Mol Gen Genet, 1998. **257**(6): p. 686-692.
36. Medzhitov R, J.C.J., *Innate immune recognition: mechanisms and pathways*. Immunol Rev, 2000. **173**(89-97).
37. Ross AL, S.J., *The catalytic activity of REV1 is employed during immunoglobulin gene diversification in DT40*. Mol Immunol, 2006. **43**(10): p. 1587-1594.
38. Masuda K, O.R., Hikida M, Kurosaki T, Yokoi M, Masutani C, Seki M, Wood RD, Hanaoka F, O-Wang J, *DNA polymerases eta and theta function in the same genetic pathway to generate mutations at A/T during somatic hypermutation of Ig genes*. J Biol Chem, 2007. **282**(24): p. 17387-17394.
39. Acharya N, J.R., Prakash S, Prakash L, *Complex formation with Rev1 enhances the proficiency of Saccharomyces cerevisiae DNA polymerase zeta for mismatch extension and for extension opposite from DNA lesions*. Mol Cell Biol, 2006. **26**(24): p. 9555-9563.
40. Liu J, P.N., Oldfield CJ, Su EW, Uversky VN, Dunker AK, *Intrinsic disorder in transcription factors*. Biochemistry, 2006. **6**(45): p. 6873-6888.
41. Bustos DM, I.A., *Intrinsic disorder is a key characteristic in partners that bind 14-3-3 proteins*. Proteins, 2006. **63**(1): p. 35-42.
42. Kumar R, T.E., *Gene regulation by the glucocorticoid receptor: structure:function relationship*. J Steroid Biochem Mol Biol, 2005. **94**(5): p. 383-394.
43. Tang M, S.X., Frank EG, O'Donnell M, Woodgate R, Goodman MF, *UmuD'(2)C is an error-prone DNA polymerase, Escherichia coli pol V*. Proc Natl Acad Sci USA, 1999. **96**(16): p. 8919-8924.
44. Jarosz DF, B.P., Cohen SE, Walker GC, *Y-family DNA polymerases in Escherichia coli*. Trends Microbiol, 2007. **15**(2): p. 70-77.
45. Sutton MD, O.T., Walker GC, *The Escherichia coli SOS mutagenesis proteins UmuD and UmuD' interact physically with the replicative DNA polymerase*. Proc Natl Acad Sci USA, 1999. **96**(22): p. 12373-12378.

46. Shinagawa H, I.H., Kato T, Nakata A., *RecA protein-dependent cleavage of UmuD protein and SOS mutagenesis*. Proc Natl Acad Sci USA, 1988. **85**(6): p. 1806-1810.
47. Burckhardt SE, W.R., Scheuermann RH, Echols H, *UmuD mutagenesis protein of Escherichia coli: overproduction, purification, and cleavage by RecA*. Proc Natl Acad Sci USA, 1988. **85**(6): p. 1811-1815.
48. Nohmi T, B.J., Dodson LA, Walker GC, *RecA-mediated cleavage activates UmuD for mutagenesis: mechanistic relationship between transcriptional derepression and posttranslational activation*. Proc Natl Acad Sci USA, 1988. **85**(6): p. 1816-1820.
49. Baynton K, B.-R.A., Fuchs RP, *Distinct roles for Rev1p and Rev7p during translesion synthesis in Saccharomyces cerevisiae*. Mol Microbiol, 1999. **34**(1): p. 123-133.

Figures and Tables

Figure 1.

Figure 1. The C-terminus of Rev1 is highly conserved in vertebrates but to a lesser extent among invertebrates. The sequence of the C-terminal 100 amino acids of Rev1 protein in various higher and lower eukaryotes is shown. Hs, *H. sapiens*; Mm, *M. musculus*; Tn, *Tetradon nigroviridis*; Rn, *Rattus norvegicus*; Gg, Gallus gallus; Xl, *Xenopus laevis*; Dm, *Drosophila melanogaster*; Sp, *Schizosaccharomyces pombe;* Ust, *Usilago maydis*; Asp, *Aspergillus fumigatus;* Sc, *Saccharomyces cerevisiae.*

Figure 2.

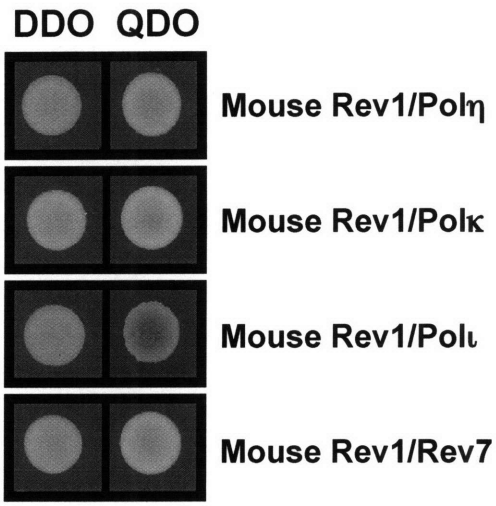
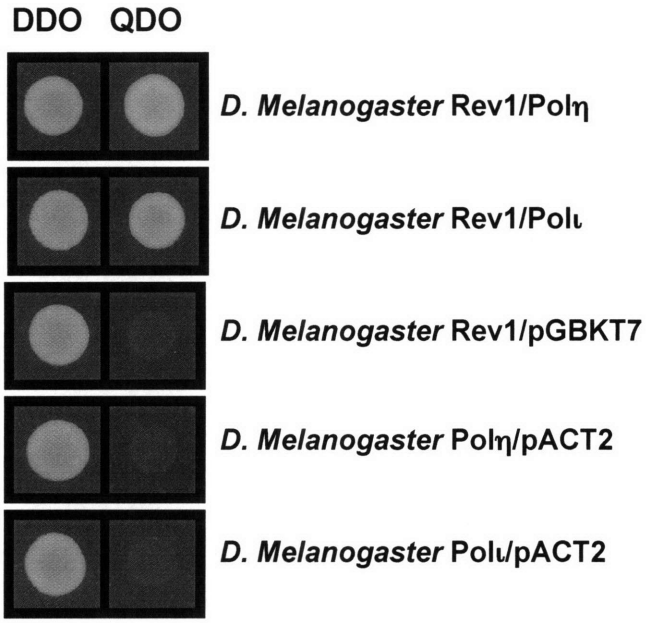


Figure 2. Rev1 interacts with specialized DNA polymerases in mice.
The C-terminus of mouse Rev1 (~120 a.a.) interacts with Polη, Polκ, Polι, and Rev7 in the yeast two-hybrid assay. Yeast transformants expressing a mouse Rev1-activation domain (AD) fusion protein and the designated polymerase (Polι, Polκ, Polη, or Rev7)-binding domain (BD) fusion protein are selected on double drop out (DDO) media (*-Trp* or *Leu)*. Positive interactions are indicated by growth on quadruple drop out (QDO) media acids (*-Trp*, *-Leu*, *-Ade*, *-His)*. Growth on QDO media indicates the two proteins physically interact, as their proximity results in the expression of proteins required for histidine and adenine biosynthesis.

Figure 3. Interactions between Rev1 protein and Y-family DNA polymerases.
(**A**) Yeast two-hybrid results for Drosophila Rev1 with Drosophila Polη and Polι. (**B**) Drosophila Rev1 co-precipitates with Polη; Lane 1: input Rev1-Myc + YFP; Lane 2: IP Rev1-Myc + YFP; Lane 3: input Rev1-Myc + Polη-YFP; Lane 4: IP Rev1-Myc + Polη-YFP. (**C**) Rev1 does not interact with Polη homologs (Rad30 or *eso1+*) in *S. cerevisiae* or *S. pombe*.

A



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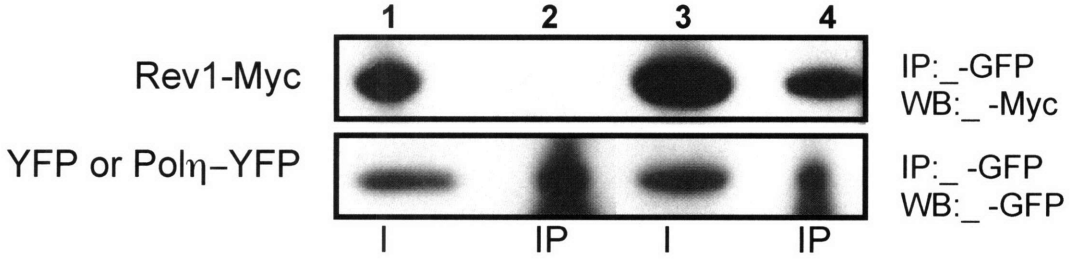


Figure 3, continued.

C

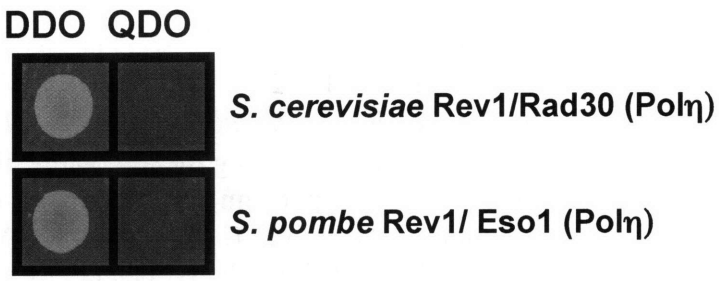
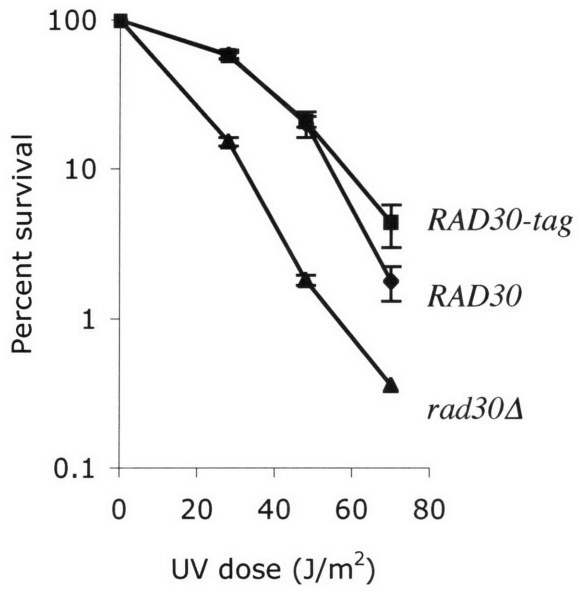


Figure 3. Interactions between Rev1 protein and Y-family DNA polymerases.
(**C**) Rev1 does not interact with Polη homologs (Rad30 or *eso1+*) in *S. cerevisiae* or *S. pombe*.

Figure 4. *S. cerevisiae* Rev1 does not immunoprecipitate Rad30.
(A) *Tagged Rad30 (Polη) is fully functional for UV radiation survival*: comparison of WT (W1588-4C), Rad30-TEV-ProA-His (RWY13), and *rad30Δ* (RWY15) strains. Error bars represent standard error. **(B)** *Rev1-HA-pAS311 can rescue the MMS sensitivity of the rev1 null mutant.* Top row: wildtype (W1588-4A + pAS311), second row: *rev1Δ* (YLW20 + pAS311) bottom row: Rev1-HA (YLW20 + pAS311-REV1-HAC). **(C)** *Rev1 and Rad30 (Polη) do not co-immunoprecipitate in the absence of DNA damage.* IgG was used to precipitate Rad30-TEV-ProA-7His protein using the alternate coIP protocol with strains RWY75 and YSD7. Lane 1: RWY75 input sample probed with PAP for Rad30-TEV-ProA-7His; Lane 2: RWY75 input; Lane 3, YSD7 IP; Lane4, RWY75 IP, showing Rad30 band only. Lanes 2-4 probed with anti-HA antibody, detects Rev1-HA present in the input and also (through the IgG-binding activity of ProA) nonspecifically detects the high concentration of Rad30-ProA in the IP. **(D)** *Rev1 and Rad30 (Polη) do not coimmunoprecipitate in the presence of DNA damage.* Yeast extracts were made from cells that had been subjected to UV radiation. IgG was used to precipitate Rad30-TEV-ProA-7His protein by the primary coIP protocol. Lane 1: Rev1-HA and Rad30-ProA (RWYRWY254 + pAS311-REV1-HAC); Lane 2: Rev1-HA only (RWY270 + pAS311-REV1-HAC); Lane 3: Rad30-ProA only (RWY254). (E) *Rev1-HA coimmunoprecipitates endogenously tagged Rev7-Myc.* Lane 1: Rev1-Cterm-HA and Rev7-13Myc (YSD5 + pAS311-REV1CT239-HAC); Lane 2: Full length Rev1-HA and Rev7-13Myc (YSD5 + pAS311-Rev1-HAC); Lane 3: Rev7-13Myc alone (YSD5 + pAS311). Full length Rev1 is produced at lower levels than the C-terminal 239 amino acid fragment, resulting in the difference in quantity of Rev7-Myc which coIPs in lane 1 compared with lane 2. **(F)** *Endogenously tagged Rev7 immunoprecipitates Rev1-HA.* Rev7-13Myc immunoprecipitates Rev1 in the presence (+) of Rev1-HA (YSD5 + pAS311-Rev1-HAC) but does not in the absence (-) of Rev1-HA (YSD5 + pAS311). Rev1-HA is undetectable in the input (not shown).

Figure 4.

A



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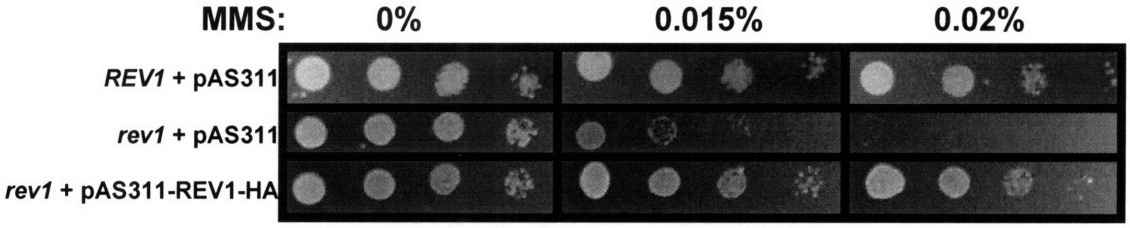

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(A) *Tagged Rad30 (Polη) is fully functional for UV radiation survival*: comparison of WT (W1588-4C), Rad30-TEV-ProA-His (RWY13), and *rad30Δ* (RWY15) strains. Error bars represent standard error. **(B)** *Rev1-HA-pAS311 can rescue the MMS sensitivity of the rev1 null mutant.* Top row: wildtype (W1588-4A + pAS311), second row: *rev1Δ* (YLW20 + pAS311) bottom row: Rev1-HA (YLW20 + pAS311-REV1-HAC).

Figure 4 (continued).

C

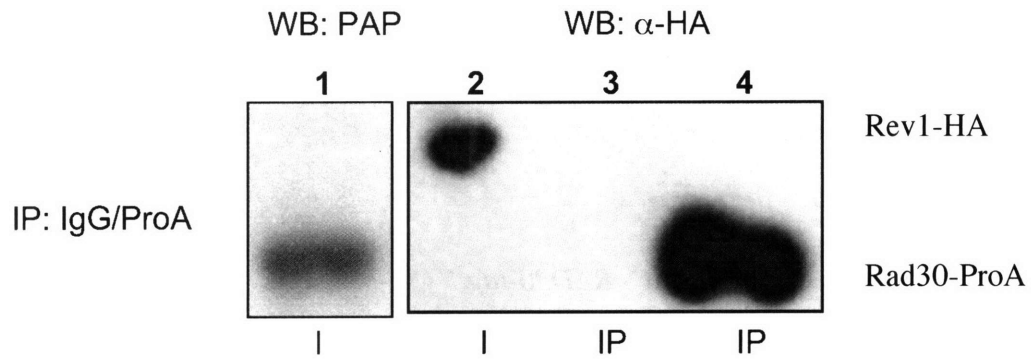

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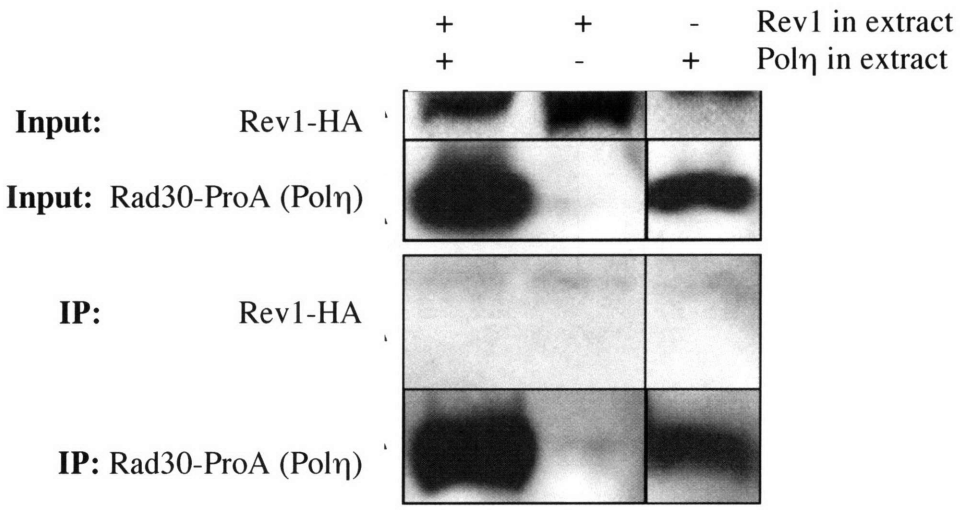
Figure 4 (continued).

D



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(D) *Rev1 and Rad30 (Polη) do not coimmunoprecipitate in the presence of DNA damage.* Yeast extracts were made from cells that had been subjected to UV radiation at a dose of 50 J/m^2. IgG was used to precipitate Rad30-TEV-ProA-7His protein by the primary coIP protocol. Lane 1: Rev1-HA and Rad30-ProA (RWYRWY254 + pAS311-REV1-HAC); Lane 2: Rev1-HA only (RWY270 + pAS311-REV1-HAC); Lane 3: Rad30-ProA only (RWY254).

Figure 4 (continued).

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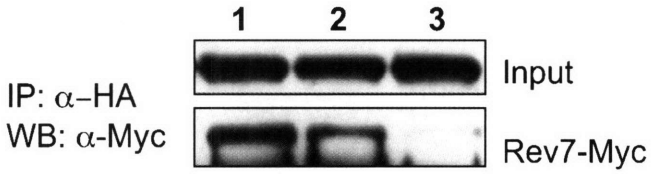
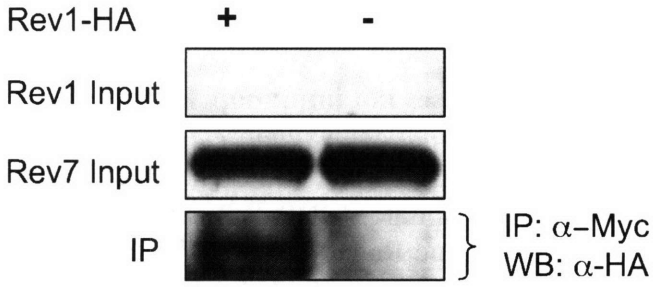


F



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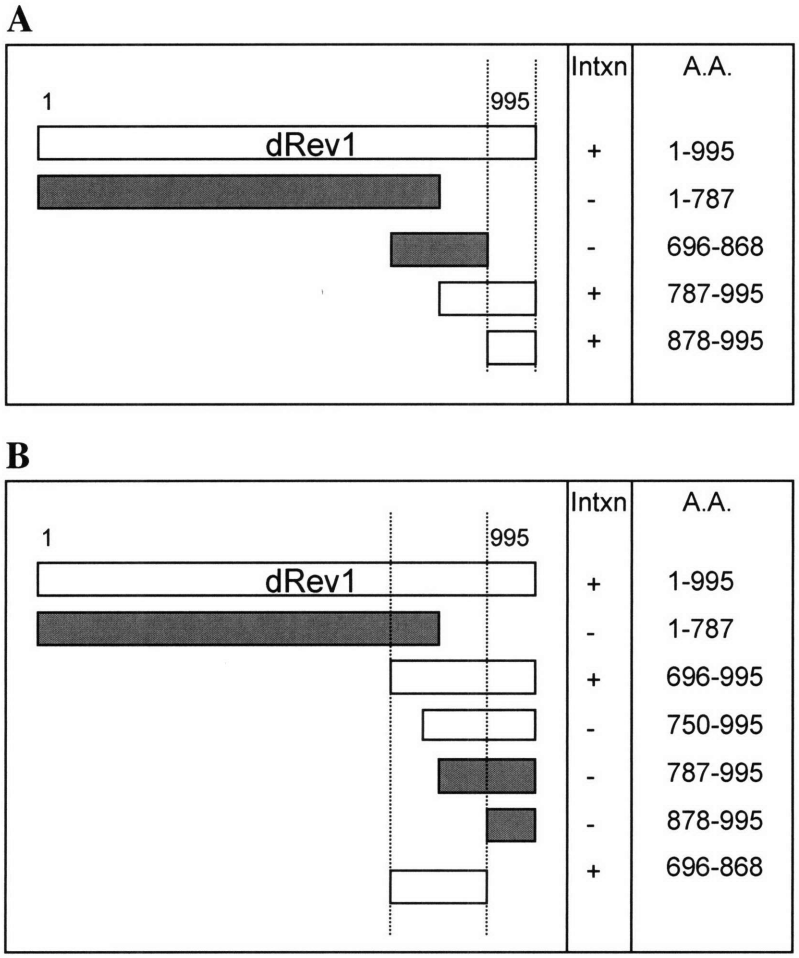
(E) *Rev1-HA coimmunoprecipitates endogenously tagged Rev7-Myc.* Lane 1: Rev1-Cterm-HA and Rev7-13Myc (YSD5 + pAS311-REV1CT239-HAC); Lane 2: Full length Rev1-HA and Rev7-13Myc (YSD5 + pAS311-Rev1-HAC); Lane 3: Rev7-13Myc alone (YSD5 + pAS311). Full length Rev1 is produced at lower levels than the C-terminal 239 amino acid fragment, resulting in the difference in quantity of Rev7-Myc which coIPs in lane 1 compared with lane 2. **(F)** *Endogenously tagged Rev7 immunoprecipitates Rev1-HA.* Rev7-13Myc immunoprecipitates Rev1 in the presence (+) of Rev1-HA (YSD5 + pAS311-Rev1-HAC) but does not in the absence (-) of Rev1-HA (YSD5 + pAS311). Rev1-HA is undetectable in the input (not shown).

Figure 5.



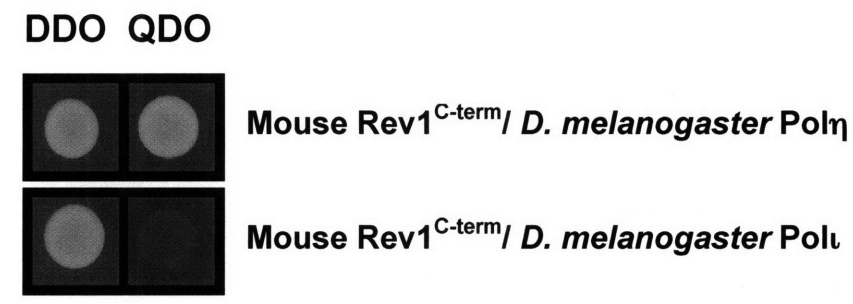
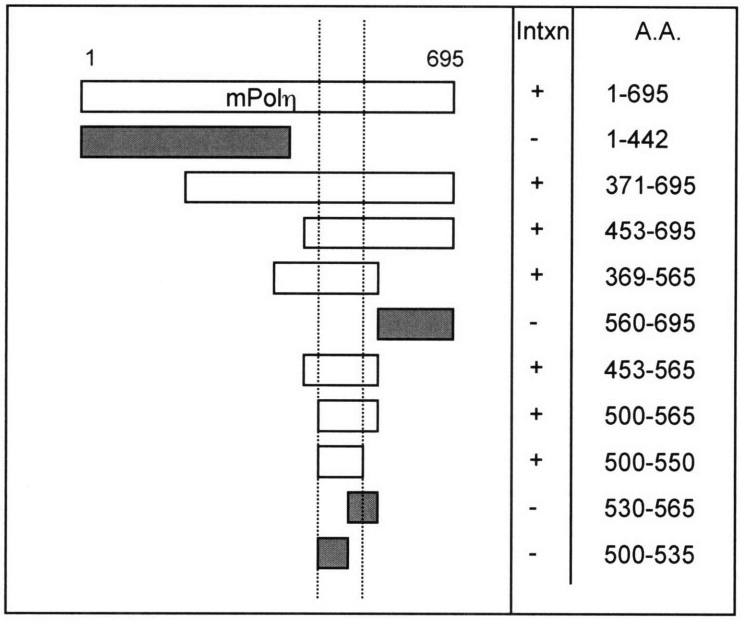


Figure 5. Drosophila Polη and Polι have different requirements for interaction with Rev1.

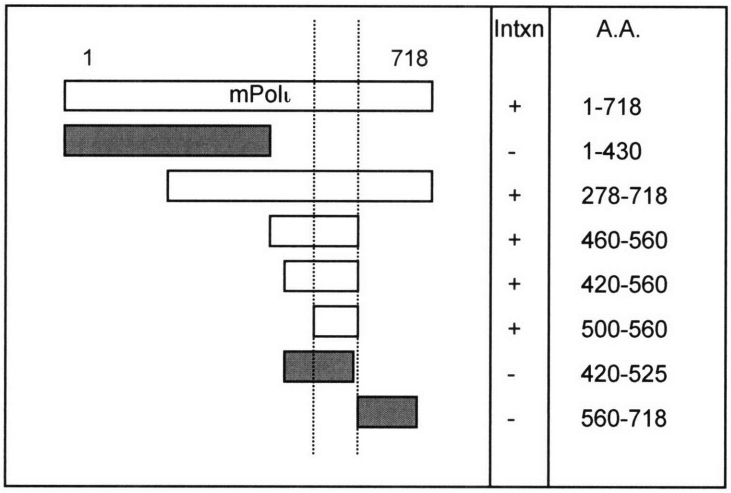
(A) Rev1 interacts with dPolη through its conserved C-terminal domain (~117a.a.). **(B)** dPolι requires amino acids upstream of the C-terminus. **(C)** *Drosophila* Polη interacts with the C-terminus (~120a.a.) of mouse Rev1, while *Drosophila* Polι does not.

Figure 6.

A


1
695
mPolη
Intxn
A.A.
+ 1-695
- 1-442
+ 371-695
+ 453-695
+ 369-565
- 560-695
+ 453-565
+ 500-565
+ 500-550
- 530-565
- 500-535

B


1
718
mPolι
Intxn
A.A.
+ 1-718
- 1-430
+ 278-718
+ 460-560
+ 420-560
+ 500-560
- 420-525
- 560-718

C

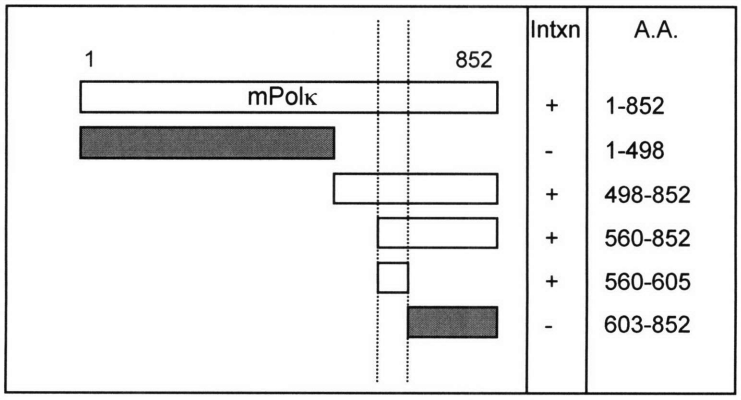
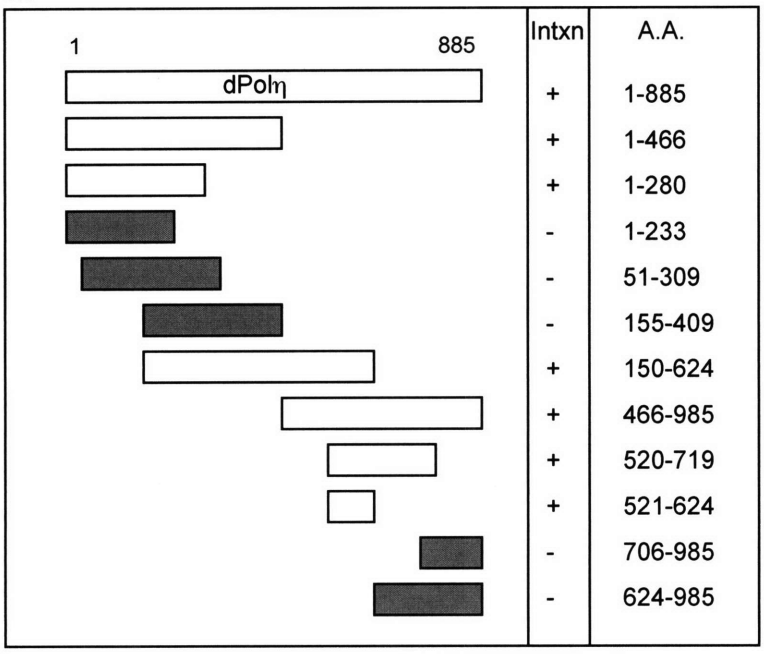

1
852
mPolκ
Intxn
A.A.
+ 1-852
- 1-498
+ 498-852
+ 560-852
+ 560-605
- 603-852

Figure 6. Mapping of mouse Rev1-interaction domains in Y-family DNA polymerases.

The interaction between mouse Rev1 and the Y-family polymerases requires a region spanning ~50 a.a. in the C-terminal half of **(A)** Polη (500-550), **(B)** Polι (500-560), and **(C)** Polκ (560-605).

Figure 7.

A



B

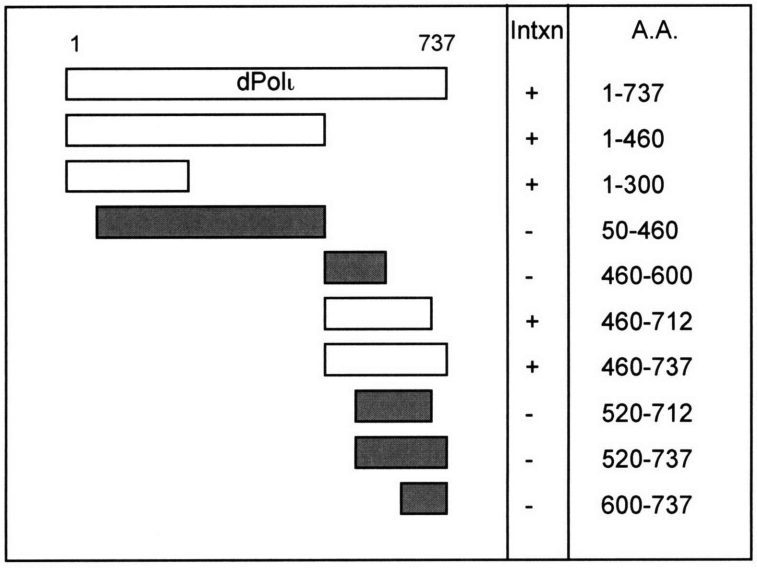
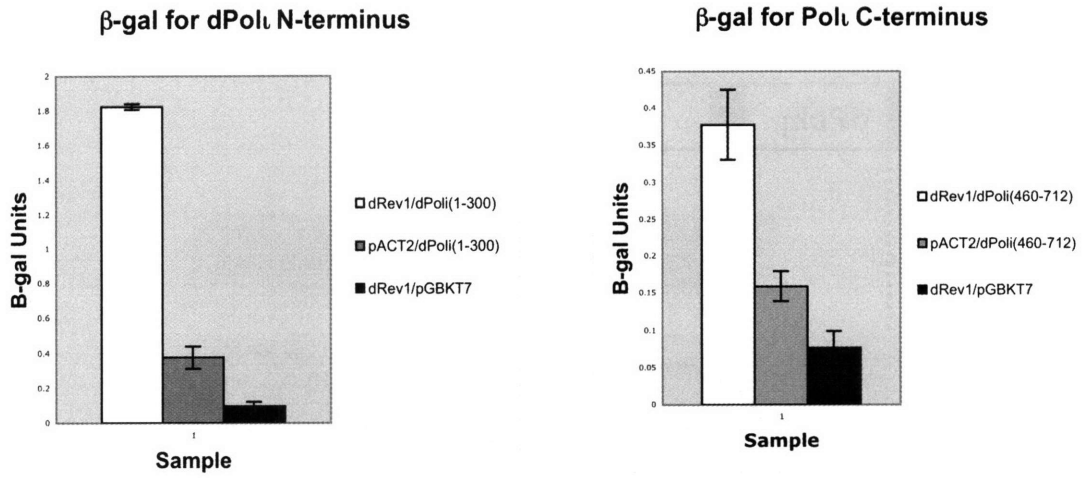


Figure 7. Drosophila Polη and Polι bind Rev1 with two independent regions.
(A) dPolη and **(B)** dPolι interact with Rev1 via an N-terminal peptide as well as a region located in the C-terminal half of each protein.

Figure 8.

A



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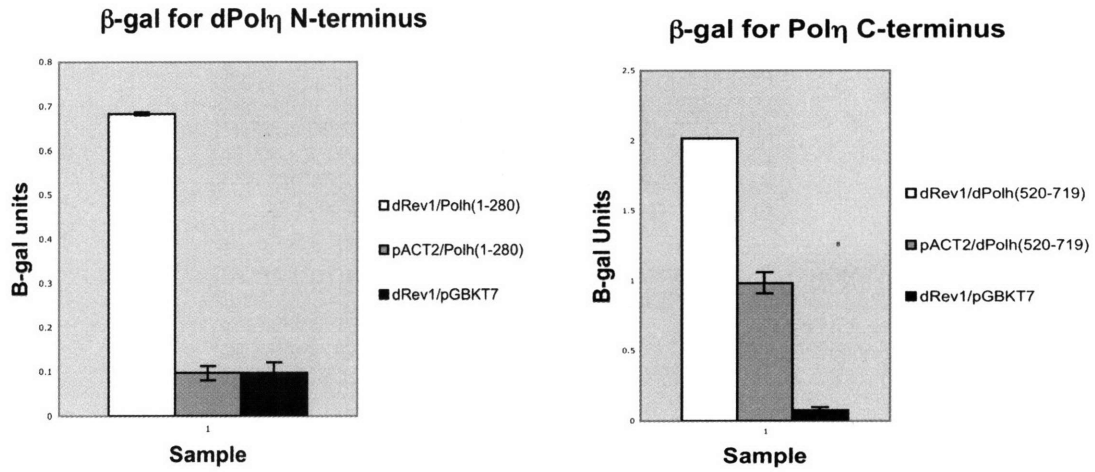

Figure 8. Expression of β-galactosidase confirms two Rev1 binding domains in *Drosophila* Polη and Polι.

(**A**) dPolι and (**B**) dPolη interact with Rev1 via an N-terminal peptide as well as a region located in the C-terminal half of each protein. Full-length protein interactions for Polη and Polι are set at a value=1 unit (not shown). All displayed values are normalized to the full-length interaction.

Figure 9.

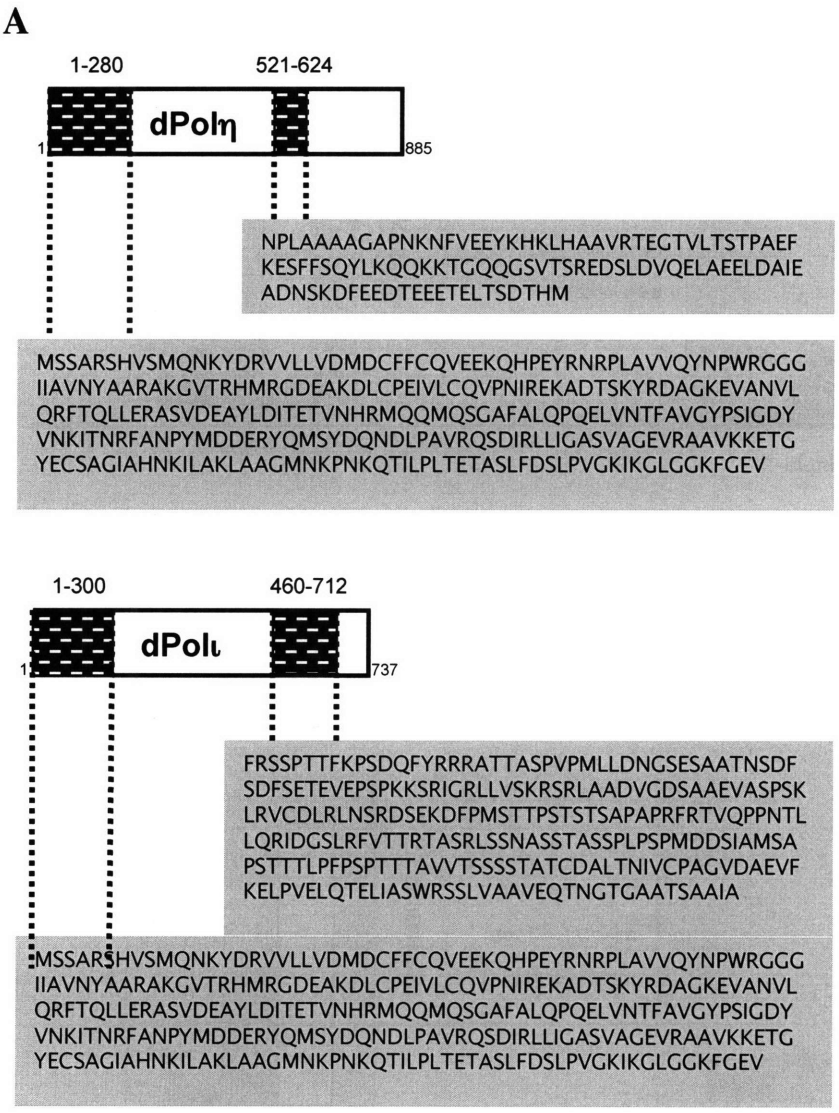

Figure 9. The amino acid sequences of Rev1-binding regions Drosophila and mouse Y-family polymerases.

Similarly located binding domains in (**A**) Drosophila and (**B**) mouse Y-family DNA polymerases comprise a disordered hinge between the N-terminal polymerase domain and the C-terminal Zn-finger. These sequences reveal no reliable alignment outside closely related species. Drosophila Polη and Polι N-terminal fragments comprise a second binding domain not observed in mouse.

Figure 9 (continued).

B

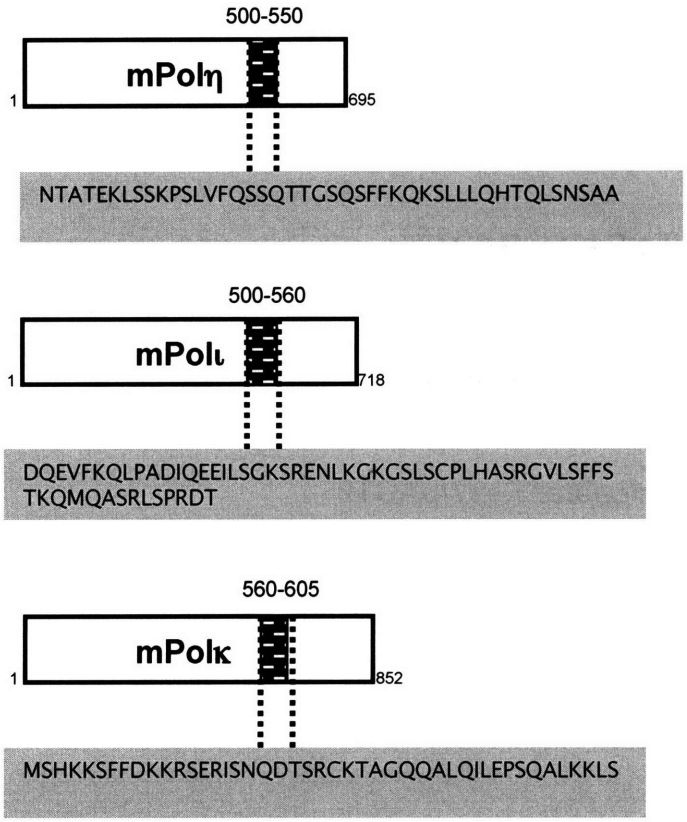

Figure 10.

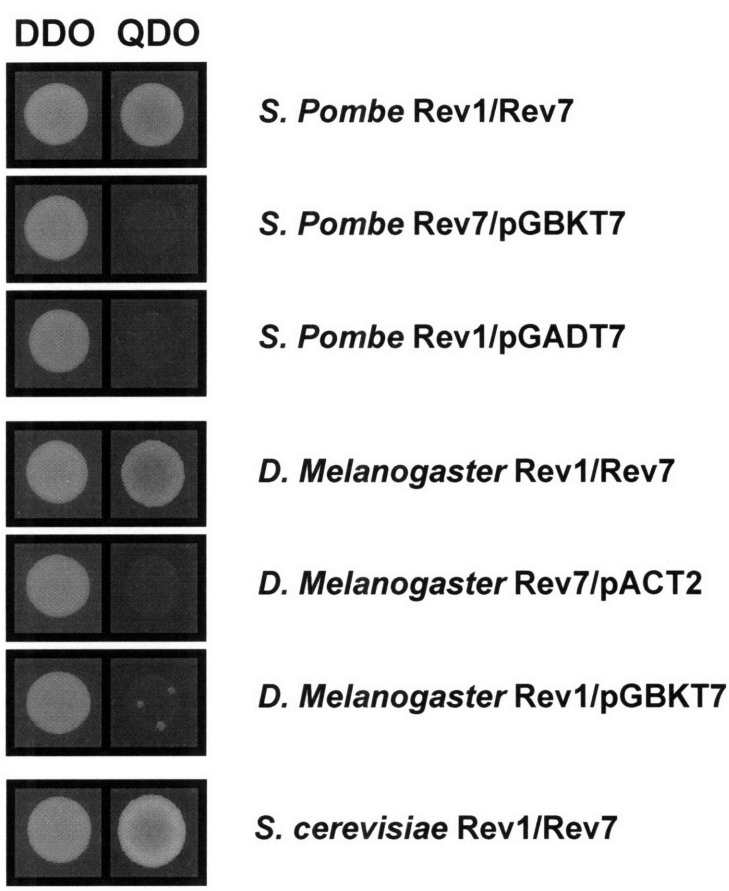

Figure 10. Rev1 interacts with Rev7 in *S. pombe*, *D. melanogaster*, and *S. cerevisiae*. Homologs of (**A**) *S. pombe* Rev1 and (**B**) Drosophila Rev1 interact with Rev7 homologs in the yeast two-hybrid assay. *S. cerevisiae* Rev1 and Rev7 also interact (**C**).

Table 1.

	Mouse	Fly	Worm	Budding yeast	Fission yeast
Polη	+	+	-	-	-
Polι	+	+			
Polκ	+		-		-
Rev7	+	+		+	+

Table 1. The conservation of TLS polymerase interactions with Rev1 protein within different species. The presence (+) or absence (-) of a DNA polymerase interaction with Rev1 (as determined by the yeast two-hybrid or other methods described here) is indicated. Shaded boxes indicate that the polymerase has not been identified in the species.

Table 2. S. cerevisiae strains used in this study.

Strain	Genotype
W1588-4C	*MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5*
W1588-4A	*MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5*
YSD5	*MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5 bar1::LEU2 REV7-13MYC::HIS3MX6*
YLW20	*MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5 rev1::KanMX*
YLW70	*MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5 bar1::LEU2*
RWY13	*MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5 RAD30-TEV-ProA-7His::HIS3MX*
RWY15	*MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5 rad30::KanMX*
RWY254	*MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5 bar1::LEU2 RAD30-TEV-ProA-His::HISMX*
RWY270	*MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5 bar1::LEU2 pep4::KanMX*

Chapter 4:
Genetic clues to the regulation of translesion synthesis in *Saccharomyces cerevisiae*

Rachel Woodruff and Graham C. Walker

ABSTRACT

This genetic study elucidates nuances of the translesion synthesis (TLS) subpathways of post-replication repair (PRR) in *Saccharomyces cerevisiae.* The four TLS genes of *S, cerevisiae, RAD30* (encoding pol eta), *REV1, REV3* and *REV7* (together encoding pol zeta), are all regulated by the *RAD6/RAD18* PRR pathway. Longstanding genetic evidence divides the TLS polymerases among two independent subpathways: Rev1 and pol ζ (zeta) in one, and Rad30 in the other. However, recent findings have suggested that in some species, pol η (eta) physically interacts, and can function cooperatively, with Rev1 and pol ζ. I present data consistent with conditional cooperation between pol η and the other TLS polymerases. I also address the distinct shapes of the UV killing curves of the TLS mutants. I find that pol η protein levels are not induced in response to DNA damage, ruling out the previously proposed model that such induction accounts for the complex shapes of the killing curves of TLS mutants. I suggest that they may be explained either by a persistence-like phenomenon, or by the timing and regulation of TLS.

INTRODUCTION

Polymerase (pol) η *(RAD30)* acts as part of the *RAD6* post replication repair (PRR) epistasis group [1], as do the other translesion synthesis (TLS) DNA polymerases of *S. cerevisiae*, Rev1 and Pol ζ (encoded by *REV3* and *REV7).* The PRR pathway is initiated by the action of Rad6 and Rad18, which ubiquitinate PCNA (and possibly other targets), leading to the activation of TLS and other DNA damage tolerance mechanisms.

The activities of the TLS polymerases differ with respect to the efficiency and accuracy of bypass of specific lesions. For instance, the cyclobutane pyrimidine dimers (CPDs) caused by UV radiation are bypassed efficiently and accurately by pol η (Rad30), while the other major type of UV-induced lesion, the (6-4) photoproduct, is bypassed predominantly by the combined activities of Rev1 and pol ζ [2-4]. In contrast, pol η, Rev1 and pol ζ each contribute to the bypass of abasic sites [5].

Rev1 and pol ζ function cooperatively in mutagenesis [6], and therefore define an "error-prone" subpathway of PRR. PRR also includes at least two "error-free" subpathways which do not involve translesion synthesis [7] [8]. Although Rev1 has deoxycytidyl transferase activity, which is important for the survival of some lesions ([9], Wiltrout and Walker, unpublished results), it also plays a major non-catalytic role in TLS [10]. Its physical interaction with pol ζ [11] enhances pol ζ's TLS activity [12]. The classic error-free pathway involves *RAD5, MMS2*, and *UBC13,* which avoids both point mutations and gross chromosomal rearrangements by a TLS-independent mechanism [8]. *RAD30* resists classification into either "error-free" or "error-prone" branches of PRR, both because its effect on mutagenesis is subtle and context-dependent [1, 13] and because it is genetically independent of *RAD5*. Therefore, *RAD30* may define its own distinct sub-pathway.

However, the functional interaction of pol η with Rev1 and pol ζ is intriguingly complex. Initial studies of *S. cerevisiae RAD30* found that it was genetically independent of *REV1* [1, 13]. However, it was more recently discovered that, in many species, polymerase η physically interacts with Rev1 protein [14](see Chapter 3). In *S. pombe,* pol η does not interact directly with Rev1 (see Chapter 3), but it does interact with pol ζ (Kosarek and Friedberg, personal communication). Since pol ζ and Rev1 stably associate, it is possible that an interaction between pol η and pol ζ could functionally

replace the interaction between pol η and Rev1 in some species. The function of this physical association is unknown, but one hypothesis, consistent with Rev1's many interactions and the finding that much of Rev1's activity is independent of its catalytic function, is that Rev1 plays a structural role in the recruitment of the other TLS polymerases to the DNA.

In this study, I have used genetics to query the functional relationship between pol η and the other TLS polymerases of *S. cerevisiae*, Rev1 and pol ζ. I address the complex effects of UV dose on survival of TLS-deficient strains. I find that there is no induction of Rad30 protein abundance in response to DNA damage, such that another effect must account for the complex and distinct shapes of the *rad30* and *rev1* killing curves. I present models that can account for these effects based either on pre-existing physiological heterogeneity of yeast cultures, or on complex and damage-dependent functional interactions among the TLS polymerases.

MATERIALS AND METHODS

Strains and Plasmids:

I used derivatives of W1588-4C (*MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5*), which is a W303 strain whose *rad5* mutation is replaced by the wildtype *RAD5* sequence [15]. Deletion of *RAD30* was constructed by gene replacement using PCR amplified *rad30::KanMX* from the Saccharomyces Genome Deletion Project strain 4255. Deletion of *REV1* was similarly constructed using the Saccharomyces Genome Deletion Project strain 1643. The *bar1Δ* strains were constructed by insertion of linearized pZV77 (gift of Steve Bell lab) to disrupt *BAR1*. Double knockout strains were constructed by mating of *rad30* and *rev1, rev3,* or *rev7* spores and using PCR to test for disruption of both genes. Clones of three separately isolated spores of this genotype were used for the epistasis assays. To produce the TEV-ProA-7His tagged Rad30 fusion protein used in the UV induction assay, the tag cassette was amplified from pYM10 [16] and inserted by homologous recombination to replace the stop codon of *RAD30*. The Rad30-TAP tag [17] was PCR amplified out of the Rad30-TAP strain (Open Biosystems) and transformed into our strain background. Chris Kaiser kindly provided pMRT7 [18]. See strain list (Table 1) and plasmid list (Table 2) for more details.

UV sensitivity assays:

For asynchronous assays, cultures were grown to saturation for 3 days at 30 degrees C. For the cell-cycle specific assays, overnight cultures were arrested with either 50 ng/ml alpha-factor or 15 ug/ml nocodazole for at least 3 hours at 30 degrees C, then washed twice with water (for alpha-factor) or 1% DMSO (for nocodazole). Microscopic analysis of cells confirmed arrest. Synchronous or asynchronous cultures were then diluted in water and spread on selective minimal medium plates. To avoid interference with the UV from the plate walls, cells were never spread out to the edges of the plates. Plates were irradiated within 20 minutes using a G15T8 UV lamp (General Electric) at 254nm, 1 J/m^2 per second for varying amounts of time. After irradiation, plates were kept in the dark at 30 degrees C for 3 days before colonies were counted. For the assay in Figure 7,

all cultures were grown overnight in 1 micromolar copper sulfate prior to the assay, and 1 micromolar copper sulfate was added to the plates as well, to induce ubiquitin expression.

MMS sensitivity assays:

Cultures were grown as for UV sensitivity assays, diluted in water, and plated on YPD plates containing the indicated percentage of methyl methanesulfonate (MMS). Plates were incubated at 30 degrees Celsius for 3-4 days before colonies were counted.

UV induction time-course:

Exponentially growing cultures were collected by centrifugation, washed once in water, and resuspended at OD_{600}~1. After they were sonicated briefly to separate clumps of cells, cultures were poured into petri dishes in 15 mL batches and irradiated using a G15T8 UV lamp (General Electric) at 254nm, at 1 J per square meter per second, for either ten or eighty seconds. Based on plating of cells before and after irradiation, ~90% survive the low dose, while ~30% survive the high dose of UV. After irradiation, cells were immediately diluted in 2 x YPD growth medium and incubated at 30 degrees C until their harvest. Before irradiation, and at 15, 40, 75, 105 and 270 minutes after irradiation, equivalent amounts of culture (based on OD_{600}) were collected, and whole cell extract was prepared from them by trichloroacetic acid precipitation. Samples were analyzed by immunoblotting.

Immunoblotting. Whole cell extracts were prepared by trichloroacetic acid precipitation [16]. Protein samples were separated on SDS-polyacrylamide gels, transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore), and probed with appropriate antibodies. ProA-tagged protein was detected using rabbit peroxidase anti-peroxidase (PAP) antibody diluted 1:5,000 (Sigma).

RESULTS

UV survival curves consistent with two modes of UV damage tolerance. In order to gain insight into the functional relationships of Rad30 (pol η) with Rev1 and pol ζ (Rev3 and Rev7), I compared the UV sensitivity of wildtype, *rad30,* and *rev1,* strains (Figure 1). The UV sensitivities of *rev3* and *rev7* strains are very similar to *rev1* (data not shown), consistent with evidence that they can function in the same genetic pathway [19, 20]. For these assays, yeast cultures were grown for 3 days into stationary phase, diluted, and spread on plates shortly before exposure to UV. A comparison of the killing curves of the single mutant strains alone reveals a significant difference between *rad30* and *rev1*. The *rev1* strain is dramatically sensitive to the low dose (10 J/m^2), but its sensitivity is much less affected by increasing UV between 35 and 55 J/m^2 than between 0 and 35 J/m^2. This cannot be due to edge effects from the plate, because cultures were not spread to the edge of the plates, and the colonies that survive high doses are found across the surface area on which cells were spread.

In contrast, the killing curve for the *rad30* strain exhibits a shoulder. At 10 J/m^2, *rad30* is as UV-resistant as the wildtype, but at higher doses, the *rad30* strain is increasingly sensitive to changes in dose (Figure 1). At a high dose, 55 J/m^2 in this assay, the *rev1* and *rad30* strains reach a state of identical sensitivity (Figure 1). At still higher doses (not shown here), it is possible to observe a reversal of the relative UV sensitivities of the *rad30* mutant and *rev3* mutant (which we assume is similar to *rev1)* [21].

The change in slope of the *rev1* mutant's survival relative to UV dose could result from a phenomenon similar to bacterial persistence. Persisters are a small subpopulation within a bacterial (or yeast, [22]) culture whose distinct physiological state makes them unusually resistant to stress [23]. This data could be explained by a the presence of a persister population, making up about 1% of the culture before irradiation, whose UV-resistance is uncovered when high levels of damage kill off the rest of the culture in the *rev1* strain. Stationary yeast cultures are physiologically quite heterogeneous and asynchronous, and therefore could reasonably harbor such a subpopulation.

Alternatively, this pattern may be accounted for by TLS promoting survival of UV-induced DNA damage by more than one mode. A simple model is that a primarily

Rev1/pol ζ-dependent mode is used at low levels of damage, and a more pol η-dependent mode contributes to survival at higher levels of damage, with the transition between the modes resulting in the killing curve shapes that have been observed. For example, Pol ζ and Rev1 might have a constitutive, low level of activity, which can be saturated by high levels of DNA damage, while Rad30 (pol η) is activated in response to a higher threshold level of DNA damage or replication stress. Such an arrangement is unexpected because pol η is generally less mutagenic than Rev1 and pol ζ. Therefore, constitutive activity by Rev1 and pol ζ would promote use of the more mutagenic activity as a first response to low levels of damage, rather than only as the last resort under extreme stress.

Effect of UV-induced DNA damage on Rad30 protein abundance. It has been proposed that the increasing significance of pol η at higher doses results from its activation, in response to DNA damage, by a damage-induced increase in pol η concentrations in the cell [21]. This would be consistent with the observation of a damage-induced increase in *RAD30* message [1, 13, 24]. Maintaining pol η at low levels except in the presence of significant DNA damage would minimize the potentially mutagenic effects of pol η. Pol η activity can be mutagenic [25]; for instance, it is highly error-prone when replicating undamaged DNA [26], and can also promote mutagenesis by incorporating oxidized dNTPs into the nascent DNA [27, 28].

To determine whether there is such a dose-dependent increase in the abundance of Rad30 protein (pol η), I prepared protein from cells after either treatment with UV or mock treatment, and compared the amounts of Rad30 protein at specific times after treatment. Results of a representative assay are shown in Figure 2a. For this assay, I fused the YM10 tag [16] into the genomic *RAD30* locus, producing a C-terminal TEV-ProA-7His tagged Rad30 protein which is fully competent for UV survival (Figure 2c). Cells were irradiated in water at two doses: a low dose which killed 10% of the cells, and a high dose which killed 70%. Total protein was TCA precipitated, and tagged Rad30 was detected by immunoblot, using PGK as a loading control (not shown). In multiple experiments with various UV doses, no significant change in Rad30 protein was observed in response to UV.

In contrast to our observations, a modest change in Rad30 protein levels in response to DNA damage was observed in one recent study [29]. In this study,

polyubiquitinated Rad30 was observed, and Rad30 protein was found to have a short half-life, suggesting that Rad30 protein is subject to proteolytic digestion, and that its increased abundance after DNA damage is the result of stabilization of this otherwise unstable protein. However, other researchers have more recently reported a much longer half-life for Rad30 protein ([24]; M. Wiltrout and G. Walker, unpublished results) and have failed to observe polyubiquitinated Rad30 [24, 30]. The discrepancy between these results may be due to Skoneczna et al. [29] having used a C-terminal TAP-tag to detect the short-lived Rad30 protein. I have shown that this tag abolishes Rad30's *in vivo* function (Figure 2b), and a very recent study reports a similar finding [24]. In contrast, the C-terminal TEV-ProA-7His tag I used did not interfere with Rad30's function (Figure 2c). I found that the TAP-tagged and TEV-ProA-His-tagged versions of Rad30 were detected at the same levels by western blotting against the ProA epitope common to both tags (data not shown), demonstrating that there is not a problem with expression of the TAP-tagged protein. Therefore, it is likely that the TAP tag is responsible for destabilizing the protein in the previous study, resulting in altered degradation kinetics. I conclude that Rad30 protein abundance does not normally increase after UV irradiation, and therefore is not the cause of the biphasic contribution of TLS to survival of UV-induced DNA damage. During the preparation of this manuscript, a similar analysis using Myc-tagged pol η was reported and confirmed no change in protein abundance in response to DNA damage [24].

Epistasis analysis. There may, however, be a different conditional change in the regulation of pol η. Rev1's putative structural role in TLS [10] and its cell cycle dependence [31] led us to ask whether pol η can conditionally participate in a pathway with Rev1 and pol ζ. Initial studies concluded that *RAD30* does not belong to the same epistasis group as *REV1* or *REV3* with respect to UV survival [1, 13]. However, more recent studies have shown that Rev1, pol ζ and pol η are all required for G-to-T mutagenesis at the site of a benzyoapyrene adduct *in vivo* [32]. In addition, the finding that pol η interacts physically with Rev1 in some species [14] (See Chapter 3) and with pol ζ in others (Kosarek and Friedberg, personal communication), implies that the two TLS pathways might not be entirely independent.

To assess the possibility that pol η can conditionally cooperate with Rev1 and pol ζ, epistasis analysis was performed using cultures grown asynchronously to stationary phase as before, and treated with low (10 J/m^2) and high (55 J/m^2) UV doses (Figure 3a and b). At the low dose, 86% of wildtype cells survive, and all three *rev* mutant strains are dramatically more sensitive than *rad30*. At this dose, I find that *rad30* is synergistic with respect to all three of the other mutants, *rev1, rev3 and rev7* (Figure 3a). At the high dose, which 13% of wildtype cells survived, the *rev1* and *rad30* strains were similarly UV sensitive, and *rev3* is slightly more sensitive than either (at the high dose, *rev7* is not shown), possibly representing a Rev1-independent function of pol ζ. In contrast to the results from the low dose, at 55 J/m^2, *rev1* and *rad30* have additive effects on UV killing, and *rev3* and *rad30* appear epistatic.

This is consistent with a model in which pol η participates in the same pathway with pol ζ to promote survival of moderately high levels of DNA damage, while acting in a separate, and partially redundant, pathway from Rev1 and pol ζ for survival of lower levels of DNA damage. A complex, dose-dependent genetic relationship between *rev3* and *rad30* [1, 21], and between *rev1* and *rad30* [1] with respect UV survival has been observed in the past, but the complexity has never been explained. The previous studies found that a synergistic relationship reappeared at doses higher than assayed here, but did not observe the synergy between *rev3* and *rad30* at low doses [1, 21].

To determine whether *RAD30* (pol η) and *REV1* might act in the same epistasis group to promote survival of a different type of DNA damage, I assayed the sensitivities of *rev1* and *rad30* mutants to long-term DNA damage by spreading them on plates containing the alkylating agent methyl methanesulfonate (MMS) at a concentration of 0.02% (Figure 3c). At this dose, the relationship between the two genes appears to be additive (Figure 3c). It should be noted that this assay is significantly different from the UV survival assay, not only because of the types of DNA damage produced, but also because in this case the cells must grow in the continuing presence of the damaging agent to be counted as survivors. I therefore compared these results with those of another study, in which yeast cultures were treated by short-term (30 minute) exposure to MMS at higher concentrations [5], resulting in *rad30* and *rev1* killing curve shapes similar to

those observed for UV-induced damage [5]. However, in this study also, *rev1* and *rad30* were not found to be epistatic [5].

UV sensitivity upon release from G1 arrest: Pol η and Pol ζ. Because Rev1 is cell-cycle regulated [31], I reasoned that assays of synchronized cultures might both provide insight into the complex relationship between pol η *(RAD30)* and pol ζ *(REV3, REV7),* and reveal any cell-cycle specific genetic interaction between *RAD30* (pol η) and *REV1*. The cleanest synchronization of yeast cultures is achieved by arresting with alpha-factor in G1. Cultures were arrested in G1 before irradiating, replicating the condition under which Rev1 has the greatest effect on UV sensitivity [31]. Cells were released from arrest by washing, diluting, and spreading them on minimal plates. The plates were then irradiated within 20 minutes of spreading, in order to expose the cells to DNA damage just as they exit the G1 arrest.

The UV survival phenotypes of wildtype, *rad30, rev1* and *rad18* strains are shown in Figure 4a. The basic shapes of dose dependent killing of WT, *rad30* and *rev1* strains by UV upon G1 release are similar to those observed for asynchronous cells (Figure 4a). Even though Rev1 is disproportionately important to UV survival under these conditions [31], a mutation in *rad18*, which is epistatic to both *rev1* and *rad30* [1], causes greater UV-sensitive than the *rev1*mutation alone (Figure 4a). Interestingly, the UV sensitivity of the *rad18* strain forms a similar pattern to, a *rev1* mutant's, rather than forming a shoulder like *rad30.*

The UV survival phenotypes of the pol ζ (*rev3* and *rev7)* and pol η *(rad30)* mutants are addressed in Figure 4, while assays relevant to the cell cycle specificity of Rev1 function will be shown in Figure 5. Figure 4b shows the UV survival of all strains when treated with a low UV dose upon release from G1 (*rev1* is shown here for comparison). At a low UV dose, the results are similar to what was observed for asynchronous cultures: *rad30* deletion alone has little effect on UV sensitivity, but in a *rev1, rev3,* or *rev7* background, additional deletion of *rad30* causes a synergistic defect in UV survival (Figure 4b). At a higher UV doses, I found that *rev7* was additive with *rad30* (Figure 4c), and *rev3,* as in the asynchronous assay, was epistatic to *rad30* (Figure 4d). It is intriguing that *rev3* and *rev7* mutants differ, since both genes encode subunits of pol ζ. These data imply that the catalytic subunit, encoded by *REV3,* may have a

damage-induced function independent of Rev7, and cooperative with Rad30. These results are consistent with a model in which pol η (Rad30) dose-dependently acts in the same pathway with pol ζ to promote survival of UV-induced damage in either asynchronous or synchronized culture.

Cell cycle specific UV assay of genetic interaction between *S. cerevisiae REV1* and *RAD30*. Previous studies have shown that *S. cerevisiae* polymerase η and Rev1 have a non-epistatic genetic relationship, and therefore act independently with respect to UV induced DNA damage [1, 4]. However, such experiments were performed only with asynchronously growing yeast cultures. More recent work demonstrated that Rev1 protein abundance is strongly cell cycle regulated, and that its relative importance in survival of UV-induced DNA damage is cell cycle dependent [31, 33]. Therefore, I addressed the possibility that the use of asynchronous cultures has obscured a cell-cycle specific genetic interaction between *RAD30* and *REV1*. To learn whether the cell cycle has any effect on the functional relationship between Rev1 and Rad30, I assayed the UV survival of WT, *rev1, rad30* and *rev1rad30* strains under the same conditions used previously to demonstrate the cell cycle dependence of Rev1 function [31]. Cultures were arrested with alpha-factor in G1 or with nocodazole in G2, and were then exposed to DNA damage upon release from arrest. As noted previously, all strains are more UV sensitive when irradiated in G1 than when irradiated in G2, and *rev1's* UV sensitivity is particularly enhanced by irradiation upon release from G1 [31].

In the cultures irradiated upon release from G2 arrest (Figure 5a), I found that the *rev1rad30* double knockout was substantially more sensitive to killing by UV radiation than either single mutant, indicating a synergistic relationship between *REV1* and *RAD30,* in which the two genes act independently, and are partially redundant, in promoting UV survival. I observed that irradiation upon release from G2 does not result in a complex, dose-dependent genetic relationship between *rad30* and *rev1* such as was observed in asynchronous cultures for *rad30* with *rev3* and possibly *rev1,* or in G1-synchronous cultures for *rad30* with *rev3* and *rev7*. However, the relationship between the killing curves of the single mutants is similar to what was observed previously in that the *rad30* strain has a shoulder at a low dose of 20 J/m^2 while the *rev1* strain is more sensitive, but at a higher dose, *rev1* and *rad30* strains are similarly sensitive to UV (Figure 5a). It

should be noted that, due to the reduced sensitivity of all strains when irradiated in G2, effects that depend on high stress or lethality would only be revealed at much higher doses than were used here.

Epistasis analysis with respect to UV irradiation on release from G1 shows a complex, dose-dependent relationship between *REV1* and *RAD30* (Figure 5b). At a low dose of 5 J/m^2, the *rev1rad30* mutant is synergistically more UV-sensitive than either the *rev1* or *rad30* strain, but at a dose of 10 J/m^2, the difference in survival between the *rev1rad30* and the *rev1* strain decreases. At high doses of 15-20 J/m^2, *REV1* appears to be epistatic to *RAD30* (Figure 4b). Notably, this is the only condition under which I have observed apparent epistasis between *REV1* and *RAD30*, suggesting that the two genes may function together in a cell cycle-dependent and dose-dependent pathway.

At higher doses (not shown here), I observed greater variability in survival frequency among individual cultures, but the general trend was reproducible; i.e., a reduced slope for both *rev1* and *rev1rad30* killing with respect to dose at high doses. This is unlikely to be the effect of mutagenesis, as the UV-induced mutation frequency of a *rev1* strain are substantially lower than the approximately 1% survival frequencies observed [34]. The leveling off of killing at higher doses could, however, be due to uncovering a small population of asynchronous cells which were not arrested in G1 phase prior to DNA damage, and which would therefore have a higher UV resistance than the arrested cells. Although microscopic analysis was used to confirm the arrest of the culture in G1 or G2 for these experiments, arrest is confirmed for only 95-100% of the population. However, the recurrence of a decreased slope in the *rev* and *rad18* killing curves at higher doses suggests that this is not simply an artifact of cell cycle synchronization. It may, however, be explained by a physiologically heterogeneous cell culture in a which a small sub-population is more UV-resistant than the bulk of the cells, similar to persistence in bacterial cultures. This possibility is addressed in the discussion. The exposure of a more UV-resistant sub-population, rather than an actual epistatic relationship between the two genes, might cause the overlap observed between the survival of *rev1rad30* and *rev1* strains.

Alternatively, the apparent dose-dependent epistasis between *rev1* and *rad30* in G1 but not in G2 suggests that the functional interactions of Rad30 with Rev1 and pol ζ

vary depending on both the stage of the cell cycle and the level of DNA damage experienced by the cell. While *RAD30* and *REV1* act in independent pathways in cells irradiated upon release from G2, they may act together in a common pathway when the cell is subject a heavy load of DNA lesions just before S phase. These are not the first data to suggest that pol η and Rev1 work together for specific types of bypass [32].

Cell cycle specific MMS assay of genetic interaction between *S. cerevisiae* *REV1* and *RAD30*. To learn whether the cell cycle specificity observed for UV sensitivity is independent of the type of DNA damage, I performed a similar assay using the methylating agent methyl methanesulfonate (MMS) to introduce DNA damage (Figure 6). Cultures were synchronized as for the UV assay, washed, and plated on plates containing 0.01% or 0.02% MMS. Note that in this assay, exposure to DNA damage is over an extended period of time, whereas in the UV assay, all exogenous lesions were caused simultaneously upon release from cell cycle arrest.

I observed that the MMS sensitivity of the *rad30* strain is scarcely distinguishable from the wildtype at either stage of the cell cycle (Figure 6). The *rev1* mutation, however, causes a dramatic increase in MMS sensitivity, particularly when MMS exposure began on release from G1 (Figure 6b), consistent with the model that Rev1's function is cell cycle dependent. The MMS treatments at either stage of the cell cycle cannot distinguish between an additive and epistatic relationship between *rev1* and *rad30* because of the low level of sensitivity exhibited by the *rad30* mutant at these doses (Figure 6a). However, there is a clear absence of the strong synergy that was observed between the two genes for UV sensitivity at the low doses to which *rad30* cells are as resistant as wildtype (Figures 3a, 4b, 6b). This indicates either a lesion-dependent difference in the role or the regulation of TLS by pol η, or a difference between long-term and short-term exposure to DNA damage, as discussed previously.

Effect of overproduction of ubiquitin on UV sensitivity. Epistasis assays have suggested the possibility of a conditional pathway in which pol η (Rad30) depends on Rev1 for its function. The functions of both pol η and Rev1 depend partially on ubiquitinated PCNA [35-37]. One hypothesis is that Rev1 may be required for a context-dependent function of pol η which is independent of ubiquitinated PCNA. If so, Rev1 would be partially redundant to ubiquitinated PCNA with respect to the final outcome of

survival. In this case, ubiquitinated PCNA, and therefore ubiquitin, should be more important for survival in a *rev1* background. I overexpressed ubiquitin in WT, *rev1*, and *rad30* backgrounds, and measured the UV sensitivity with or without excess ubiquitin (Figure 7). Consistent with the hypothesis that Rev1 may be partially redundant with ubi-PCNA, I found that in the *rev1* strain, and not the WT or *rad30* strains, overexpression of ubiquitin did confer an increase in UV resistance, implying that ubiquitin is more necessary for survival of DNA damage in the absence of Rev1 protein. This is consistent with Rev1 promoting a less ubiquitin-dependent mode of DNA damage tolerance.

CONCLUSIONS

I have shown that biphasic killing curves of TLS mutant strains to UV survival cannot be explained by induction of Rad30 (polymerase η) protein levels in response to DNA damage. The biphasic killing curves could reflect either the physiological state of the cell culture, or a dose-dependent regulation of TLS.

Persister-like sub-population. One explanation of the biphasic pattern of UV sensitivity of asynchronous *rev* strains could be that the cultures used in the assay are physiologically heterogeneous, containing a small subpopulation analogous to the persisters which have been observed in bacterial culture [23]. In this case, the cultures used in this study, which are grown to stationary-phase from single colonies before plating and UV irradiation, would contain at least two subpopulations distinguished by their physiological states. The majority population is highly UV sensitive in the absence of Rev1 or pol ζ, and is more dependent on Rev1 and pol ζ than pol η for survival of UV-induced DNA damage. These cells account for the behavior seen at low doses, but completely die off at higher doses, revealing the greater UV resistance of the minority population. A persistence-like phenomenon has been observed with respect to drug-resistance in biofilms of *C. albicans* [22]. Although a physiologically resistant subpopulation has not been observed in stationary cultures of haploid *S. cerevisiae* [38], such cultures are quite heterogeneous, and therefore might include such a population [39]. In addition, similar killing curve shapes were observed for the single mutants whether the irradiated cells were stationary phase cultures or cultures synchronized in G1

phase or G2 phase, although the flattening of the *rev1* curve is seen at a much higher survival frequency in G2-synchronized cells. Thus, if there is a more resistant sub-population, it would be more abundant in G2-synchronized culture. Such a sub-population might result from the advantageous induction of a DNA damage repair pathway or checkpoint prior to UV irradiation in a small percentage of cells.

Damage-induced changes in TLS regulation. Instead of (or in addition to) a resistant sub-population, there may be dose-dependent damage-induced Rad30 regulation at one or more levels. The dose-dependent epistasis between *rev3* and *rad30* suggests the possibility of a damage-induced pathway involving both pol ζ and pol η. One potential mediator of damage-induced regulation is PCNA (proliferating cell nuclear antigen) [40, 41]. Pol η, Rev1 and pol ζ are all regulated by PCNA [30, 42-45], but differences between the PCNA-mediated regulation of the different polymerases could account for their dose-dependent effects on survival of UV-induced DNA damage. PCNA activates distinct pathways depending on whether it is mono-ubiquitinated, SUMOylated, or polyubiquitinated [40, 46]. PCNA is monoubiquitinated by Rad6/Rad18 in response to DNA damaging agents such as UV or MMS [40]. Rad30 interacts physically with both PCNA itself and the ubiquitin moiety (See Chapter 2) [30, 47], and either interaction alone is sufficient to partially rescue the UV sensitive phenotype of a *rad30* null [37]. A mutation which prevents PCNA ubiquitination, *pol30(K164R)*, has been shown to be epistatic to both *rad30* and *rev3* with respect of survival of high levels (50-150 J/m^2) of UV-induced DNA damage [46, 48]. Because the *pol30(K164R)* mutant also prevents accumulation of pol η foci in response to DNA damage, the interaction of pol η with ubiquitinated PCNA is thought to be required to recruit pol η to the sites of DNA damage. While an extensive study of the dose-dependence of PCNA modification state has not been performed, one study found ubiquitinated PCNA after moderate amounts of DNA damage but not after a very high level of DNA damage (due to exposure to 0.3% MMS) [40]. At least some pol ζ functions are independent of PCNA ubiquitination [49-51]. The BRCT domain of Rev1 is required for interaction with unmodified PCNA but not with ubiquitinated PCNA [35]. The formation of Rev1 foci in the absence of DNA damage is almost entirely dependent on Rev1's BRCT domain, suggesting that it is predominantly independent of PCNA monoubiquitination. Pol η may be similarly

regulated both by interaction with unmodified PCNA, in the absence of exogenous damage, and by a more robust interaction with monoubiquitinated PCNA which causes the increased formation of pol η foci at sites of DNA damage.

Another possible mode of regulation could be a damage-threshold-induced pathway in which Rev1 and Rad30 act together. This is consistent with our observations of conditional epistasis between *RAD30* and *REV1*. The repeated observation of complexity in the genetic relationships between *RAD30* and the *REV* genes suggests a complex functional relationship between Rad30 and the error-prone TLS pathway, consistent with this hypothesis.

Timing is everything. All our observations can be understood as the result of Rev1, Pol ζ, and Rad30 having differential effects on survival of UV-induced DNA damage. This can be accounted for by supposing that they function predominantly at different times during the cell cycle. TLS is thought to occur both during S phase, to allow continuous DNA replication, and during G2, to fill lesion-containing single-stranded gaps produced in the DNA during replication or excision repair [31]. The regulation and relative contributions of the different polymerases in each context may vary among species, but in *S. cerevisiae,* the cell cycle regulation of Rev1 protein abundance implies that it may be most active in gap-filling TLS [31, 33, 52]. As pol ζ's function is largely dependent on Rev1, I will assume for this model that pol ζ also functions primarily in gap-filling in yeast. The observation that *rad30* cells are much more likely than *rev1* cells to arrest as unbudded cells after UV-induced DNA damage suggests that Rad30 is more frequently required than Rev1, to allow passage through S phase [31]. This is consistent with a Rev1-independent role for pol η in TLS during S phase. Therefore, any Rev1-dependent function of pol η is more likely to occur later in the cell cycle as part of gap-filling TLS.

At low doses in asynchronous or G1 cells, I observed that *rev1, rev3,* or *rev7* strains were dramatically more sensitive to UV than the *rad30* strain, but that the double mutants display a synergistic defect in survival, suggesting that Rad30 becomes important for survival only in the absence of Rev1 or pol ζ. This can be explained by supposing that the gap-repair function of Rev1and pol ζ, since it acts temporally later than S-phase TLS, can compensate for the *rad30* strain's deficiency in S-phase TLS at

low doses. The absence of Rev1 or pol ζ, in a *rev1, rev3* or *rev7* strain at low doses, uncovers the importance of the S-phase TLS for survival, resulting in the observed synergy. The failure to observe the same synergistic effect of low doses in our MMS assay is because in that assay, the cells are chronically exposed to DNA damage, removing the influence of the order of events in the cell cycle. The results of a short-term MMS exposure assay in another study are consistent with this hypothesis [5].

At the higher levels of DNA damage in asynchronous or G1 cells, I found that the *rad30* strain is not less sensitive than the *rev1, rev3* or *rev7* strains, and the sensitivity of the double mutants suggests an epistatic relationship between *RAD30* and the *REV* genes. This may result from Rad30 (pol η) being recruited to play a damage-induced role in gap-filling TLS. If gap-filling TLS is dependent on pol η at high doses, it can no longer compensate for the loss of *RAD30,* and therefore the *rad30* strain is as sensitive to killing at higher doses as the *rev* strains. This could be equivalent to, or involve, a damage-induced function of pol η in a Rev1-dependent pathway, as discussed above, which would result in the epistasis observed. The observation of synergy at still higher doses in other studies [1, 21] suggests that at very high levels of damage, Rad30's independent function may again become pre-eminent, possibly due to the loss of ubiquitinated PCNA or some other regulatory signal.

FIGURES

Figure 1.

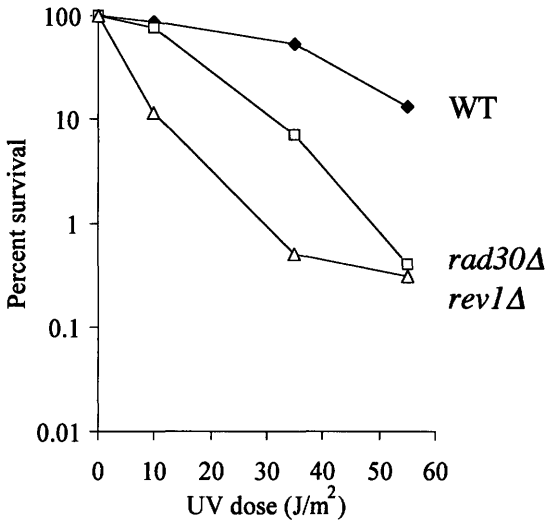

Figure 1. UV sensitivity of TLS mutants. UV sensitivity of asynchronous cultures. Wildtype (filled diamonds), *rad30* (open squares), *rev1* (open triangles). Error bars represent standard error.

Figure 2.

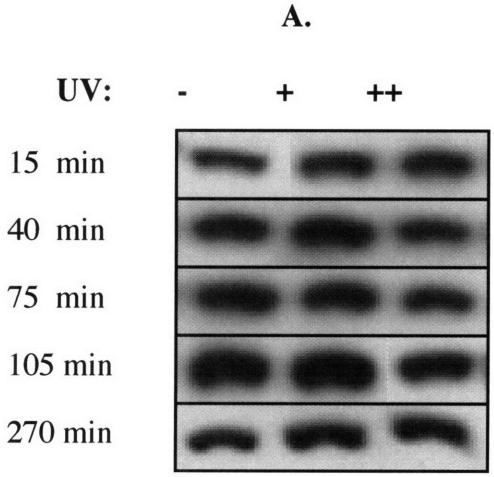

Figure 2. Rad30 protein abundance does not change in response to UV damage. A, Immunoblots of endogenously expressed Rad30-TEV-ProA-7His at various time points after UV treatment or mock treatment. The low UV dose killed 12% of cells, while the high UV dose killed 70%. **B**, Endogenously expressed TAP-tagged Rad30 was not used because it is not functional. UV survival assay of: Wildtype, diamonds; *rad30,* triangles; *RAD30-TAP*, open squares. Error bars represent standard error. **C**, Untagged Rad30 and Rad30-TEV-ProA-7His are similarly proficient for bypass of UV-induced DNA lesion. UV survival assay of: Wildtype, squares; *rad30,* triangles; *RAD30-YM10*, diamonds. Error bars represent standard error.

Figure 2, condinued.

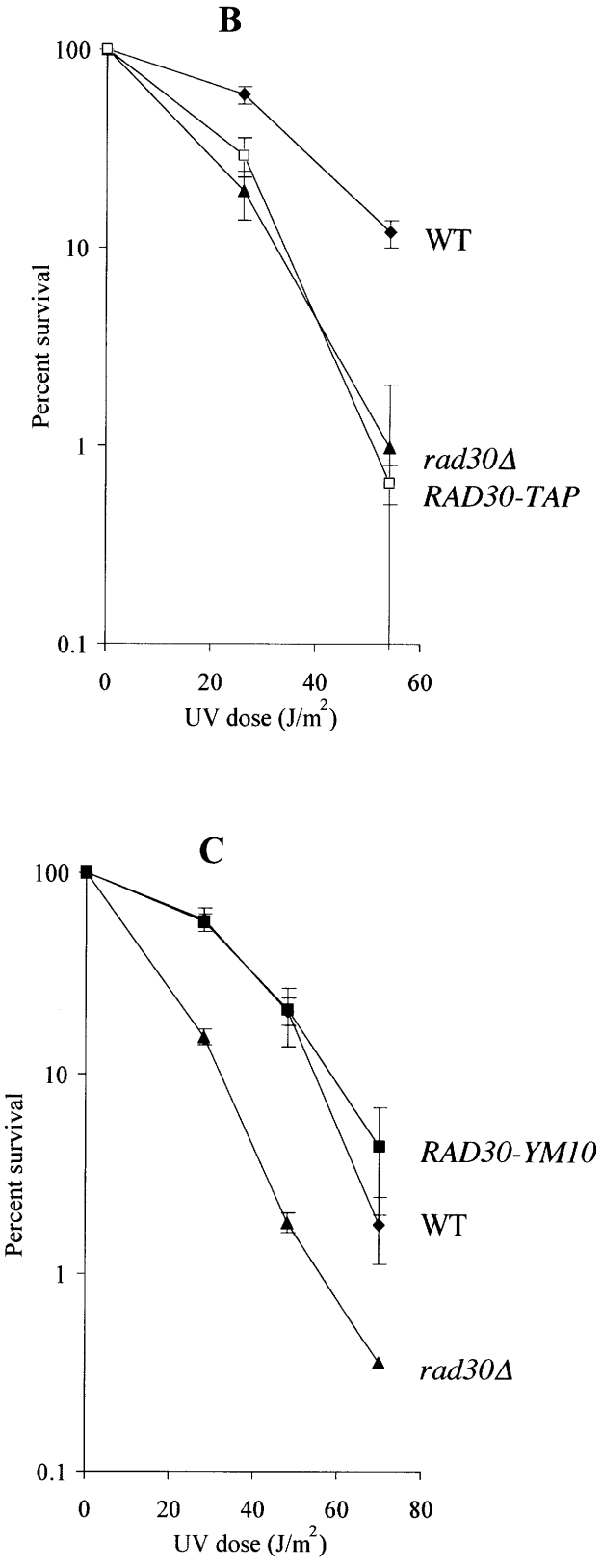

Figure 3.

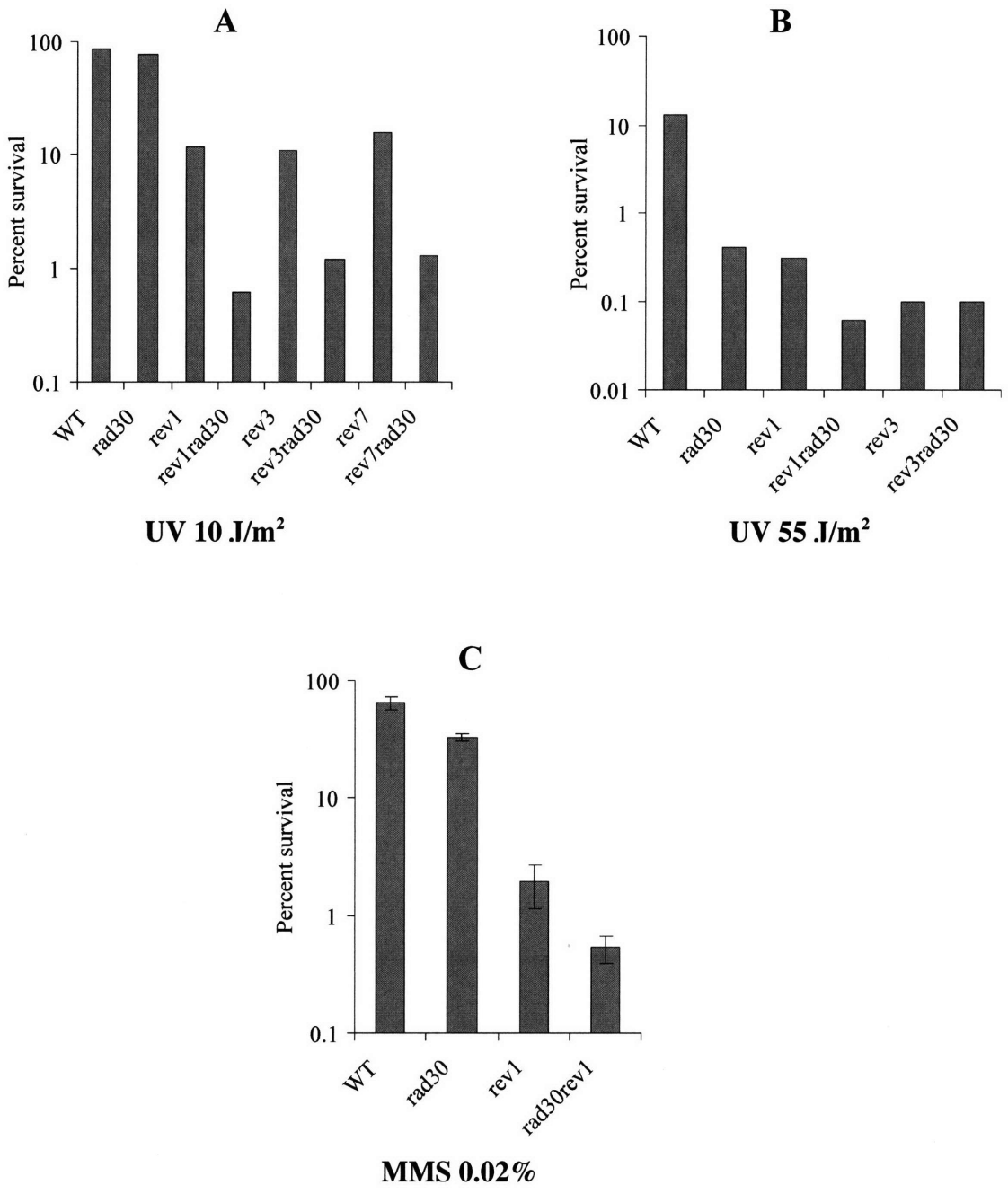

Figure 3. Genetic relationships assayed in asynchronous cultures. A, Sensitivity of asynchronous cultures to low dose of UV, 10 J/m^2. **B,** Sensitivity of asynchronous cultures to a high dose of UV, 55 J/m^2. **C,** Survival of asynchronous cultures plated on 0.02% MMS. Error bars represent standard error.

Figure 4.

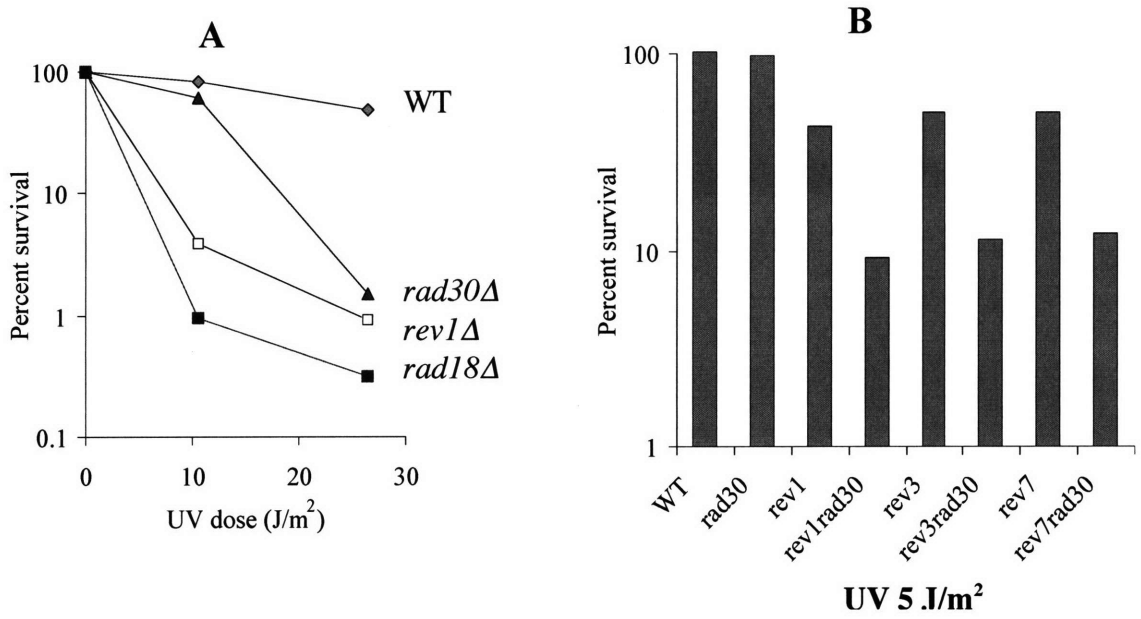

Figure 4. Genetic relationship of *RAD30* with Pol ζ *(REV3* and *REV7)* assayed upon release from G1. All cultures were arrested with alpha-factor and released from arrest just prior to UV irradiation. **A,** UV survival of wildtype (diamonds), *rad30* (triangles), *rev1* (open squares), and *rad18* (filled squares) cultures. **B,** Epistasis analysis at low dose. Sensitivity to treatment with a UV dose of 5 J/m^2 upon release from G1.

Figure 4, continued.

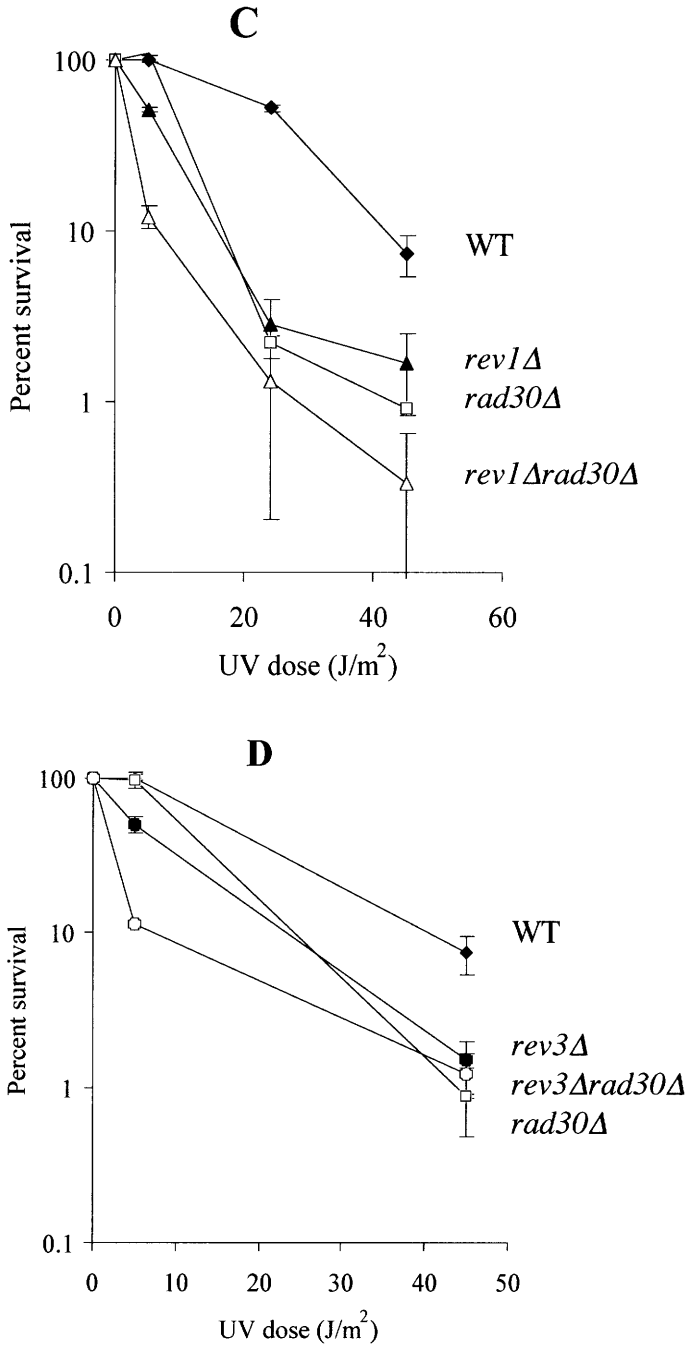

Figure 4. Genetic relationship of *RAD30* with Pol ζ *(REV3* and *REV7)* assayed upon release from G1. All cultures were arrested with alpha-factor and released from arrest just prior to UV irradiation. **C,** Genetic relationship between *REV7* and *RAD30.* Wildtype (filled diamonds), *rad30* (open squares), *rev7* (filled triangles), and *rev7rad30* (open triangles). **D,** Genetic relationship between *REV3* and *RAD30.* Wildtype (filled diamonds), *rad30* (open squares), *rev3* (filled circles), and *rev3rad30* (open circles). Error bars represent standard error.

Figure 5.

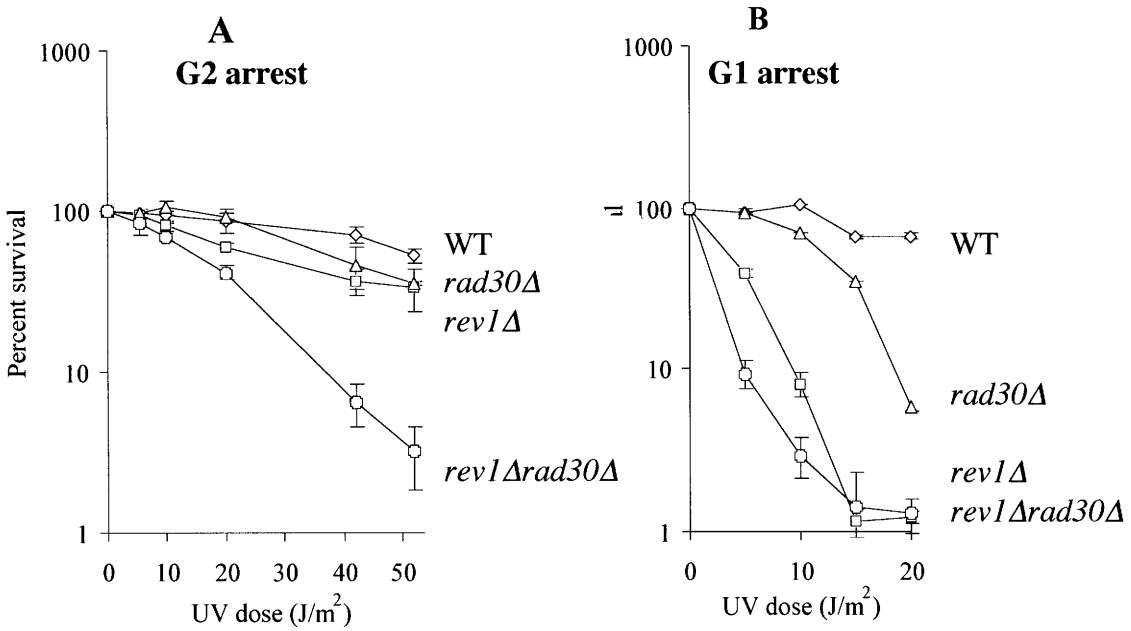

Figure 5. Cell cycle specific assays of *REV1* and *RAD30* with respect to UV sensitivity. Percent survival of UV-induced DNA damage for the wildtype (diamonds), *rad30* (triangles), *rev1* (squares) and *rev1rad30* (circles) strains **A,** after release from G2 arrest in nocodazole, or **B,** after release from G1 arrest in alpha-factor. Error bars represent standard error.

Figure 6.

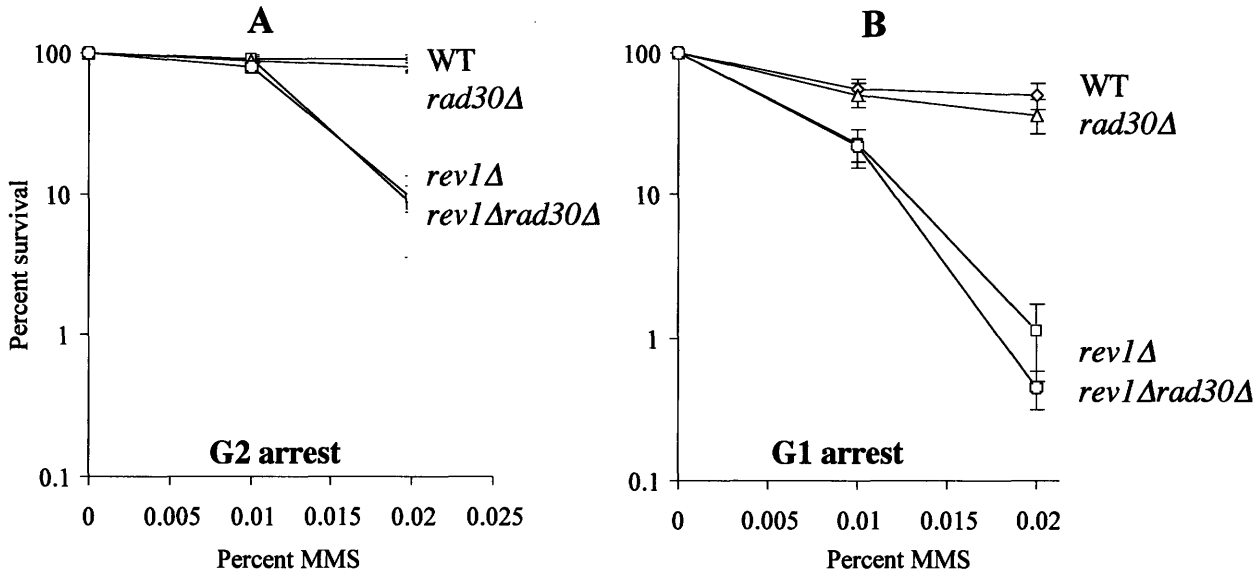

Figure 6. Cell cycle specific assays of *REV1* and *RAD30* with respect to MMS sensitivity. Wildtype (diamonds), *rad30* (triangles), *rev1* (squares) and *rev1rad30* (circles). **A,** MMS treatment upon release from G2. **B,** MMS treatment upon release from G1. Error bars represent standard error.

Figure 7.

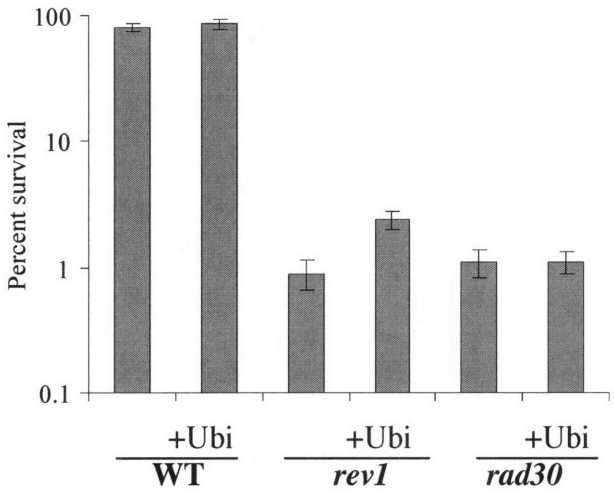

Figure 7. Protective effect of ubiquitin overexpression. Sensitivity to UV at a dose of 21 J/m^2. Ubiquitin is overexpressed from the plasmid pMRT7. All cultures were grown overnight in the presence of 1 micromolar copper sulfate to effect the induction of ubiquitin expression in strains carrying pMRT7. Error bars represent standard error.

Table 1.

Strain	Genotype
W1588-4C	*MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5*
RWY13	*MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5 RAD30-TEV-ProA-7His::HIS3MX*
RWY15	*MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5 rad30::KanMX*
LSW6	*MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5 bar1::LEU2*
LSW7	*MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5 bar1::LEU2 rev1::kanMX*
LSW8	*MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5 bar1::LEU2 rad30::KanMX*
RWY229	*MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5 bar1::LEU2 rev1::KanMX rad30::KanMX*
RWY252	*MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5 bar1::LEU2* pAS311-REV1-HAC
RWY249	*MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5 bar1::LEU2* pAS311-REV1ΔN-HAC
LSW9	*MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5 bar1::LEU2 rev3::kanMX*
LSW10	*MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5 bar1::LEU2 rev7::kanMX*
RWY116	*MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5 bar1::LEU2 rev3::kanMX rad30::kanMX*
RWY114	*MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5 bar1::LEU2 rev7::kanMX rad30::kanMX*

Table 2.

Plasmid	Relevant gene	Description/Comments
pJM96	*6His-RAD30*	pRS415 derivative containing *RAD30*
pRW1031	*RAD30-YM10*	pJM96 derivative with tag: -TEV-ProA-7His
pMRT7	*UBI-C-MYC*	2micron vector with Pcup1-Ubi-c-myc
pRW1003	*RAD30-TAP*	pJM96 derivative with TAP tag

REFERENCES

1. McDonald, J.P., A.S. Levine, and R. Woodgate, *The Saccharomyces cerevisiae RAD30 gene, a homologue of Escherichia coli dinB and umuC, is DNA damage inducible and functions in a novel error-free postreplication repair mechanism.* Genetics, 1997. **147**(4): p. 1557-68.
2. Johnson, R.E., et al., *Role of DNA polymerase zeta in the bypass of a (6-4) TT photoproduct.* Mol Cell Biol, 2001. **21**(10): p. 3558-63.
3. Guo, D., et al., *Translesion synthesis by yeast DNA polymerase zeta from templates containing lesions of ultraviolet radiation and acetylaminofluorene.* Nucleic Acids Res, 2001. **29**(13): p. 2875-83.
4. Gibbs, P.E., et al., *The relative roles in vivo of Saccharomyces cerevisiae Pol eta, Pol zeta, Rev1 protein and Pol32 in the bypass and mutation induction of an abasic site, T-T (6-4) photoadduct and T-T cis-syn cyclobutane dimer.* Genetics, 2005. **169**(2): p. 575-82.
5. Zhao, B., et al., *Role of DNA polymerase eta in the bypass of abasic sites in yeast cells.* Nucleic Acids Res, 2004. **32**(13): p. 3984-94.
6. Heidenreich, E., H. Eisler, and F. Steinboeck, *Epistatic participation of REV1 and REV3 in the formation of UV-induced frameshift mutations in cell cycle-arrested yeast cells.* Mutat Res, 2006. **593**(1-2): p. 187-95.
7. Erlich, B., Danielle, Lahue, Samson, *Anc1, a protein associated with transcription, defines a novel branch of the postreplication repair pathway.*
8. Smith, S., et al., *Mutator genes for suppression of gross chromosomal rearrangements identified by a genome-wide screening in Saccharomyces cerevisiae.* Proc Natl Acad Sci U S A, 2004. **101**(24): p. 9039-44.
9. Ross, A.L. and J.E. Sale, *The catalytic activity of REV1 is employed during immunoglobulin gene diversification in DT40.* Mol Immunol, 2006. **43**(10): p. 1587-94.
10. Nelson, J.R., et al., *Evidence for a second function for Saccharomyces cerevisiae Rev1p.* Mol Microbiol, 2000. **37**(3): p. 549-54.
11. Murakumo, Y., et al., *Interactions in the error-prone postreplication repair proteins hREV1, hREV3, and hREV7.* J Biol Chem, 2001. **276**(38): p. 35644-51.
12. Acharya, N., et al., *Complex formation with Rev1 enhances the proficiency of Saccharomyces cerevisiae DNA polymerase zeta for mismatch extension and for extension opposite from DNA lesions.* Mol Cell Biol, 2006. **26**(24): p. 9555-63.
13. Roush, A.A., et al., *Deletion of the Saccharomyces cerevisiae gene RAD30 encoding an Escherichia coli DinB homolog confers UV radiation sensitivity and altered mutability.* Mol Gen Genet, 1998. **257**(6): p. 686-92.
14. Guo, C., et al., *Mouse Rev1 protein interacts with multiple DNA polymerases involved in translesion DNA synthesis.* Embo J, 2003. **22**(24): p. 6621-30.
15. Zhao, X., E.G. Muller, and R. Rothstein, *A suppressor of two essential checkpoint genes identifies a novel protein that negatively affects dNTP pools.* Mol Cell, 1998. **2**(3): p. 329-40.
16. Knop, M., et al., *Epitope tagging of yeast genes using a PCR-based strategy: more tags and improved practical routines.* Yeast, 1999. **15**(10B): p. 963-72.

17. Ghaemmaghami, S., et al., *Global analysis of protein expression in yeast.* Nature, 2003. **425**(6959): p. 737-41.
18. Helliwell, S.B., S. Losko, and C.A. Kaiser, *Components of a ubiquitin ligase complex specify polyubiquitination and intracellular trafficking of the general amino acid permease.* J Cell Biol, 2001. **153**(4): p. 649-62.
19. Hirano, Y. and K. Sugimoto, *ATR homolog Mec1 controls association of DNA polymerase zeta-Rev1 complex with regions near a double-strand break.* Curr Biol, 2006. **16**(6): p. 586-90.
20. Lawrence, C.W., *Cellular functions of DNA polymerase zeta and Rev1 protein.* Adv Protein Chem, 2004. **69**: p. 167-203.
21. Abdulovic, A.L. and S. Jinks-Robertson, *The in vivo characterization of translesion synthesis across UV-induced lesions in Saccharomyces cerevisiae: insights into Pol zeta- and Pol eta-dependent frameshift mutagenesis.* Genetics, 2006. **172**(3): p. 1487-98.
22. LaFleur, M.D., C.A. Kumamoto, and K. Lewis, *Candida albicans biofilms produce antifungal-tolerant persister cells.* Antimicrob Agents Chemother, 2006. **50**(11): p. 3839-46.
23. Balaban, N.Q., et al., *Bacterial persistence as a phenotypic switch.* Science, 2004. **305**(5690): p. 1622-5.
24. Pabla, R., D. Rozario, and W. Siede, *Regulation of Saccharomyces cerevisiae DNA polymerase eta transcript and protein.* Radiat Environ Biophys, 2007.
25. Pavlov, Y.I., D. Nguyen, and T.A. Kunkel, *Mutator effects of overproducing DNA polymerase eta (Rad30) and its catalytically inactive variant in yeast.* Mutat Res, 2001. **478**(1-2): p. 129-39.
26. Matsuda, T., et al., *Low fidelity DNA synthesis by human DNA polymerase-eta.* Nature, 2000. **404**(6781): p. 1011-3.
27. Shimizu, M., et al., *Efficient and erroneous incorporation of oxidized DNA precursors by human DNA polymerase eta.* Biochemistry, 2007. **46**(18): p. 5515-22.
28. Shimizu, M., et al., *Erroneous incorporation of oxidized DNA precursors by Y-family DNA polymerases.* EMBO Rep, 2003. **4**(3): p. 269-73.
29. Skoneczna, A., et al., *Polymerase eta is a short-lived, proteasomally degraded protein that is temporarily stabilized following UV irradiation in Saccharomyces cerevisiae.* J Mol Biol, 2007. **366**(4): p. 1074-86.
30. Parker, J.L., et al., *Contributions of ubiquitin- and PCNA-binding domains to the activity of Polymerase eta in Saccharomyces cerevisiae.* Nucleic Acids Res, 2007. **35**(3): p. 881-9.
31. Waters, L.S. and G.C. Walker, *The critical mutagenic translesion DNA polymerase Rev1 is highly expressed during G(2)/M phase rather than S phase.* Proc Natl Acad Sci U S A, 2006. **103**(24): p. 8971-6.
32. Zhao, B., et al., *Poleta, Polzeta and Rev1 together are required for G to T transversion mutations induced by the (+)- and (-)-trans-anti-BPDE-N2-dG DNA adducts in yeast cells.* Nucleic Acids Res, 2006. **34**(2): p. 417-25.
33. Sabbioneda, S., et al., *Yeast Rev1 is cell cycle regulated, phosphorylated in response to DNA damage and its binding to chromosomes is dependent upon MEC1.* DNA Repair (Amst), 2007. **6**(1): p. 121-7.

34. Lawrence, C.W. and R. Christensen, *UV mutagenesis in radiation-sensitive strains of yeast.* Genetics, 1976. **82**(2): p. 207-32.
35. Guo, C., et al., *REV1 protein interacts with PCNA: significance of the REV1 BRCT domain in vitro and in vivo.* Mol Cell, 2006. **23**(2): p. 265-71.
36. Guo, C., et al., *Ubiquitin-binding motifs in REV1 protein are required for its role in the tolerance of DNA damage.* Mol Cell Biol, 2006. **26**(23): p. 8892-900.
37. Bienko, M., et al., *Ubiquitin-binding domains in Y-family polymerases regulate translesion synthesis.* Science, 2005. **310**(5755): p. 1821-4.
38. Yang, H., Q. Ren, and Z. Zhang, *Chromosome or chromatin condensation leads to meiosis or apoptosis in stationary yeast (Saccharomyces cerevisiae) cells.* FEMS Yeast Res, 2006. **6**(8): p. 1254-63.
39. Allen, C., et al., *Isolation of quiescent and nonquiescent cells from yeast stationary-phase cultures.* J Cell Biol, 2006. **174**(1): p. 89-100.
40. Hoege, C., et al., *RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO.* Nature, 2002. **419**(6903): p. 135-41.
41. Haracska, L., et al., *Interaction with PCNA is essential for yeast DNA polymerase eta function.* Mol Cell, 2001. **8**(2): p. 407-15.
42. Kannouche, P., et al., *Domain structure, localization, and function of DNA polymerase eta, defective in xeroderma pigmentosum variant cells.* Genes Dev, 2001. **15**(2): p. 158-72.
43. Plosky, B.S., et al., *Controlling the subcellular localization of DNA polymerases iota and eta via interactions with ubiquitin.* Embo J, 2006. **25**(12): p. 2847-55.
44. Garg, P., et al., *Proliferating cell nuclear antigen promotes translesion synthesis by DNA polymerase zeta.* J Biol Chem, 2005. **280**(25): p. 23446-50.
45. Garg, P. and P.M. Burgers, *Ubiquitinated proliferating cell nuclear antigen activates translesion DNA polymerases eta and REV1.* Proc Natl Acad Sci U S A, 2005. **102**(51): p. 18361-6.
46. Stelter, P. and H.D. Ulrich, *Control of spontaneous and damage-induced mutagenesis by SUMO and ubiquitin conjugation.* Nature, 2003. **425**(6954): p. 188-91.
47. Kannouche, P.L., J. Wing, and A.R. Lehmann, *Interaction of human DNA polymerase eta with monoubiquitinated PCNA: a possible mechanism for the polymerase switch in response to DNA damage.* Mol Cell, 2004. **14**(4): p. 491-500.
48. Haracska, L., et al., *Opposing effects of ubiquitin conjugation and SUMO modification of PCNA on replicational bypass of DNA lesions in Saccharomyces cerevisiae.* Mol Cell Biol, 2004. **24**(10): p. 4267-74.
49. Zhang, H., P.E. Gibbs, and C.W. Lawrence, *The Saccharomyces cerevisiae rev6-1 mutation, which inhibits both the lesion bypass and the recombination mode of DNA damage tolerance, is an allele of POL30, encoding proliferating cell nuclear antigen.* Genetics, 2006. **173**(4): p. 1983-9.
50. Chen, C.C., et al., *Genetic analysis of ionizing radiation-induced mutagenesis in Saccharomyces cerevisiae reveals TransLesion Synthesis (TLS) independent of PCNA K164 SUMOylation and ubiquitination.* DNA Repair (Amst), 2006. **5**(12): p. 1475-88.

51. Zhang, H., P.E. Gibbs, and C.W. Lawrence, *The Saccharomyces cerevisiae rev6-1 mutation, which inhibits both the lesion bypass and the recombination mode of DNA damage tolerance, is an allele of POL30*. Genetics, 2006.
52. Uppuluri, P. and W.L. Chaffin, *Defining Candida albicans stationary phase by cellular and DNA replication, gene expression and regulation*. Mol Microbiol, 2007. **64**(6): p. 1572-86.

CHAPTER 5:

SUMMARY AND PERSPECTIVES

My dissertation research has focused on the regulation of polymerase eta (pol η) in *Saccharomyces cerevisiae* by protein expression, localization, and protein-protein interactions. In Chapter 2, I have analyzed the ubiquitin-interacting domain and assayed the chromatin-association state of yeast pol η. In Chapter 3, I tested the hypothesis that Rev1 and pol η physically interact in yeast. In Chapter 4, I examined the genetic relationships of pol η *(RAD30)* with the other TLS polymerases Rev1 and pol zeta (ζ), and determined that pol η protein abundance does not vary significantly in response to DNA damage.

In Chapter 2, I uncovered a significant difference between regulatory domains of yeast and mammalian pol η. I had initially observed, within the sequence of *S. cerevisiae* pol η, a conserved region including a motif notated as a zinc finger [1]. However, the zinc finger motif is degenerate in *S. cerevisiae* pol η, lacking one of the key zinc-coordinating cysteine residues. Further sequence analysis revealed that other pol η homologs had complete zinc finger motifs, unlike the degenerate motif of *S. cerevisiae* pol η, but also that the region surrounding the putative zinc finger was highly conserved, even in *S. cerevisiae* pol η.

Although its function was not yet known, I predicted that mutation of this highly conserved region would interfere with pol η activity by disrupting a protein-protein interaction. I also wondered whether the degenerate zinc finger could possibly coordinate a zinc ion. By mutational analysis, I found that the conserved region is required for UV resistance, but that substitutions can be made at the putative zinc-coordinating residues without phenotypic consequences. Therefore, I hypothesized that *S. cerevisiae* pol η does not bind zinc.

In the course of this work, other studies identified this conserved region as a novel ubiquitin-interaction domain, the UBZ (Ubiquitin Binding Zinc Finger) domain, which mediates an interaction with ubiquitin in both yeast and mammalian pol η homologs [2-4]. Recently, the NMR structure of the UBZ domain from human pol η was solved, showing that it is a zinc-coordinated zinc finger [5]. In human pol η, substitutions at the zinc coordinating residues resulted in loss of function and disrupted the interaction with ubiquitin [2, 4]. My results, together with these findings, suggest that the ubiquitin-interacting function of the UBZ domain of yeast pol η is conserved between yeast and

human. In contrast, neither the sequence, nor the functional significance, of the ability to coordinate zinc are conserved in *S. cerevisiae* pol η.

I propose that the UBZ domain of yeast pol η forms a structure similar to that of its human homolog but, unlike the human homolog, does not require zinc binding to stabilize its structure. I plan to test this hypothesis directly by assaying the structural, zinc- and ubiquitin-binding properties of a purified UBZ domain from *S. cerevisiae* pol η. I expect the UBZ domain of *S. cerevisiae* pol η to form a stable structure and bind ubiquitin in the absence of zinc. If this is the case, *S. cerevisiae* pol η will be the second reported naturally occurring "zincless finger" [6]. Such structures have successfully been engineered twice in the past as well [7].

As I was beginning to write this thesis, a study of mutations very similar to my own was published [8]. The authors of this study assume that the UBZ domain of *S. cerevisiae* pol η contains a normal zinc finger. This leads them to conclude that because zinc finger residues can be mutated without affecting pol η's function, the UBZ domain's interaction with ubiquitin is not important for pol η function in *S. cerevisiae*. Instead, they suggest that an unknown, zinc-independent function accounts for the conservation of the UBZ domain in pol η homologs. By determining directly whether the UBZ domain of *S. cerevisiae* pol η binds zinc, I will clarify what conclusions can be drawn about the functions of the UBZ domain.

In mammals, pol η's UBZ domain is required for the formation of pol η foci in response to DNA damage [4]. Focus formation is thought to result from the interaction of pol η with ubiquitinated PCNA at sites of DNA damage [4, 9]. Although the UBZ domain of *S. cerevisiae* pol η has been shown to interact with ubiquitin [3], there had been no previous studies of focus formation or DNA association in *S. cerevisiae* to determine whether the biological significance of the ubiquitin interaction is the same in yeast and mammals. Toward this end, I performed the first study of the chromatin-association state of pol η in yeast. I found that the wildtype pol η protein is chromatin-associated even in the presence of DNA damage, but the amount of chromatin-associated pol η increases by approximately 2-fold after the cells are treated with UV to induce DNA damage. The increase was not observed when cells were harvested immediately after UV treatment, but was seen after 35 or 120 minutes (data shown is for 120 minutes).

Furthermore, I found that the damage-induced increase in chromatin-association of yeast pol η is dependent upon its UBZ domain. The UBZ domain mutants which cause a defect in UV-survival also cause the amount of chromatin-associated pol η to decrease, rather than increase, in response to DNA damage. Intriguingly, the UBZ mutant pol η is chromatin-associated in the absence of exogenous damage, and differs from wildtype only in its response to UV-induced DNA damage. My results are consistent with a similar roles for the UBZ domain in *S. cerevisiae* and in mammals.

The chromatin-association assays I performed measure DNA-association more inclusively than do the focus-counting assays used in mammalian cells. The chromatin-associated pol η should include not only the focus-forming pool of pol η, as well as any additional pol η which may have access to the DNA. It has not been determined whether focus-formation necessarily correlates with activity for pol η in mammalian cells. My results imply that chromatin-association is not sufficient for pol η activity, because the UBZ mutation, without impairing pol η's catalytic activity, causes a severe loss of function in pol η, but is still detectably chromatin-associated after DNA damage. Therefore, the UBZ domain itself must be required for a more specific function beyond general chromatin association to promote pol η function. I hypothesize that the UBZ domain-mediated interaction with ubiquitinated PCNA is required for precise placement of pol η in an orientation to enable it to bypass DNA lesions.

With respect to the function of the UBZ domain, I was also intrigued by the presence of two different ubiquitin-interaction motifs, the UBM and the UBZ domain, in the Y family of DNA polymerases. In particular, although pol iota (ι) has greater overall sequence similarity to pol η than to Rev1, it includes a UBM, like Rev1, while pol kappa (κ) and pol η contain UBZ domains. This pattern suggests that the two ubiquitin interaction domains may have evolved for distinct functions. I searched, by homology, for UBM- and UBZ-containing proteins in the database in order to compare and contrast their functions. I identified a new UBZ-containing protein, Pcf11, a transcription termination factor. It will be interesting to see what functional differences further studies may uncover between these two related domains.

There is still much to be learned about the function of the UBZ domain in general and with respect to the activity of polymerase η. One reminder of the incompleteness of

our understanding is that the UBZ domain interacts better with polyubiquitin than monoubiquitin [4, 10], while pol η's function is supported only by monoubiquitinated PCNA, and not polyubiquitinated PCNA. It may be that pol η does interact with polyubiquitinated PCNA, but that this interaction sequesters pol η in an inactive position, while the interaction with ubiquitinated PCNA helps place pol η in a position where it is TLS-enabled.

I also investigated the modification state of pol η itself. By mutation analysis, I found several potential phosphorylation sites in the C-terminal region of polymerase η whose mutation affects UV survival. I also identified a functionally significant potential SUMOylation site or ubiquitination site located very close to the ubiquitin-interacting UBZ domain. Since the activities of Rev1 and pol ζ are known to be influenced by kinases [11, 12] and Rev1 itself not only contains a potential phosphoprotein-binding motif (the BRCT) [13], but can also be phosphorylated itself [12], phosphorylation of pol η in yeast may regulate any physical interaction it has with Rev1 and/or pol ζ.

In Chapter 3, I have demonstrated another significant difference between yeast pol η and its mammalian counterpart: by coimmunoprecipitation I was unable to detect an interaction between pol η and Rev1 in yeast. My collaborator, Nicole Kosarek, in Errol Friedberg's laboratory, used a different technique, yeast 2-hybrid, to assay the interactions of Rev1 with pol η and other polymerases from several species. She was able to detect an interaction between the Rev1 and pol η homologs from *Drosophila melanogaster,* but not from *S. cerevisiae* or *Schizosaccharomyces pombe*. We concluded that there is no direct physical interaction between pol η and Rev1 in *S. cerevisiae*. However, a study was just published in which an interaction between the purified yeast pol η and Rev1 proteins is observed [14]. The fact that this interaction has only been observed for the purified proteins, and not under more physiological conditions, could imply that it is not physiologically relevant. However, even if we assume that it is a physiologically relevant physical interaction, there remains a striking difference between the species in which the interaction is robustly detectable by 2-hybrid and/or coIP, and organisms such as yeast, in which the interaction is significantly weaker or more conditional. There may be a physiologically relevant interaction in yeast which is

dependent on the presence of other factors such as additional interactions, or the modification states of the proteins involved.

Another intriguing finding of Chapter 3 is that sequence analysis could not identify a conserved Rev1-interacting motif shared among the regions from different proteins which were shown to interact with Rev1. Instead, the Rev1-interacting regions were all predicted to be disordered. Because of the work of a recent graduate of my lab [15], which demonstrated that the UmuD protein which regulates translesion synthesis in *E. coli* is an intrinsically unstructured protein, I recognized this as a finding of potential functional significance. Interactions with disordered regions have distinct properties, making them ideal for highly specific and reversible interaction, or for allosteric coupling. Therefore, we propose that Rev1 is able to interact specifically with its many interaction partners, and to quickly switch between them and adapt to changing conditions.

It is unknown what function a physical association between pol η and Rev1/pol ζ might have. One possibility is that Rev1 might be involved in recruiting pol η to sites of DNA damage. An entirely different possibility is that their association allows pol η (the more abundant protein) to be associated with every Rev1/pol ζ in order to compete with them. Genetic analysis could help determine whether pol η acts conditionally in the same pathway with Rev1/polζ, or instead acts in a competing pathway, but has not been done in those species in which Rev1 and pol η are known to physically interact.

In Chapter 4, I have undertaken the most thorough analysis to date of the genetic interactions between pol η and Rev1 and pol ζ in yeast, to elucidate the functional interaction between these proteins. I demonstrated that pol η protein abundance does not change significantly in response to UV-induced DNA damage in yeast, and therefore cannot account for a putative damage-induced activation of pol η function. The complex results of my genetic analysis have led me to ask whether there may be multiple overlapping modes of regulation of pol η in yeast. One possibility is a cell-cycle specific role for Rev1 in pol η's regulation.

Pol η has additional known and possible protein interaction partners which have not been discussed in this dissertation, but which may contribute to its multi-faceted regulation. While TLS is clearly promoted by PCNA monoubiquitination, it is not

exclusively dependent on this signal. An allele of *POL30* (encoding PCNA) which abolishes ubiquitination of PCNA and SUMOylation at K164 by K164R substitution is epistatic to *rad30*, *rev1*, *rev3* and *rev7* with respect to UV killing [9, 16]. However, pol ζ has some mutagenic activity independent of PCNA modification [16-18], and SUMOylation can also promote spontaneous mutagenesis [9].

A small proportion of mammalian cells, about 10%, do have pol η foci in the absence of PCNA ubiquitination, or in the absence of DNA damage (when ubiquitinated PCNA is undetectable) [4, 19]. The same is true of Rev1 foci [20], which also form in response to DNA damage [21]. The BRCT domain of Rev1 is required for interaction with unmodified PCNA but not with ubi-PCNA [20]. The formation of Rev1 foci in the absence of DNA damage is almost entirely dependent on Rev1's BRCT domain, suggesting that it is predominantly independent of PCNA monoubiquitination. Pol η may be similarly regulated both by interaction with unmodified PCNA, in the absence of exogenous damage, and by a more robust interaction with ubi-PCNA which causes the increased formation of pol η foci at sites of DNA damage.

There is also the possibility of PCNA-independent DNA association. One way this may be achieved is by interaction with an alternative clamp. The 9-1-1 complex (named for the *S. pombe* genes encoding it) is a heterotrimeric ring protein with structural similarity to PCNA [22]. This complex binds the DNA, where it acts as a DNA damage sensor in conjunction with the damage-induced checkpoint response (see section 1b), and also contributes to DNA repair [23]. In *S. cerevisiae* the 9-1-1 complex is composed of Ddc1, Rad17 and Mec3 proteins [24]. A recent study found that Ddc1 is involved in pol ζ's association with chromatin, specifically in response to UV radiation, and that pol ζ-dependent mutagenesis is partially dependent on Ddc1 [25].

Another factor that may help to recruit pol η, and which physically interacts with it, is an alternate PCNA-loading complex, Ctf18-RFC [26]. PCNA is normally loaded onto the DNA by RFC, but this process can also be performed by Ctf18-RFC, a variant complex which is involved in sister chromatid cohesion [26]. Ctf18-RFC was found to stimulate pol η activity *in vitro* by both PCNA-dependent and PCNA-independent means, suggesting that its interaction with pol η may help recruit the polymerase to the DNA [26]. Additional evidence of a connection between cohesion proteins and pol η has been

found in *S. pombe,* where the pol η homolog is Eso1, a protein made up of both pol η and a Ctf7-homolog, which is required for establishment of sister chromatid cohesion [27, 28].

TLS polymerases may also be activated through distinct mechanisms in response to specific types of damage or situations. One example is the observation that pol ζ-dependent ionizing radiation (IR) induced mutagenesis in yeast is only partially suppressed in a *pol30K164R* background [16-18]. Since IR produces double strand breaks (DSBs) in the DNA, the observation of a Rad18- and Ddc1-independent means of recruiting Rev1 and pol ζ to DSBs in another study [11] may be part of the same phenomenon. The association of Rev1 and pol ζ with DSBs (monitored by ChIP), requires the checkpoint kinase Mec1 (ATR homolog); failure to identify a specific Mec1 target suggests that the phosphorylation of multiple proteins promotes the TLS polymerases' association with DSBs [11].

Although pol η has not been shown to associate with DSBs, it is involved in DNA replication under a variety of different conditions, for which it may be regulated by distinct mechanisms. At a very basic level, TLS during gap-filling occurs at a different stage of DNA replication and the cell cycle than initiation of TLS by polymerase switching), so that different protein/protein interactions may be involved in the two modes of TLS. Pol η also participates in several known specialized functions, including somatic hypermutation of Ig variable regions and homologous recombination.

The role of TLS in somatic hypermutation (SHM) is thought to be regulated by PCNA modification, because *pol30(K164R)* and *rad18* mutations greatly reduce SHM [29, 30]. However, SHM is not abolished by these mutations [30]. Furthermore, a physical and functional interaction between pol η and the mismatch repair protein heterodimer MSH2-MSH6 contributes specifically to pol η's role in SHM. MSH2 physically interacts with pol η and stimulates its catalytic activity [31], while MSH2-MSH6 is partially required for pol η's contribution to SHM [32, 33].

A specific protein interaction partner, which is not involved in the response to UV-induced DNA lesions, also mediates pol η's role in homologous recombination (HR). This is the recombination factor Rad51, a DNA-binding protein involved in D-loop formation and cancer inhibition [34]. Rad51 helps to recruit pol η to D loop

structures *in vitro*, either by direct interaction with pol η or by alteration of the DNA structure [35].

Ultimately, the results of my dissertation research and of all the studies of TLS, have merely characterized the tip of an iceberg. As we expand our collective understanding of the nuances of DNA damage tolerance mechanisms and their regulation, I hope the results will lead not only to a better understanding of the early events that lead to carcinogenesis, but also perhaps to more effective ways of targeting cancer therapies to kill tumor cells specifically.

REFERENCES

1. Gerlach, V.L., et al., *Human and mouse homologs of Escherichia coli DinB (DNA polymerase IV), members of the UmuC/DinB superfamily*. Proc Natl Acad Sci U S A, 1999. **96**(21): p. 11922-7.
2. Bienko, M., et al., *Ubiquitin-binding domains in Y-family polymerases regulate translesion synthesis*. Science, 2005. **310**(5755): p. 1821-4.
3. Parker, J.L., et al., *Contributions of ubiquitin- and PCNA-binding domains to the activity of Polymerase eta in Saccharomyces cerevisiae*. Nucleic Acids Res, 2007. **35**(3): p. 881-9.
4. Plosky, B.S., et al., *Controlling the subcellular localization of DNA polymerases iota and eta via interactions with ubiquitin*. Embo J, 2006. **25**(12): p. 2847-55.
5. Bomar, M.G., et al., *Structure of the ubiquitin-binding zinc finger domain of human DNA Y-polymerase eta*. EMBO Rep, 2007. **8**(3): p. 247-51.
6. Doublie, S., et al., *The crystal structure of human endonuclease VIII-like 1 (NEIL1) reveals a zincless finger motif required for glycosylase activity*. Proc Natl Acad Sci U S A, 2004. **101**(28): p. 10284-9.
7. Struthers, M.D., R.P. Cheng, and B. Imperiali, *Design of a monomeric 23-residue polypeptide with defined tertiary structure*. Science, 1996. **271**(5247): p. 342-5.
8. Acharya, N., et al., *Mutations in the ubiquitin binding UBZ motif of DNA polymerase {eta} do not impair its function in translesion synthesis during replication*. Mol Cell Biol, 2007.
9. Stelter, P. and H.D. Ulrich, *Control of spontaneous and damage-induced mutagenesis by SUMO and ubiquitin conjugation*. Nature, 2003. **425**(6954): p. 188-91.
10. Bish, R.A. and M.P. Myers, *Werner helicase-interacting protein 1 binds polyubiquitin via its zinc finger domain*. J Biol Chem, 2007. **282**(32): p. 23184-93.
11. Hirano, Y. and K. Sugimoto, *ATR homolog Mec1 controls association of DNA polymerase zeta-Rev1 complex with regions near a double-strand break*. Curr Biol, 2006. **16**(6): p. 586-90.
12. Sabbioneda, S., et al., *Yeast Rev1 is cell cycle regulated, phosphorylated in response to DNA damage and its binding to chromosomes is dependent upon MEC1*. DNA Repair (Amst), 2007. **6**(1): p. 121-7.
13. Rodriguez, M., et al., *Phosphopeptide binding specificities of BRCA1 COOH-terminal (BRCT) domains*. J Biol Chem, 2003. **278**(52): p. 52914-8.
14. Acharya, N., et al., *Complex formation of yeast Rev1 with DNA polymerase {eta}*. Mol Cell Biol, 2007.
15. Simon, S.M., Sousa, F. J. R., Mohana-Borges, R. S., and Walker, G. C., *Regulation of E. coli SOS mutagenesis by dimeric intrinsically disordered umuD gene products*. In Press, 2007.
16. Zhang, H., P.E. Gibbs, and C.W. Lawrence, *The Saccharomyces cerevisiae rev6-1 mutation, which inhibits both the lesion bypass and the recombination mode of DNA damage tolerance, is an allele of POL30, encoding proliferating cell nuclear antigen*. Genetics, 2006. **173**(4): p. 1983-9.

17. Chen, C.C., et al., *Genetic analysis of ionizing radiation-induced mutagenesis in Saccharomyces cerevisiae reveals TransLesion Synthesis (TLS) independent of PCNA K164 SUMOylation and ubiquitination.* DNA Repair (Amst), 2006. **5**(12): p. 1475-88.
18. Zhang, H., P.E. Gibbs, and C.W. Lawrence, *The Saccharomyces cerevisiae rev6-1 mutation, which inhibits both the lesion bypass and the recombination mode of DNA damage tolerance, is an allele of POL30.* Genetics, 2006.
19. Watanabe, K., et al., *Rad18 guides poleta to replication stalling sites through physical interaction and PCNA monoubiquitination.* Embo J, 2004. **23**(19): p. 3886-96.
20. Guo, C., et al., *REV1 protein interacts with PCNA: significance of the REV1 BRCT domain in vitro and in vivo.* Mol Cell, 2006. **23**(2): p. 265-71.
21. Murakumo, Y., et al., *Analyses of ultraviolet-induced focus formation of hREV1 protein.* Genes Cells, 2006. **11**(3): p. 193-205.
22. Majka, J. and P.M. Burgers, *Yeast Rad17/Mec3/Ddc1: a sliding clamp for the DNA damage checkpoint.* Proc Natl Acad Sci U S A, 2003. **100**(5): p. 2249-54.
23. Helt, C.E., et al., *Evidence that DNA damage detection machinery participates in DNA repair.* Cell Cycle, 2005. **4**(4): p. 529-32.
24. Parrilla-Castellar, E.R., S.J. Arlander, and L. Karnitz, *Dial 9-1-1 for DNA damage: the Rad9-Hus1-Rad1 (9-1-1) clamp complex.* DNA Repair (Amst), 2004. **3**(8-9): p. 1009-14.
25. Sabbioneda, S., et al., *The 9-1-1 checkpoint clamp physically interacts with polzeta and is partially required for spontaneous polzeta-dependent mutagenesis in Saccharomyces cerevisiae.* J Biol Chem, 2005. **280**(46): p. 38657-65.
26. Shiomi, Y., et al., *A second proliferating cell nuclear antigen loader complex, Ctf18-replication factor C, stimulates DNA polymerase eta activity.* J Biol Chem, 2007. **282**(29): p. 20906-14.
27. Madril, A.C., et al., *Fidelity and damage bypass ability of Schizosaccharomyces pombe Eso1 protein, comprised of DNA polymerase eta and sister chromatid cohesion protein Ctf7.* J Biol Chem, 2001. **276**(46): p. 42857-62.
28. Tanaka, K., et al., *Fission yeast Eso1p is required for establishing sister chromatid cohesion during S phase.* Mol Cell Biol, 2000. **20**(10): p. 3459-69.
29. Bachl, J., I. Ertongur, and B. Jungnickel, *Involvement of Rad18 in somatic hypermutation.* Proc Natl Acad Sci U S A, 2006. **103**(32): p. 12081-6.
30. Arakawa, H., et al., *A Role for PCNA Ubiquitination in Immunoglobulin Hypermutation.* PLoS Biol, 2006. **4**(11): p. e366.
31. Wilson, T.M., et al., *MSH2-MSH6 stimulates DNA polymerase eta, suggesting a role for A:T mutations in antibody genes.* J Exp Med, 2005. **201**(4): p. 637-45.
32. Delbos, F., et al., *Contribution of DNA polymerase eta to immunoglobulin gene hypermutation in the mouse.* J Exp Med, 2005. **201**(8): p. 1191-6.
33. Delbos, F., et al., *DNA polymerase eta is the sole contributor of A/T modifications during immunoglobulin gene hypermutation in the mouse.* J Exp Med, 2007. **204**(1): p. 17-23.
34. Costa, S., et al., *DNA repair polymorphisms might contribute differentially on familial and sporadic breast cancer susceptibility: a study on a Portuguese population.* Breast Cancer Res Treat, 2006.

35. McIlwraith, M.J., et al., *Human DNA polymerase eta promotes DNA synthesis from strand invasion intermediates of homologous recombination.* Mol Cell, 2005. **20**(5): p. 783-92.