Novel Regulatory Mechanisms of Mutagenic Translesion DNA Synthesis: Characterization of *REV1* in *Saccharomyces cerevisiae*

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ABSTRACT

Cells are constantly subjected to DNA damage from endogenous and exogenous sources. Spontaneous DNA damage alone accounts for ~30,000 DNA lesions per day in a mammalian cell. Cells are also exposed to an enormous variety of environmental agents that can cause a wide range of modified bases and aberrant DNA structures. To respond to the large diversity of DNA lesions that can be produced, cells possess a host of DNA repair and damage tolerance systems. The majority of these processes operate with exquisite accuracy to restore the correct DNA sequence and structure to maintain genomic stability. However, in some cases, DNA damage induces a mutagenic response and these mechanisms are responsible for the active introduction of mutations into the genomes of all organisms.

Since the discovery in 1999 of a novel superfamily of error-prone translesion DNA polymerases, we have gained substantial insight into the biochemical mechanisms of DNA damage tolerance and mutagenesis. Translesion polymerases are specialized to insert a nucleotide opposite to DNA lesions and often produce mutations during the replication of undamaged DNA. It is now appreciated that the regulation of DNA damage tolerance systems at multiple levels is critical to the appropriate deployment of these potentially mutagenic translesion polymerases to prevent rampant mutagenesis.

In particular, this thesis has focused on determining the regulation of the translesion polymerase Rev1 in the model organism *Saccharomyces cerevisiae*. The *REV1* gene is responsible for the vast majority of spontaneous and damage-induced mutagenesis in all eukaryotes, from unicellular yeast to multicellular humans. Thus, an understanding the regulation and molecular mechanisms of *REV1* activity will provide critical insight into the processes of mutagenesis underlying disease and evolution.

The studies described here provide evidence supporting a new model of translesion synthesis, based on the observation of the cell-cycle regulation of the Rev1 protein. Additionally, mutations in conserved motifs in Rev1 have allowed characterization of the protein-protein interactions critical for *REV1* activity in survival and mutagenesis. Taken together, the data presented here argue for a cellular response mediated through Rev1 regulation which temporally and spatially restricts potentially mutagenic translesion synthesis such that it is employed only when necessary.

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Title: Professor of Biology

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"I will not cease from mental fight..." —W. Blake
"Suffer, suffer into truth." —Aeschylus

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Introduction

Chapter One—Introduction

Overview

The faithful replication of DNA and proper transmission of chromosomes is essential to inherit an accurate and complete genome, which encodes the information necessary for life. Ironically, the process of living itself generates reactive metabolites that can cause DNA damage. Cells are also exposed to a vast array of exogenous stresses that can directly or indirectly lead to DNA damage. Although cells contain multiple, highly complex systems to faithfully restore DNA to its original sequence and structure, at times distinct mechanisms are required to temporarily tolerate DNA damage without mediating repair of a lesion. These DNA damage tolerance processes contribute to survival after DNA damage and, in some situations, also actively promote the generation of mutations. The factors responsible for spontaneous and damage-induced mutagenesis are now known to include specialized DNA polymerases, termed translesion polymerases. Understanding of these potentially dangerous, yet highly conserved, polymerases is critical to a complete knowledge of cell stress responses, mechanisms of genomic integrity, cell death after DNA damage, induction of mutations, disease development, and the processes of adaption and evolution. My thesis work has focused on the regulation of one of these translesion DNA polymerases, *S. cerevisiae* Rev1, and the molecular mechanisms of its function.

Here I will provide an introduction to the many strategies a cell may employ to allow survival in the face of DNA damage. I focus on the contribution of damage tolerance mechanisms, in particular translesion synthesis, and review our current understanding of the regulation of translesion synthesis, including a discussion of the prevailing model and the emergence of new paradigms. I describe the DNA polymerases that mediate translesion synthesis and highlight the unique properties of the Rev1 family. To further understand the contribution of *REV1* to translesion synthesis and mutagenesis, I have characterized its regulation, discussed in Chapter Two. Additionally, I have undertaken structure-function studies, described in Chapter Three, to elucidate the role of the several domains of Rev1 in its function *in vivo*.

1.1 Types of DNA damage

DNA damage represents a major obstacle to proper cellular functions. It has been estimated that there are approximately ~30,000 lesions generated spontaneously in a mammalian cell per day (114). DNA damage can occur both spontaneously and from environmental sources. Spontaneous sources of DNA damage include reactive oxygen species generated primarily during aerobic metabolism; base deamination, especially of cytosine to uracil; and the inherent lability of DNA to depurinations and depyrimidinations (47, 114). Additionally, many environmental factors can cause DNA damage, such as ionizing or ultraviolet (UV) radiation and chemical agents including methyl methanesulfonate (MMS), cisplatin, and benzo[*a*]pyrene (47). Reflecting the diversity of DNA damaging agents, there are a wide variety of DNA lesions which can adversely affect the cell. These fall broadly into two classes of DNA damage: modifications to the nitrogenous base or alterations in the sugar-phosphate backbone. Many types of base modifications can take place, including: base loss by cleavage of the N-glycosidic bond; adduction by aldehydes, alkylation, or other electrophilic additions to any of various reactive positions on the base; oxidative damage, mostly by addition of hydroxyl to double bonds; covalent linkages between adjacent bases, such as *cis-syn* and 6-4 TT dimers, to form intrastrand crosslinks; and interstrand crosslinks caused by bifunctional alkylating agents (47). Alterations in the sugar-phosphate backbone can take the form of single or double strand breaks generated by cleavage at abasic sites, by free radical abstraction of a hydrogen from the ribose moiety, or by inhibition of a DNA nicking activity such as topoisomerase I (47).

1.2 DNA repair

1.2.1 Repair of base modifications

The diversity of DNA lesions produced necessitates a panoply of cellular responses to DNA damage. Accordingly, cells have developed multiple DNA repair pathways. The repair of base modifications constitutes one class of DNA repair pathways. A modified base may be repaired by direct reversal, such as the enzymatic cleavage of UV photoproducts by photolyase (47, 175). Alternatively, a lesion or improper base pair may be excised and the DNA

resynthesized by several excision repair pathways: nucleotide excision repair (NER) which mainly acts on large, bulky lesions that distort the DNA helix (39, 47, 174), base excision repair (BER) which predominantly repairs alkylation damage and depurinations (9, 47, 174), or mismatch repair (MMR) which recognizes DNA mismatches produced during DNA replication or by deamination events (47, 97, 176). All of these pathways function by a conceptually similar strategy. First, a DNA damage recognition factor binds to a DNA lesion and recruits endonucleases which nick the DNA flanking the lesion. In the case of BER, this recognition factor is a glycosylase that removes the damaged base while leaving the DNA backbone intact (9, 47, 174). For NER and MMR, the recognition factor does not cleave the DNA itself but instead recruits other endonucleases (39, 47, 97, 174, 176). A helicase or other enzyme will remove the intervening damage, creating a gap which is then filled in by a DNA polymerase using the undamaged strand as a template. The remaining nick is sealed by a DNA ligase. Long-patch and short-patch, and in some cases very-short-patch, variations of these pathways exist and there is considerable overlap and cross-talk among the excision repair pathways (47). Moreover, these pathways are to some extent redundant with each other, reflecting the cell's dedication to ensuring genomic integrity, as well as the severity of potentially lethal effects of unrepaired DNA damage.

1.2.2 Repair of strand breaks

1.2.2.1 Double strand break repair

DNA strand breaks constitute a distinct form of DNA damage handled by another set of DNA repair pathways. Double-strand breaks (DSBs) are repaired by three separate pathways: non-homologous end-joining (NHEJ), homologous recombination (HR), and single strand annealing (SSA). In highly repetitive vertebrate genomes or when a homologous sequence is not available such as in G1 haploid yeast, repair of DSBs occurs by the end joining of non-homologous chromosomes (47, 68). Briefly, Ku proteins bind to the ends of DSBs and recruit end-bridging factors—DNA-PK and Artemis in vertebrates, the Mre11/Rad50/Xrs2 complex in yeast—which process the ends of the breaks (47, 68). This is necessary since DSBs rarely have clean ends, but usually have broken ribose-phosphate moieties which impede ligation. DNA

ligase IV and X-family DNA polymerases then fill in any gaps and seal the DNA backbone to restore an intact chromosome (47, 68). Prokaryotes also have a recently discovered NHEJ pathway which recapitulates the enzymatic steps outlined above in eukaryotes, but using multifunctional proteins which contain DNA polymerase, nuclease, and ligase activities in a single polypeptide (20, 68).

When a homologous sequence is available, such as during S-phase and G2 while the preferred substrate of a sister chromatid is present, the cell will repair a DSB through homologous recombination (Fig. 1) (47). The first step of HR is exonucleolytic 5′ to 3′ resection of the ends to generate ssDNA with free 3′ ends which invade the homologous region of the double strand substrate (47, 163, 206). This substrate can be a sister chromatid, a homologous chromosome, a different chromosome, or even a repeated region on the same chromosome (47, 163). Strand invasion displaces the original complementary strand of the substrate, forming a D-loop structure containing a primed template for DNA polymerase to extend (47, 163, 206). At this point, HR can proceed through any of three subpathways (47, 163, 186). In the classical double-strand break repair (DSBR) pathway, the 5′ end of the DNA can pair with the substrate in the D-loop bubble, generating a double Holliday junction (Fig. 1A). Holliday junctions can translocate over many kilobases by branch migration. Once a DNA polymerase has filled in the gap, Holliday junction resolution separates the two chromosomes, in either a crossover or non-crossover manner (47, 163, 206). The DSBR pathway has been well-established for meiotic recombination in eukaryotes. However, mitotic recombination may occur predominantly through alternative subpathways of HR. In the synthesis-dependent strand annealing (SDSA) pathway, following the initiation of DNA synthesis after strand invasion, the invading strands separate from the substrate and re-anneal to each other to complete DNA synthesis (Fig. 1B) (47, 163). A form of SDSA is also thought to occur during template switching mode of DNA damage tolerance (see section 1.3.1). Additionally, HR may occur through break-induced replication (BIR), in which one half of the broken chromosome is lost and the replication fork reconstituted by strand invasion recapitulates the entire rest of the chromosome (Fig. 1C) (47, 163).

Fig. 1

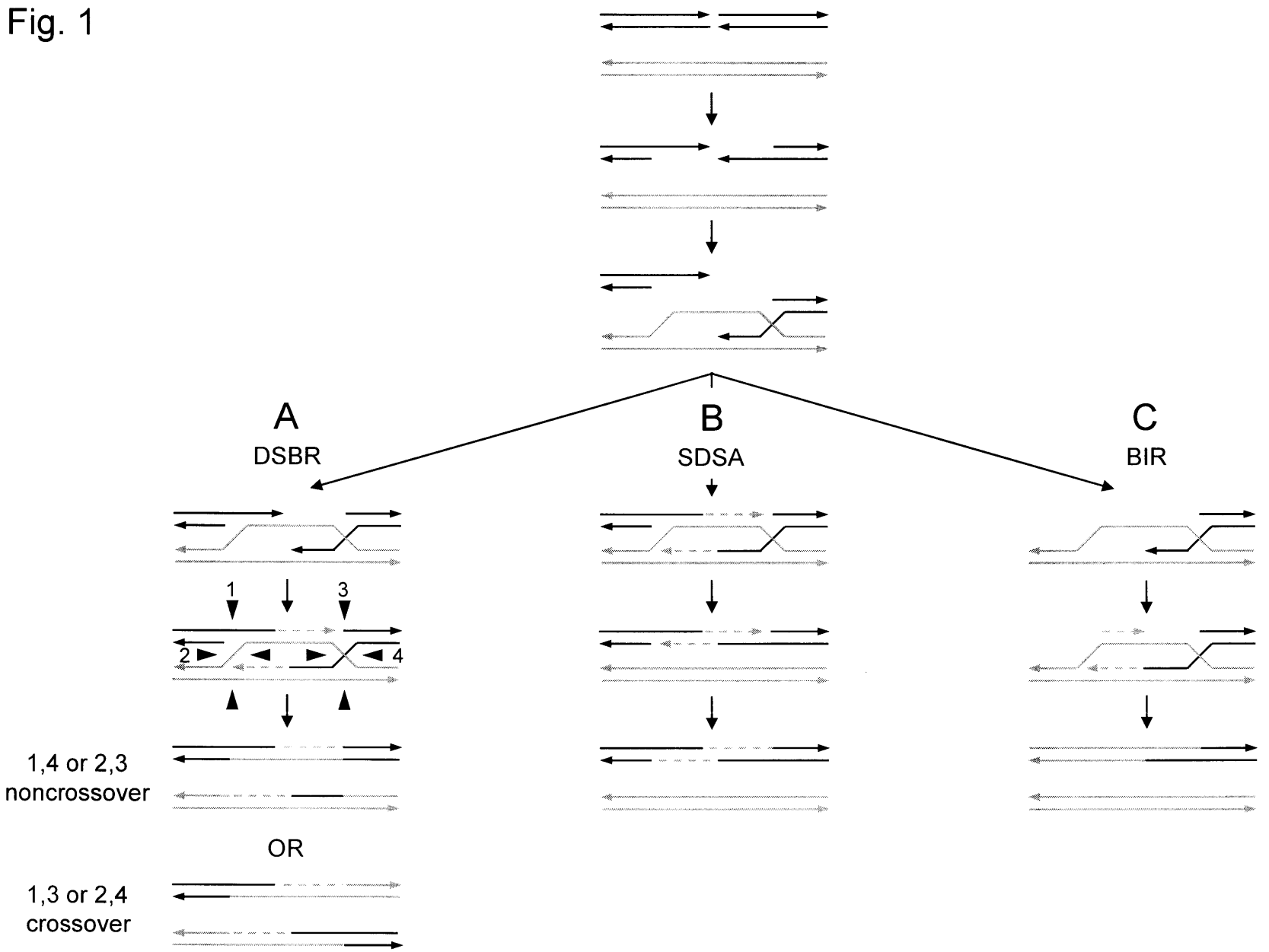
A
DSBR
B
SDSA
C
BIR
1
3
2
4
1,4 or 2,3
noncrossover
OR
1,3 or 2,4
crossover

Fig. 1 Pathways of repair of DSB by homologous recombination. [Adapted from (186).] Black lines and grey lines indicate two different chromosomes. Arrows indicate the 3′ end of the DNA strand and triangles indicate cleavage events. A DSB can be repaired through three subpathways. First, the 5′ ends are resected, allowing the free 3′ strands to invade a region of homology. The displaced strand forms a D-loop structure. (*A*) In the classic DSBR pathway, annealing of the other half of the broken chromosome generates a double Holliday junction which can translocate to increase the region of DNA transferred from one chromosome to another (not shown). Cleavage can occur in one of two ways (at arrows 1, 3 and 2, 4 or 1, 4 and 2, 3) to resolve the Holliday junctions in a crossover or non-crossover manner respectively. (*B*) Alternatively, annealing of the other half of the broken chromosome can be disrupted after DNA synthesis has generated complementary ends, such that the two nascent strands can now pair with each other to finish filling in the remaining gaps. (*C*) If the other half of the broken chromosome is lost, establishment of two replication forks can resynthesize the lost DNA using the other chromosome as a template.

Finally, a DSB can be repaired by single-strand annealing (SSA). This pathway relies on annealing of direct repeats, usually on the same DNA molecule (47, 163). In this situation, exonucleolytic processing of the DSB to produce 3′ ssDNA uncovers regions of homology that can then pair. Cleavage of the 3′ heterologous tails allows ligation after a DNA polymerase has filled in the gaps. SSA results in deletion of any intervening sequence and is therefore highly mutagenic, however SSA appears to be an important DSB repair pathway in higher eukaryotes (163).

How a cell chooses between the NHEJ and HR modes of repair is not clear and is currently a focus of ongoing research. In vertebrates, NHEJ predominates over HR while in yeast, HR is more frequent than NHEJ (47, 68). If multiple breaks are present, NHEJ can result in chromosomal translocations and even when presented with only one DSB, NHEJ can cause loss of information at the break site due to end processing which may remove damaged nucleotides. Despite this mutagenic potential, vertebrates may preferentially use NHEJ since their genomes contain many repeated regions such that HR could attempt to synapse repeats on two different chromosomes, causing a genomic rearrangement (47). Additionally, end joining factors are thought to be recruited very rapidly to hold closely opposed ends of the DSB together to avoid translocations. In contrast, HR uses a sister chromatid or homologous chromosome as a template to repair the DSB, resulting in less potential for genomic rearrangements and information loss. However, the break-induced replication (BIR) mode of HR has been associated with an increase in point mutations generated by mutagenic translesion polymerase mediated DNA synthesis (74, 166). Cell-cycle stage contributes to DSB repair choice, as does the nature of the DSB, but overall the mechanisms controlling this decision are not currently well understood (47, 206).

1.2.2.2 Single strand break repair

Single-strand break repair (SSBR) requires additional DNA repair components (24, 47). Although a simple nick can easily be ligated, a strand break often has complex ends composed of broken ribose or phosphate moieties and may also contain a region of ssDNA. In mammalian cells, nick recognition is accomplished primarily by poly(ADP-ribose) polymerase (PARP). The single strand ends can be processed to remove blocked 5′ and 3′ termini either by the AP-lyase

activity of DNA polymerase β or by polynucleotide kinase. Gap filling is usually accomplished by an X-family polymerase such as polymerase β, but the replicative DNA polymerases may substitute if needed. The scaffolding protein XRCC1 coordinates SSBR by stimulating polynucleotide kinase, as well as interacting with DNA polymerase β and DNA ligase III. Finally DNA ligase III or DNA ligase I seals the nick (24, 47). Many of the components of SSBR are unique to mammals and at this time, it is not clear how or if SSBR occurs in lower eukaryotes.

1.3 DNA damage tolerance

In addition to DNA repair processes, cells have mechanisms to temporarily tolerate DNA damage encountered during transactions with DNA. Although not generally considered the first line of defense after DNA damage, tolerance mechanisms are crucial for cellular survival under conditions when a DNA lesion is encountered during DNA metabolism (47). Inhibition of DNA metabolism by a lesion may arise due to mutational inactivation of one of the previously described DNA repair pathways, exposure to high levels of DNA damage, a DNA lesion which is particularly recalcitrant to DNA repair or one which is present in an inaccessible region of the chromosome, or simply by chance. Damage tolerance is particularly important during DNA replication, although it may also play a role during transcription (S. Cohen, unpublished data) and, theoretically, in other situations where DNA and possibly RNA is processed in a sequence-specific manner. When the replication machinery encounters a modified base that cannot be used as a template by the highly stringent replicative DNA polymerase, DNA synthesis can temporarily halt (47, 100). Such replication fork stalling can generate ssDNA stretches due to the uncoupling of leading and lagging strand synthesis (118, 153, 195). DNA damage tolerance mechanisms facilitate the restoration of DNA replication at stalled replication forks and also promote resolution of aberrant DNA structures left behind when replication forks reprime downstream of a DNA lesion (47, 107). Conceptually, DNA damage tolerance is very different from DNA repair in that, rather than restoring the DNA to its proper sequence and structure, tolerance pathways help the replication machinery bypass a DNA lesion, leaving the lesion still present in the DNA after replication (47). This is accomplished by two main modes of DNA

damage tolerance: recombinational bypass and translesion synthesis (TLS), which is the focus of this thesis, in particular the TLS polymerase Rev1.

1.3.1 Recombinational bypass

Recombinational bypass of a DNA lesion uses information from another, undamaged strand as a template to synthesize DNA past a region containing the lesion. This occurs by dissociation of the nascent DNA strand from its template to allow pairing with an undamaged template (Fig. 2). The presence at the replication fork of two double-stranded helices comprising four strands of homologous DNA can lead to a variety of complex DNA structures during bypass of a lesion. For simplicity, linear pathways are described, but it is important to note that in the cell, combinations of different topological conformations can occur sequentially. Several non-exclusive models have been proposed for recombinational bypass (47). The major distinctions in the pathways rely on the identity of the undamaged template and the timing of the bypass synthesis relative to encountering the lesion, i.e. whether the DNA opposite the lesion is synthesized at the replication fork through fork regression or after the fork has passed leaving a gap that can be filled in later by daughter strand gap repair.

To bypass a DNA lesion at a fork, the stalled replication fork may roll backwards to reanneal the two parental strands, allowing pairing of the two daughter strands and DNA synthesis past the site of the lesion using one newly synthesized daughter strand as a template for the other (Fig. 2A) (37, 47). Multiple resolutions of this "chicken foot" Holliday junction structure are possible, including reverse branch migration to restore semiconservative replication (Fig. 2A) or cleavage of the regressed fork to yield a broken chromosome which can then invade to form a D-loop and restart replication (Fig. 2B) (37, 47). This model is most frequently termed replication fork regression or template switching.

Alternatively, instead of fork regression, DNA synthesis may reprime downstream of a lesion, leaving behind a lesion-containing ssDNA gap that can be processed by the recombination machinery (Fig. 2C) (47, 69). In this case, the template can be an already replicated homologous sequence from a sister chromatid, a homolog, another chromosome, or a repeated region on the same chromosome. Pairing, strand exchange, branch migration, and Holliday junction resolution are envisioned to occur similarly to normal homologous

recombination (see Section 1.2.2.1) (47, 182). This mode of recombinational bypass is known as daughter strand gap repair, which, despite its name, is a form of damage tolerance since the lesion remains in the replicated double helix. The term "repair" refers to the gap, which is removed during the bypass. Though long considered unlikely, it is now appreciated that daughter strand gap repair could operate well after replication has been completed (107).

Fig. 2

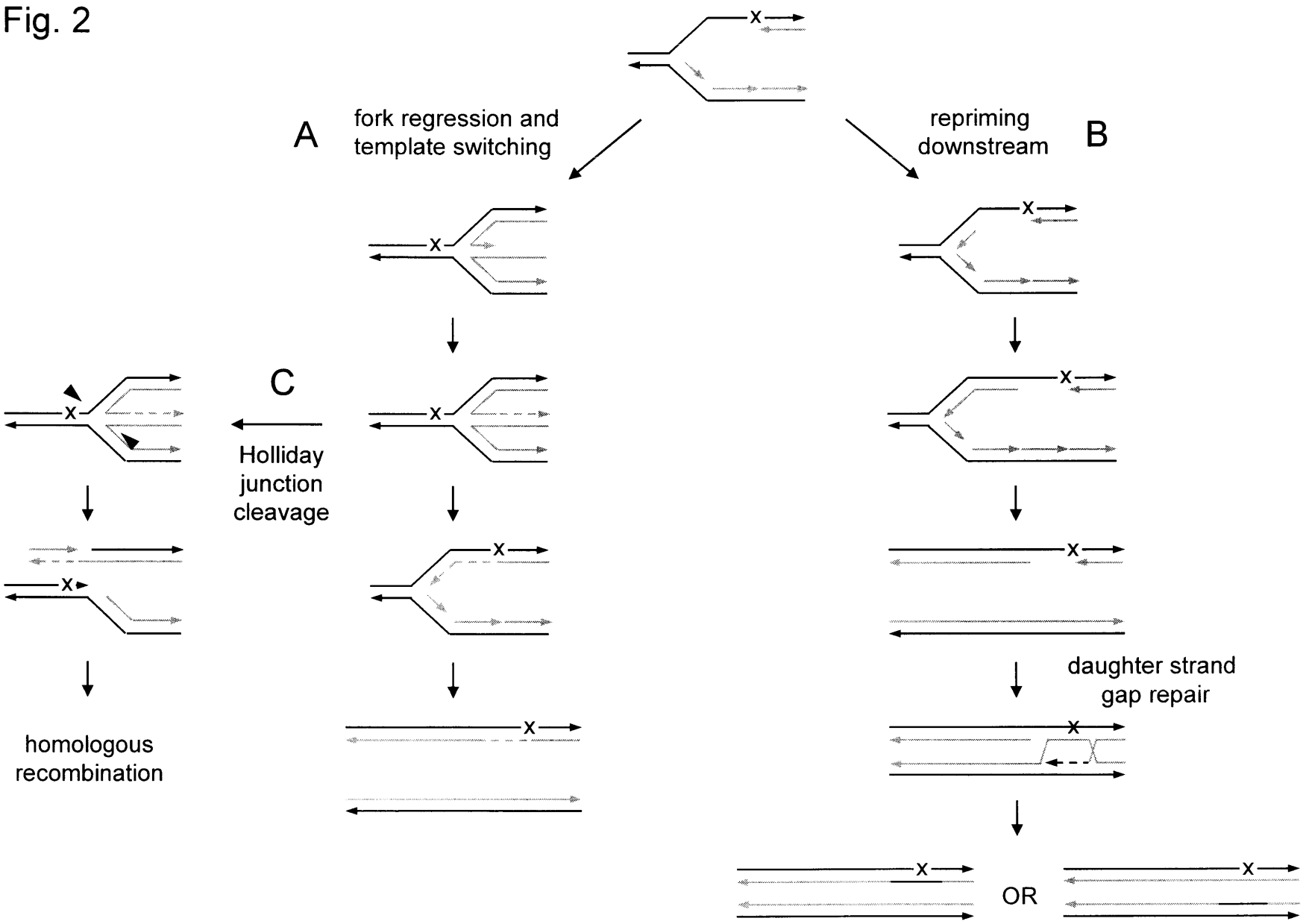
x
A
fork regression and
template switching
repriming
downstream
B
C
Holliday
junction
cleavage
homologous
recombination
daughter strand
gap repair
OR

Fig. 2 Pathways of recombinational bypass. Arrows indicate the 3′ end of the DNA strand, triangles indicate the cleavage events, and dashed lines indicate DNA synthesis. Black lines show the parental DNA strand; grey lines indicate the daughter strand. A DNA lesion which blocks the leading strand replication fork can be bypassed in several ways. (*A*) Regression of the replication fork results in pairing of the two nascent daughter strands by a template switch. DNA synthesis using the undamaged daughter strand can then proceed past the site of the lesion. Reverse branch migration of the Holliday junction reanneals the daughter strands to their original template strands to allow semiconservative replication to resume. (*B*) Alternatively, once the fork has regressed to form a Holliday junction, cleavage could occur to generate two linear molecules which would be substrates for homologous recombination, as described in section 1.2.2.1. (*C*) Repriming of the DNA polymerase downstream of the site of the lesion creates a gap in the daughter strand. The recombination machinery can then pair this ssDNA with an undamaged template and the gap can be filled in using the sister chromatid, or another DNA molecule with homology.

1.3.2 Translesion synthesis

Translesion synthesis (TLS) is the process by which a DNA lesion is bypassed by inserting a nucleotide opposite to the lesion (47). Many DNA lesions cannot be used as a template for the highly stringent replicative DNA polymerases, which are optimized to replicate the entire genome with high accuracy and efficiency (7, 47). A specialized class of DNA polymerases, known as TLS polymerases, can use various damaged bases as templates and insert nucleotides opposite to lesions despite the conformational constraints many modified bases may impose (47, 56, 164). Most TLS polymerases are members of the Y family of DNA polymerases (148), with the notable exception of DNA polymerase ζ, a member of the B family of replicative DNA polymerases in eukaryotes (132). In addition to polymerase ζ, TLS polymerases include five major types of Y family polymerases (148). Briefly, they include: 1) the prokaryotic polymerase V, composed of UmuC and a dimer of the accessory factor UmuD′, which bypasses a variety of DNA lesions in a mutagenic manner; 2) polymerase IV, also known as DinB, Dpo4, and polymerase κ in prokaryotes, archaea, and eukaryotes respectively, which functions in a relatively error-free manner in cells to bypass adducted G residues; 3) polymerase η, found only in eukaryotes, which is specialized to bypass *cis-syn* TT dimers produced by UV irradiation and 4) its paralog polymerase ι whose biological function is unknown; and finally 5) Rev1, found only in eukaryotes, which has limited polymerase activity, restricted to inserting primarily C residues across from G's and certain lesions (47). (Refer to section 1.3.6 for more details.) In this thesis, I have focused on understanding the regulation and function of Rev1.

1.3.2.1 Physical features of TLS polymerases

Several crystal structures have provided insight into the architectural features that confer unique properties to the Y family members (115, 116, 137, 138, 180, 189, 190, 216) and recently many new structures have been elucidated which refine our understanding. Despite a complete lack of primary sequence homology with all other known DNA polymerases, Y family members share the classic "right-hand" DNA polymerase fold (7, 164, 212). Like replicative polymerases, the catalytic aspartate and glutamate residues, which coordinate the divalent magnesium ions that stabilize the triphosphate group of the incoming dNTP, are located in the central palm region (7,

164, 211). Though the secondary structure of the domains is different in Y-family polymerases, thumb and fingers domains analogous to those in replicative polymerases grip the DNA and make specific contacts to the primer and template strands respectively (Fig. 3) (7, 164, 211).

Although they share a common overall architecture, Y family polymerases differ from replicative polymerases in certain key ways to allow them to perform translesion synthesis. At a whole-structure level, this can be seen by the presence and absence of entire domains relative to replicative polymerases. Importantly, Y family polymerases lack the 3′ to 5′ exonuclease domain of replicative DNA polymerases which functions to proofread the newly replicated strand (56, 212). This domain contributes a ~10^2 fold increase in fidelity to replicative DNA polymerases (96). It should be noted that there is precedent for extrinsic proofreading of mismatches inserted by non-replicative polymerases by the exonuclease subunits of replicative polymerases (127). A novel little fingers domain (also called the polymerase associated domain (PAD) or the wrist) in Y family polymerases extends from the classical fingers domain and makes extra contacts with the DNA (211, 212). This additional DNA binding region provides important stability for the ternary complex, since Y family members have short, stubby thumb and fingers domains which make few contacts with the DNA backbone (164, 211). Y family polymerases generally have an open grip on the DNA (Fig. 3) and a greatly reduced processivity relative to replicative DNA polymerases (48, 164); truncations of the little fingers domain reduces DNA binding and processivity even further (164). The little finger domain appears to contact the region of the template containing the lesion (Fig. 3) and has been implicated in lesion specificity (19).

Closer inspection of the active site of Y family and replicative polymerases also reveals significant differences. Particularly for the archaeal and prokaryotic Y family polymerases, the active site is larger and more open (Fig. 3) (164, 211, 212). This more spacious active site allows accommodation of large bulky adducts (117), and even two covalently linked bases in a thymine-thymine dimer (115). Also, Y family polymerases make fewer contacts to the forming base pair (211, 212) and, in particular, lack the O-helix of replicative DNA polymerases which, upon binding of a dNTP, rotates ~40° to sterically check the forming base pair (Fig. 3) (155). Based on crystallographic analysis, the Y family polymerases may not exhibit an induced fit upon binding of the incoming dNTP, which contributes to the replicative fidelity of replicative polymerases (212), however this seems not be true of DNA polymerase η (198).

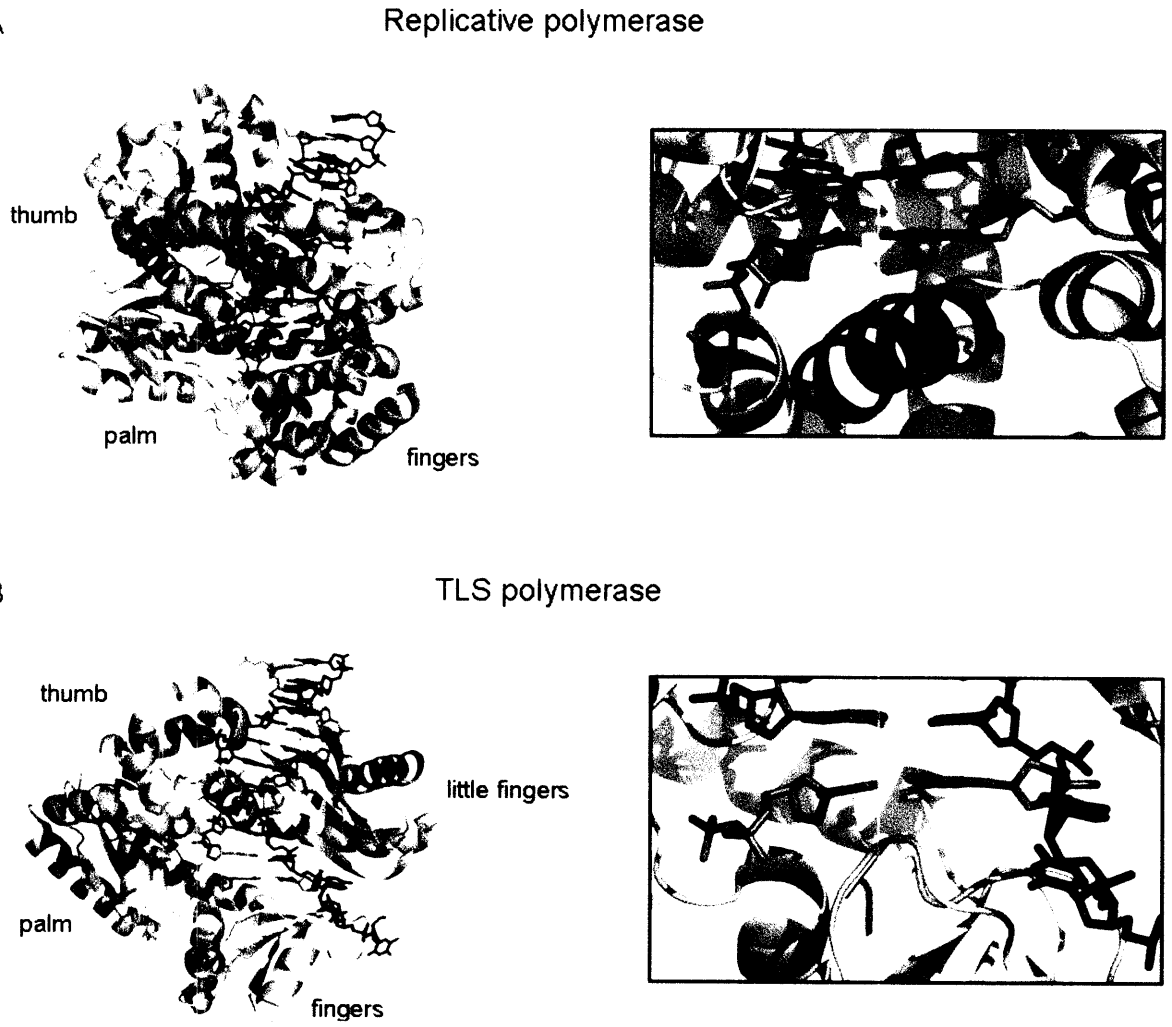

Fig. 3 Comparison of the structures of replicative and TLS polymerases. The template DNA strand is shown in red, the primer strand in green, and the forming base pair in blue. (*A*) The crystal structure of a replicative DNA polymerase lacking its exonuclease domain (83) reveals extensive contacts with the DNA, both at the whole structure level and in a close-up of the active site. In particular, the O-helix (shown in orange on the right) is intimately involved in interactions with the forming base pair. The right hand fold may not be apparent due to the orientation required to indicate the incipient base pair. (*B*) In contrast, TLS polymerases, as shown by archaeal Dpo4 (116), have a substantially more open structure with fewer contacts to the DNA, especially in the active site. Note the lack of interactions with the template strand (in red) which allows accomodation of aberrant base modifications into the active site.

At the current time, only three Y family polymerases have been co-crystallized with DNA: archaeal Dpo4 with a variety of damaged and undamaged templates, human polymerase ι (138), and yeast Rev1 (137). Intriguingly, Rev1 displays a unique catalytic activity restricted to inserting only dCMP nucleotides (140). This specialized activity is explained by a novel mechanism of base pairing using a conserved arginine residue that forms a hydrogen bond with the incoming nucleotide. The template G is flipped out of the active site by contacts with other conserved residues, allowing bypass of bulky G adducts (137). Since Rev1 has an unusual bypass mechanism and polymerase ι may use an unusual Hoogsteen base pairing mechanism (138), more classical translesion synthesis has only been directly observed with Dpo4. Thus, it is unclear how particular features of different polymerases may contribute to lesion specificity. It will be of great interest to determine how the molecular architecture of TLS polymerase active sites is coordinated with specificity of lesion bypass.

1.3.2.2 Fidelity of TLS polymerases

TLS polymerases exhibit a markedly low accuracy of base pair insertion on undamaged DNA templates. Compared to replicative DNA polymerases, which incorporate the wrong nucleotide only once for every ~10^7 bases replicated, TLS polymerases display error rates of approximately one incorrect nucleotide for every 10 to 10,000 bases (56, 96, 164). The novel features of Y family DNA polymerases that allow them to use an increased variety of base structures as templates also confer a decreased replication fidelity. Therefore, TLS polymerases have potentially mutagenic activity inside the cell (46). The lack of a 3′ to 5′ proofreading domain partially explains the reduced fidelity of TLS polymerases (56, 212). However, the loss of up to four additional orders of magnitude is likely due to the lack of induced fit and fewer contacts that TLS polymerases make with the template base and incoming nucleotide (211, 212). Additionally, some TLS polymerases, like DNA polymerase ι, may operate through a Hoogsteen base pairing mechanism rather than the canonical Watson-Crick pairing (164).

However, despite their low fidelity on undamaged DNA, a recent paradigm shift has reclassified TLS polymerases from simply being considered as "error-prone" polymerases (56) to a more nuanced understanding of their role as lesion-specific bypass polymerases (46). Originally called "sloppier copiers" and lauded for their "benefits of infidelity", it is now

appreciated that certain TLS polymerases have a high degree of fidelity opposite their cognate lesions (80, 81, 196). Cognate lesions have been defined for several TLS polymerases both by their ability to bypass the lesion accurately *in vitro* and *in vivo* as well as by their ability to insert nucleotides with equal or higher efficiency opposite the lesion than on undamaged DNA (80, 81, 124, 196). This is most notably seen in the case of DNA polymerase η, which is specialized to bypass *cis-syn* TT dimers caused by UV irradiation (81). In humans, mutations of DNA polymerase η result in the disease Xeroderma Pigmentosum Variant which is characterized by a high frequency of sunlight-induced skin cancers (106, 120). Thus, although DNA polymerase η exhibits among the lowest fidelities of any TLS polymerase on undamaged DNA ($\sim 10^{-1}$) (121), opposite its cognate lesion it is highly accurate and when inactivated, the polymerases that substitute for it *in vivo* are much less accurate (106).

1.3.2.3 Disadvantages and benefits conferred by TLS polymerases

Although they may have high accuracy opposite certain lesions, the fact that TLS polymerases have poor fidelity on undamaged DNA suggests that they are tightly regulated *in vivo* to avoid rampant mutagenesis. When overexpressed or misregulated due to mutations compromising key regulatory motifs, certain TLS polymerases confer a hypermutator phenotype (10, 13, 92, 144). Additionally, even when not accompanied by an increased level of mutagenesis, mild overexpression of TLS polymerases could result in alterations in replication fork speed due to the slow polymerization rate of TLS polymerases (7, 49, 78, 110). However, the risk to the cell of potential mutations and replication perturbation is presumably outweighed by the fact that TLS polymerases confer a measure of resistance to DNA damaging agents. In the absence of TLS polymerases, cells are moderately sensitized to various DNA damaging agents (46, 47) and, importantly, often contain higher levels of genomic rearrangements (10, 32, 35, 58, 79, 149, 204). Especially in the genomes of higher eukaryotes, which contain a large amount of noncoding DNA and many repetitive elements, TLS-induced base pair substitutions would seem to be preferable to aberrant or collapsed replication fork structures or gapped DNA which occur in the absence of TLS. These structures can elicit recombinational repair, triggering translocations and other gross chromosomal rearrangements. Even frameshift mutations, which have a much higher potential to inactivate protein function than a missense mutation, would be

less catastrophic for a cell than chromosomal rearrangements. This may explain why, despite the carcinogenic potential of mutator polymerases, their overexpression has not, to date, been clearly correlated with oncogenesis. This is in contrast to the X family DNA polymerase β, overexpression of which is strongly linked to cancer (28).

In addition to promoting survival after DNA damage, TLS polymerases can provide other benefits to cells. In unicellular organisms, TLS polymerases have been implicated in adaptive mutagenesis—the ability to induce mutations upon cellular stress (43, 46). In higher eukaryotes, TLS polymerases play a critical role in the generation of mutations in the variable regions of antibodies produced by B cell lymphocytes in a process known as somatic hypermutation (SHM) (26). Thus, despite potentially deleterious mutagenic effects, TLS polymerases presumably provide more benefits than disadvantages to cells, consistent with the observation that TLS polymerases have been found in all organisms sequenced to date.

1.3.3 Regulation of TLS

In order to limit potentially mutagenic translesion synthesis such that it is employed only when needed, a set of regulatory mechanisms ensure that TLS polymerases function only at sites of DNA damage, presumably preferentially at their cognate lesions, or in situations when mutations would be advantageous, such as during cell stress or antibody generation. TLS polymerases are regulated at multiple levels—in fact, almost every mechanism the cell has at its disposal to control the activity of protein is used. In this thesis, I have focused on the regulation of Rev1, discussed further in Chapter Two.

1.3.3.1 Transcriptional, translational, and degradational regulation

In bacteria, the TLS polymerases are under the well-studied transcriptional control of the SOS response (47). In eukaryotes, some TLS polymerases are induced at the mRNA level after DNA damage (126, 146, 170). Additionally, all TLS polymerases are upregulated at the mRNA levels in meiosis in yeast and mouse models (23, 34, 99, 181). In *E. coli*, UmuC protein levels are kept at a very low level relative to its transcriptionally coregulated partner UmuD by translational control (158). The human *REV1* and both yeast and human *REV3* transcripts

contain small upsteam open reading frames which presumably reduce translational efficiency of the major open reading frame encoding the Rev1 and Rev3 proteins (53, 54, 102, 111). In eukaryotes, small upstream open reading frames greatly decrease the frequency with which the ribosome reaches the main open reading frame and act as a mechanism to reduce basal expression of a protein (52). Additionally, alternative splicing of the human *REV3* gene produces an in-frame stop codon in ~50% of *REV3* transcripts, further reducing the levels of Rev3 protein (102). Timed degradation of TLS polymerase subunits upon completion of the SOS response also contributes to control of their activity (44). Additionally, in recent work from our lab, 26S proteasomal degradation of Rev1 and Rev3 proteins may be involved in keeping the levels of these mutagenic polymerases low inside the cell (M.E. Wiltrout, L. Waters, unpublished data; see Appendix B).

1.3.3.2 Post-translational regulation

A variety of post-translational modifications control TLS polymerases. In many bacteria, UmuD, the UmuC accessory protein, undergoes auto-proteolytic cleavage liberating the N-terminal 24 amino acids. This cleavage produces the shorter protein UmuD′, which is required to stimulate the TLS polymerase activity of UmuC (22, 142, 178). In eukaryotes, PCNA, the eukaryotic sliding processivity clamp, becomes ubiquitinated upon DNA damage (71). PCNA ubiquitination is thought to recruit TLS polymerases (17, 161) and stimulate catalytic activity of DNA polymerase η (50), as well as possibly DNA polymerase ζ and Rev1 (50, 67). Additionally, many of the TLS polymerases become ubiquitinated themselves, which is thought to promote recruitment to sites of DNA damage (17). It is unknown at this time if phosphorylation plays a role in TLS polymerase regulation, however there is some evidence to support the idea that the various DNA damage checkpoints may, directly or indirectly, affect translesion synthesis (4, 15, 70, 84). Additionally, genetic interactions between *CDC7* kinase and TLS polymerases may indicate a role for cell-cycle-dependent phosphorylation events regulating TLS [(159); L. Waters, unpublished data; see Appendix B]. Finally, in principle, post-translational modification of histones or the action of other chromatin remodeling factors may be involved in regulating access of the TLS polymerases to sites of DNA damage.

1.3.3.3 Regulation through subcellular localization

Subcellular localization is a major means of regulating translesion synthesis in eukaryotes. In normally growing cells, GFP fusions to TLS polymerases show diffuse nuclear localization patterns. In a small percentage of cells, TLS polymerases form punctate foci which are thought to colocalize with replication forks. Upon DNA damage, the number of cells exhibiting foci increases dramatically (1, 13, 86, 87, 133, 134, 143, 188). Mutations that abolish foci formation also impair the ability of TLS polymerases to function *in vivo* (17, 58, 89). The recruitment of TLS polymerases into foci after DNA damage has been a major direction of research in the field and has provided insight into the mechanisms of regulation of translesion synthesis. Besides monitoring survival and mutagenesis after DNA damage, which only indirectly assesses function at a physiological level, the *in vivo* assays for TLS polymerase activity are limited, mainly employing transformation of lesion-bearing plasmids. Therefore, much of our knowledge regarding the biological relevance of various mutations in TLS polymerase function has been garnered from localization studies. However, when considering these studies, it is important to keep in mind several caveats.

First, TLS polymerases in general are present at quite low levels in the cell (see section 1.3.3.1) and increasing their levels can cause spontaneous mutagenesis (13). Therefore, ectopic overexpression of TLS polymerases from GFP fusions on plasmids may represent artifacts of escape from normal cellular regulation that restrict TLS polymerases from over-access to the DNA. Indeed, in one study where Rev1 was expressed ectopically but at low levels, no foci could be observed even after DNA damage (169).

Second, focus formation has been assumed to represent recruitment to replication forks based on colocalization with PCNA. However, as PCNA interacts with over 35 proteins from many DNA replication, repair, and cell cycle pathways (119), multiple DNA metabolism events could recruit PCNA. Thus, PCNA foci may not always represent sites of active replication (134) and distinct pools of PCNA may be recruited into separate "repair foci" (42). In support of this idea, recent work indicates that monoubiquitinated PCNA has a longer residence time in foci and may persist at sites of DNA damage until after replication [(42); A. Lehmann, personal communication].

Third, GFP fluorescence can only detect complexes containing at least ~40 molecules. Sites where TLS polymerases function in only one molecule or in a small complex would not be visible by this method. In principle, association into foci may represent an inactive mode of sequestration rather than an active "replication factory".

Lastly, and perhaps most importantly, colocalization by immunofluorescence does not necessarily indicate either an actual physical interaction or a functional interaction. Although monitoring by immunofluorescence for the phosphorylation of the histone variant γH2AX has long been used as a marker for DSBs *in vivo*, it has been shown by the higher resolution approach of chromatin immunoprecipitation (ChIP) that γH2AX is actually found several kilobases away from a DSB and that colocalization of γH2AX with various DSB repair proteins does not represent an actual physical or direct functional interaction (179). Colocalization is correlative, but requires further studies to define causality. Nevertheless, recruitment of DNA damage tolerance proteins to sites of DNA damage, monitored by subcellular localization, has emerged as a major paradigm for regulation of their activity. Similar studies in prokaryotes are underway and will provide important information regarding the regulation of translesion synthesis across all organisms.

1.3.3.4 Protein-protein interactions

Translesion polymerase activity is also regulated by protein-protein interactions. All Y family polymerases interact with the sliding processivity clamp, β in prokaryotes and PCNA in eukaryotes, either through specific sequences known as β-binding motifs or PIP-boxes (11, 38, 61-63, 66, 194) or, in the case of Rev1, through a PIP-box-independent mechanism (58). Although it has not been demonstrated to directly interact with PCNA, the translesion synthesis activity of polymerase ζ is stimulated by PCNA (51). Disruption of the interaction with β or PCNA generally reduces or abolishes TLS polymerase lesion bypass *in vitro* (66, 110, 164, 193, 194) and function *in vivo* (11, 14, 58, 63, 110, 193). Notably, the PCNA-interaction region of Rev1 was recently mapped to its BRCT domain (58). In contrast to other TLS polymerases, mutating the PCNA-binding region of Rev1 does not affect its catalytic activity *in vitro* (139), although interestingly it does abolish Rev1 function *in vivo* (54, 109). In eukaryotes, all Y family polymerases contain novel ubiquitin interaction motifs known as UBMs or UBZs which

promote interaction with PCNA upon its monoubiquitination after DNA damage (17). Besides PCNA, eukaryotes also have another sliding clamp known as 9-1-1 that binds ssDNA generated after replication fork stalling and recruits checkpoint factors, DNA repair, and damage tolerance factors (85, 154). The one non-Y family TLS polymerase, DNA polymerase ζ, does not contain a UBM or UBZ motif, but rather interacts with the alternative 9-1-1 clamp through its Rev7 subunit (172). The 9-1-1 complex has also been shown to interact with DNA polymerase κ in *S. pombe* (84). Interestingly, strains bearing deletions of the alternative clamp or the large subunit of the alternative clamp loader display reduced levels of mutagenesis (156). Thus, interactions with sliding clamps contribute to localization TLS polymerases to sites of DNA damage, particularly at stalled replication forks, as well as stimulating their catalytic activities.

Additionally, in eukaryotes the TLS polymerases interact with each other. DNA polymerase η interacts directly with polymerase ι to bring it to sites of DNA damage (87). Rev1 interacts with all of the TLS polymerases and may serve as an organization center for lesion bypass (57, 135, 147, 187, 188). As certain polymerases are specialized for particular lesions, whereas others seem to extend mismatches preferentially, interactions between TLS polymerases may allow coordination of bypass in a two-step manner (165). Finally, each TLS polymerase has a set of unique interaction partners that may serve to provide specificity to their functions. For example, in prokaryotes, UmuD′ interacts with UmuC to stimulate its function (167) whereas UmuD′ interaction with DinB appears to inhibit its activity (V. Godoy, D. Jarosz, S. Simon, unpublished data). In eukaryotes, DNA polymerase η interacts with the Rad51 recombinase to perform its unique role in D-loop extension during homologous recombination (91, 128).

1.3.3.5 Kinetic regulation

Finally, biochemical characterization of the kinetic parameters of TLS polymerases has revealed yet another level of regulation of their activity. For both polymerase η and the archaeal, prokaryotic, and eukaryotic homologs of DinB, incorporation of a nucleotide opposite a cognate DNA lesion occurs more efficiently and accurately than opposite undamaged DNA (80, 81). Thus, certain TLS polymerases exhibit higher activity opposite DNA lesions that they are specialized to bypass, providing an elegant explanation for how a particular TLS polymerase may be selected to bypass a given lesion. These observations were extended in another set of

experiments designed to address the molecular mechanism conferring increased efficiency of bypass opposite a cognate lesion. For DNA polymerase η, the efficiency and fidelity of lesion bypass depends on its enhanced processivity on DNA containing its cognate lesion, a *cis-syn* TT dimer, relative to an undamaged DNA template (124). Moreover, polymerase η displays enhanced binding to a lesion-bearing template relative to a replicative polymerase, and this stabilization is dependent on the correct nucleotide, an A, being incorporated opposite to a *cis-syn* TT dimer (98). A few nucleotides past the lesion, polymerase η no longer exhibits enhanced binding to the primer/template and dissociates, allowing replicative polymerases to regain access to undamaged DNA (98, 122). Similar data was also obtained with prokaryotic DinB (78). As the exonuclease activity of replicative polymerases is able to remove mismatched bases, the ability of TLS polymerases to extend synthesis a sufficient distance past the lesion is critical (49). Taken together, these data strongly argue for a paradigm of a passive switch between TLS and replicative polymerases based on enzymatic efficiency.

1.3.4 Polymerase switching

The various post-translational regulatory strategies detailed above have been integrated into a model for regulation of translesion synthesis activity known as the polymerase-switching model (45, 123, 162). Briefly, it is thought that when a replicative DNA polymerase stalls at a DNA lesion, a region of ssDNA is generated by the uncoupling of the leading and lagging strand polymerases. Aberrant DNA structures and/or the architecture of the stalled polymerase holoenzyme recruit various factors, including DNA damage checkpoint proteins, replication restart proteins, DNA repair proteins, and DNA damage tolerance proteins. These factors promote multiple strategies of resuming replication. For clarity, only those pathways relevant to translesion synthesis will be discussed. The prevailing model states that access of TLS polymerases to the DNA is governed by protein-protein interactions which mediate a polymerase handoff of the primer-template terminus from the replicative polymerase to one or more translesion DNA polymerases. TLS polymerases are able to bypass the lesion and extend past the distorting mismatch. A further switch restores the replicative DNA polymerase to the primer terminus and accurate DNA synthesis resumes. Further details about the polymerase switching model are described below.

1.3.4.1 Recruitment of TLS polymerases to stalled replication forks

In prokaryotes, little is known about the mechanism of TLS polymerase recruitment. It is thought, based on visualization of certain components of the replication machinery, that the cell assembles all of the necessary factors to carry out DNA synthesis into replication factories visualizable as discrete foci (108). At the replication fork, the homodimeric β clamp appears to play a pivotal role as a "toolbelt" to bind to multiple polymerases simultaneously in order to switch the appropriate polymerase onto the primer terminus when needed (11, 78). In support of this hypothesis, it has been shown that the T7 replicative polymerase exhibits a dynamic processivity on the sub-second timescale, allowing multiple molecules of the replicative DNA polymerase to exchange with the sliding clamp without affecting processivity (210). The "toolbelt" model is attractive and is supported by the importance of the β-binding motif for TLS polymerase function, but is limited by the fact that only two polymerases could bind the β clamp at a time, while there are five DNA polymerase families in *E. coli*. Moreover, the presence of replication factories has been shown only in certain bacteria and localization studies with TLS polymerases have not been reported in any bacterial system to date. Thus, how bacterial TLS polymerases arrive at a stalled replication fork is currently not well understood.

In eukaryotes, the Rad6/Rad18 heterodimer plays a crucial role in recruiting TLS polymerases. Rad18 can bind to the ssDNA generated at a blocked replication fork bringing the E2 ubiquitin ligase Rad6 to sites of DNA damage where it can ubiquitinate targets to promote DNA damage tolerance (5, 6). Once at a stalled replication fork, Rad6/Rad18 monoubiquitinates PCNA (71), which is thought to have a negative effect on the replicative DNA polymerase *in vivo*, possibly by disengaging it from the DNA template (185). Additionally, this modification plays a role in recruiting TLS polymerases through an interaction between the monoubiquitin moiety and the recently characterized ubiquitin-binding motifs found in all Y-family polymerases (17). Eukaryotes also contain at least one other sliding processivity clamp, the alternative clamp known as 9-1-1 since it is composed of the Rad9, Rad1, and Hus1 genes in *S. pombe* (Rad17, Mec3, Ddc1 in *S. cerevisiae*). The alternative clamp is loaded onto regions of ssDNA by an alternative clamp loader, composed of Rad24 and Rfc2-5 in *S. cerevisiae*, and participates in a DNA damage checkpoint by facilitating ATR phosphorylation of Chk1 (85,

154). Additionally, the alternative clamp interacts with several DNA repair and tolerance proteins, including the Rev7 subunit of DNA polymerase ζ (172) to localize them to sites of DNA damage. Thus, sliding clamps play a key role in recruiting TLS DNA polymerases to stalled forks and/or DNA lesions in both prokaryotes and eukaryotes.

1.3.4.2 Replicative to TLS polymerase handoff and lesion bypass

Once present at the stalled replication fork, TLS polymerases require access to the primer terminus. Regulation of DNA binding is a crucial factor in controlling TLS activity and involves both extrinsic protein-protein interactions and intrinsic enzymatic properties. Beyond the protein-protein interactions described above in section 1.3.4.1, it is currently unclear what factors may actively contribute to a polymerase handoff between the replicative and TLS polymerases. One suggestion envisions that, in eukaryotes, Rev1 modulates the handoff through its ability to bind multiple TLS polymerases (57, 65, 188). However, data supporting the mechanism by which Rev1 may promote the polymerase handoffs is currently lacking. The involvement of Rev1 in the polymerase handoff is discussed further in Chapter Three. At the present time, it is believed that the major determinants of the handoff are passive contributions mediated by the relative enzymatic efficiencies of each polymerase opposite various DNA substrates (see section 1.3.3.5). Briefly, this passive component may be promoted by the dissociation of the replicative polymerase from the lesion-bearing DNA after abortive polymerization attempts. Following replicative polymerase dissociation, transient association of multiple polymerases may occur sequentially until the best suited polymerase is able to perform lesion bypass. Ultimately, the differential primer/template affinity, processivity, and bypass activities of translesion polymerases may be the primary mechanism by which a particular DNA polymerase gains access to the DNA (49, 78, 98, 122, 124).

A further question that remains unanswered at present is how the "correct" TLS polymerase is selected to bypass a particular DNA lesion. Although an active process may be involved, currently no evidence supporting such a mechanism exists. Specificity may be imparted by a passive trial and error approach based on the inherent efficiency of TLS polymerases opposite their cognate lesion, as detailed above in section 1.3.3.5. Such discrimination may be partially imposed by the little fingers domain of TLS polymerases (19,

122). Based on crystal structures of the archaeal TLS polymerase Dpo4, the little finger domain appears to interact with the lesion at the point when the polymerase has synthesized ~ 2 nucleotides past the lesion (122). This corresponds to the position relative to the lesion at which the enhanced processivity of DNA polymerase η is lost and replicative polymerases regain access to the primer terminus (98, 122, 124). Further supporting the role of the little finger domain in lesion specificity, chimeric constructs interchanging the little fingers domain between two archaeal TLS polymerases conferred the opposite lesion bypass characteristics onto the polymerase active site (19).

After insertion of a nucleotide opposite to a lesion, another TLS polymerase may be needed to extend from the mismatch or distorted base pair (164, 165). Thus, a second polymerase handoff may be required to complete bypass of the lesion. In eukaryotes, extension from a lesion is thought to be primarily mediated by polymerase ζ, and also to some extent by DNA polymerase κ (164, 165). A further consideration for completion of lesion bypass is that the length of the patch synthesized by TLS polymerases needs to be sufficient that the replicative polymerase will not be affected by the distortion produced by the lesion. In prokaryotes, this appears to be mediated by the stimulation of processivity observed when TLS polymerases interact with the β sliding clamp (49).

1.3.4.3 TLS to replicative polymerase handoff

Finally, the replicative DNA polymerase must be recruited back onto the DNA to allow completion of replication in an accurate and efficient manner. Little is known about how the handoff back to the replicative polymerase is mediated. In eukaryotes, deubiquitinating enzymes are likely involved in turning off the global cellular recruitment of TLS polymerases to stalled forks by reducing the amount of monoubiquitinated PCNA (77). Locally at a particular site of DNA damage, however, it is not known how the replicative polymerase regains access to the primer terminus, beyond the passive contributions of enzymatic efficiency (described in section 1.3.3.5.).

1.3.4.4 Limitations of polymerase switching model and emergence of new paradigms

The polymerase switching paradigm, by definition, implies an S-phase dependence (45, 106, 107). In this model, TLS polymerases gain a temporary access to the DNA during replication to restart stalled replication forks, such that replicative polymerases function both before and after the TLS polymerase bypass of a lesion. Although the replication-dependent function of TLS polymerases is implicit in the polymerase switching model, it has not been tested until recently. Instead, the S-phase function of TLS polymerases has mainly been established through multiple studies of DNA polymerase η, which, due to its role in human disease, is the most well studied TLS polymerase. Human cells lacking polymerase η proceed more slowly through S-phase than wild-type cells after DNA damage (1, 184). Additionally, extensive colocalization studies provide support to the idea that polymerase η functions mainly during S-phase (1, 86, 87, 188). However, it is important to note that each TLS polymerase has distinct properties, including both *in vitro* lesion bypass capabilities and *in vivo* phenotypic responses, suggesting that, although certain regulatory mechanisms are likely shared by all, each TLS polymerase is regulated and used inside the cell quite differently. Therefore, although polymerase η may function primarily according to the polymerase switching model, further investigation of the other TLS polymerases will provide new models for regulation of translesion synthesis outside of the polymerase switching paradigm. Already, reports have indicated that polymerase κ plays a central role in nucleotide excision repair (NER) (145), that polymerase η can extend D-loops during HR (91, 128), and that polymerase ζ is involved in break-induced replication (BIR) pathway of the HR mediated repair of DSBs (74, 166). Given the plethora of unexpected results surrounding the translesion synthesis field, further surprises are sure to come.

Despite the dominance of the polymerase switching paradigm, historical experiments have provided evidence that is inconsistent with certain assumptions of the model (107, 195). In particular, studies showing that replication is inhibited, but not completely halted, after DNA damage indicate that the cell has the ability to continue DNA synthesis at a low level despite the presence of replication-blocking DNA lesions (36, 105, 171). Recent experiments have confirmed these findings and extended other paradigms of damage tolerance. Replication in the face of DNA damage is likely to occur through repriming of the DNA polymerase downstream of a lesion, as has recently been demonstrated (69), and would leave behind ssDNA gaps

opposite lesions, which have recently been observed in yeast (118). In *E. coli* cells, the time required for translesion synthesis to bypass a lesion was observed to be on the order of ~50 minutes (153). The length of time necessary to bypass a lesion relative to the generation time of *E. coli* indicates that translesion synthesis must occur to a significant extent in parallel, rather than exclusively in series (R. Fuchs, personal communication). This suggests that, instead of TLS-mediated restart of each stalled replication fork in turn, replication can continue downstream of a lesion such that bypass of all lesions occurs simultaneously and concurrent with, or even after, replication. Additionally, the unexpected discovery of a cell-cycle dependent upregulation of Rev1 outside of S-phase supports a post-replicative function of translesion synthesis (200). The work presented in Chapter Two discusses a new model of TLS in which Rev1 is proposed to play a crucial recruitment role to facilitate gap filling during G2/M at sites of persistent DNA lesions.

In summary, the work presented in this thesis contributes to the emerging paradigm of two phases of DNA damage tolerance: one which occurs during replication to promote continuous replication past DNA lesions (Fig. 4A) and one which takes place post-replicatively to mediate filling of gaps left behind during replication (Fig. 4B).

Fig. 4

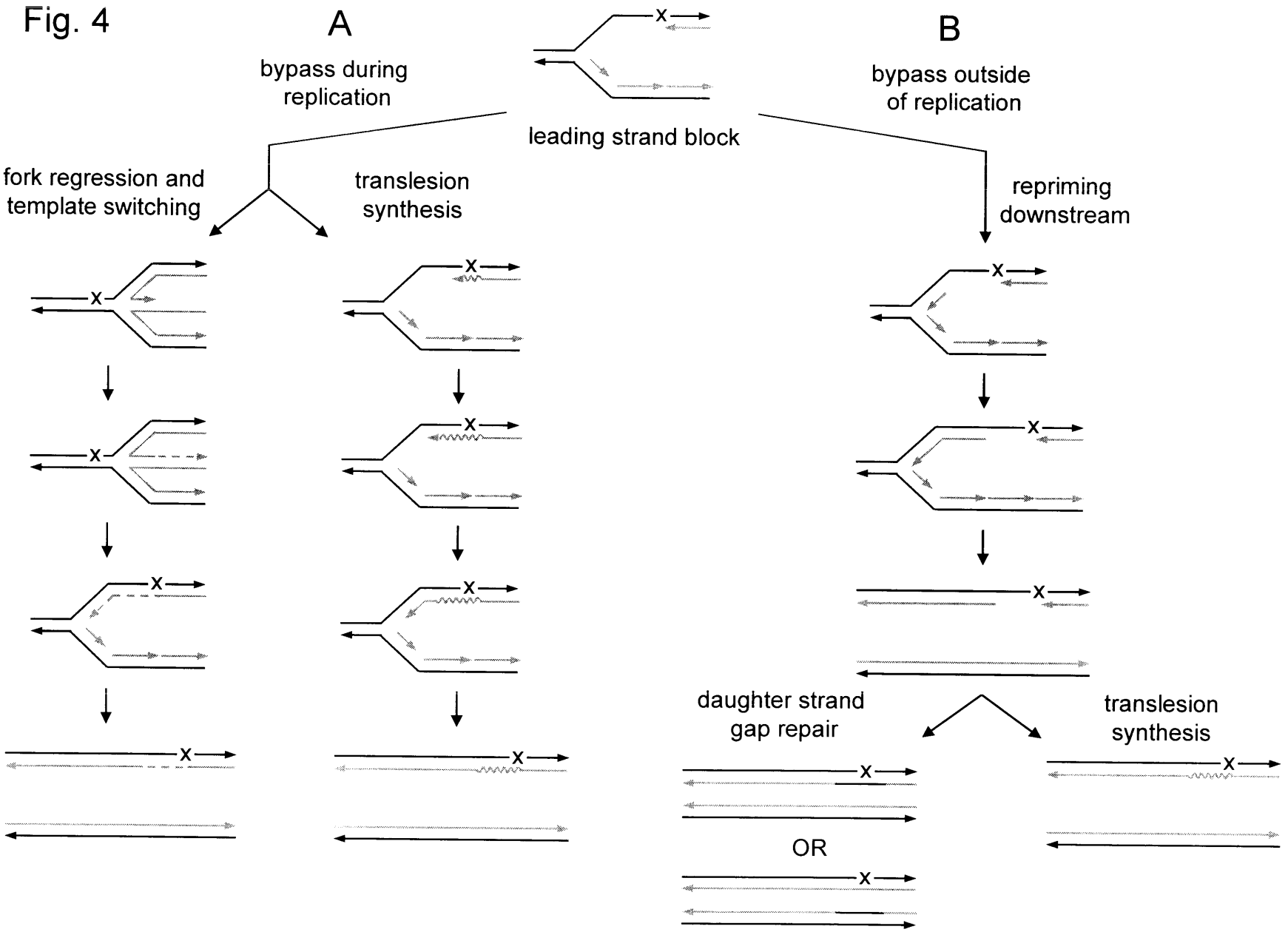
A
bypass during replication
B
bypass outside of replication
leading strand block
fork regression and template switching
translesion synthesis
repriming downstream
daughter strand gap repair
translesion synthesis
OR
X

Fig. 4 Two phases of DNA damage tolerance. Arrows indicate the 3′ end of the DNA strand, dashed lines indicate DNA synthesis, and wavy lines indicate regions replicated by TLS polymerases. Black lines indicate the parental DNA strand; grey lines indicate the daughter strand. (*A*) During replication, template switching or translesion synthesis can serve to resolve stalled replication forks and restart continuous DNA synthesis. For simplicity, alternate fork regression pathways are not shown. Bypass of the lesion by template switching would be accurate while bypass by translesion synthesis may result in mutations. (*B*) Repriming of the DNA polymerase downstream of DNA damage can generate regions of ssDNA opposite to lesions which may persist even after replication has been completed. These gaps can be filled by recombination-mediated daughter strand gap repair mechanisms in an accurate manner or by translesion synthesis which is associated with mutagenesis.

1.3.5 The eukaryotic RAD6 post-replication repair pathway

In eukaryotes, most DNA damage tolerance is mediated by the *RAD6* epistasis group (191). This set of genes was initially termed the post-replication repair (PRR) pathway, but this is a misnomer as they function to tolerate rather than repair damage and may do so during replication as well as afterwards (101). Originally, the *RAD6* pathway was defined genetically in yeast by epistasis analysis. Subsequently, additional genes have been added to the pathway based on phenotype, often without full characterization relative to other members of the epistasis group (8, 60, 76, 101, 129, 156, 159). Thus, the relationships between genes in the *RAD6* PRR pathway is poorly understood and multiple genetic interaction maps have been proposed (27, 207). To further complicate matters, the genetic interactions between components of the *RAD6* pathway can vary when different DNA damaging agents are used (159), and when characterizing spontaneous and induced responses to DNA damage (130). With these caveats, a brief explanation of each gene of the *RAD6* pathway is presented below.

1.3.5.1 RAD6, RAD18, and POL30

Together with the ssDNA-binding protein Rad18, Rad6 stands at the top of the epistasis group comprising several DNA damage tolerance mechanisms (47). Rad6 is a ubiquitin E2 ligase which is involved in several cellular stress response pathways, including N-end rule degradation, retrotransposition, sporulation, silencing, and DNA damage tolerance (47, 101). Not unexpectedly for a gene that participates in so many diverse regulatory circuits, *rad6* mutants exhibit a severe growth defect and are profoundly sensitive to a variety of DNA damaging agents (47). Rad6 requires several different effector proteins to target its ubiquitination activity towards a particular pathway (47). In order to mediate its role in DNA damage tolerance, Rad6 interacts with the DNA damage specific effector Rad18 (5). Rad18 binds to single-stranded DNA which is generated upon replication fork stalling or from replication fork repriming after DNA damage. This allows localization of the Rad6 E2 ubiquitin ligase activity to sites of DNA damage (5, 6). Rad18 also interacts with and recruits polymerase η to stalled replication forks in mammalian cells, independent of its role in the monoubiquitination of PCNA (199). Rad18 itself exhibits Rad6-dependent mono- and

polyubiquitination (131). The variously modified forms of Rad18 show differential subcellular localization, allowing regulation of the DNA damage tolerance pathways by a feedback loop controlling Rad6 and Rad18 association (131).

Although it seems likely that this elegant mechanism of coupling an ssDNA-binding protein with a post-translational regulatory protein may have many targets, currently only one downstream protein is known to be ubiquitinated in response to DNA damage by the Rad6/Rad18 heterodimer: the sliding clamp, PCNA, encoded by the *POL30* gene (71). PCNA is post-translationally modified on lysine 164 in order to mediate the majority of PCNA's regulatory effect towards DNA damage tolerance (64, 71, 185). Monoubiquitinated PCNA activates all of the downstream damage tolerance pathways and is thought to comprise the main function of the Rad6/Rad18 heterodimer (191). Given that the Y-family polymerases were recently shown to be monoubiquitinated (17, 59), it is an attractive hypothesis that Rad6/Rad18 activity has other targets as well.

As the ubiquitination of PCNA is a major mechanism (191), though not the sole means (29), for the regulation of the several PRR subpathways, it follows that the deubiquitination of PCNA would be of primary importance to maintain low background levels and to terminate the activity of DNA damage tolerance responses. Currently, little is known about how PCNA is deubiquitinated, but evidence is emerging that specific factors, themselves subject to extensive regulation, remove ubiquitin from PCNA (77) or promote the accumulation of unmodified PCNA (183). Ongoing research is dedicated towards resolving the crucial question of how the DNA damage tolerance response is reset after completion of lesion bypass.

1.3.5.2 Template switching: MMS2, UBC13, RAD5, and SRS2

One subpathway of the *RAD6* epistasis group is the template switching branch, mediated by Rad5 and the Mms2/Ubc13 heterodimer (47, 191, 192). This set of genes promotes error-free damage tolerance by replication fork regression (discussed above in section 1.3.1) (47, 191). Rad5 is a member of the Swi2/Snf2 helicase family, but lacks detectable helicase activity (82), likely because a RING finger domain is inserted into the helicase domain. In an independent function from its role as an E3 ubiquitin ligase in template switching, Rad5 also has ssDNA-dependent ATPase activity which is required for DSB repair (31). Mms2 is an E2 ubiquitin

conjugating enzyme of the UEV subtype that lack the catalytic cysteine needed to transfer ubiquitin (73). Mms2 requires the E2 ubiquitin ligase Ubc13 for activity (73). The Mms2/Ubc13 heterodimer specifically produces ubiquitin chains linked through the non-canonical lysine 63 residue which does not cause degradation of the target protein by the 26S proteasome (72, 73).

The major target of Mms2/Ubc13 is thought to be the sliding clamp PCNA (71). The Rad6/Rad18 modification of PCNA is confined to monoubiquitination, however association of Rad18 with Rad5 brings the Rad6/Rad18 heterodimer in contact with the Mms2/Ubc13 heterodimer which can then polyubiquitinate PCNA via the lysine 63 linkage (71, 192). It seems likely that the presence of K63 polyubiquitinated PCNA at the replication fork is refractory to further polymerization reactions, not only displacing the replicative polymerase but preventing TLS polymerases from accessing the replication fork as well. However, the molecular mechanisms by which K63 polyubiquitinated PCNA promotes replication fork regression and the details by which the physical DNA transactions of the exchange occur are unknown at this time.

Additionally, PCNA can be modified by the small ubiquitin-like modifier SUMO at lysine 127 or lysine 164 (71). SUMO modification of PCNA, while using the same lysine 164 residue as ubiquitination, appears to cooperate with rather than antagonize the roles of ubiquitination in DNA damage tolerance (191, 201). SUMOylated PCNA recruits the Srs2 helicase which promotes the Rad5/Mms2/Ubc13 template switching pathway by disrupting Rad51 filament formation on DNA; in the absence of *SRS2*, defects in the *RAD6* pathway are suppressed by channeling substrates into the *RAD52* HR pathway (191, 201).

1.3.5.3 Translesion synthesis

The other genes which fall into the *RAD6* epistasis group are not involved with the K63-linked polyubiquitination mediated template switching. Rather, they seem to either directly mediate translesion synthesis (*RAD30*, *REV1*, *REV3*, *REV7*, *POL32*) or to indirectly affect TLS (*CDC7*, *CDC8*, *UMP1*, *RAD24*, *RAD17*, *MEC3*, *DDC1*), presumably by regulating the TLS polymerases. The TLS branch of the *RAD6* epistasis group is well understood biochemically, but poorly understood genetically. Few of the genes that indirectly regulate TLS have been

placed into pathways or thoroughly phenotypically characterized and there are likely several more genes controlling TLS polymerase activity that have yet to be identified. For example, several genes were identified in screens for alterations in DNA damage tolerance that have never been physically mapped to a particular locus or open reading frame (*MMS3*, *NGM2*, *UMR1*, *UMR2*, and *UMR3*) (101). In yeast, the TLS polymerases themselves appear to form two distinct branches: one containing *RAD30* and the other consisting of *REV1*, *REV3*, *REV7*, and likely *POL32* (75, 126). However, the majority of the genetic analyses of the interactions among TLS polymerases have been performed in *S. cerevisiae* which lacks DNA polymerase κ and polymerase ι, two of the five major Y family polymerase families (148). Polymerase κ was lost in a group of ascomycete yeast and is also missing in some eukaryotic lineages, but is otherwise found in organisms from archaea to humans (L. Waters, unpublished observation). Many eukaryotes also contain polymerase ι, a paralog of polymerase η, not found in *S. cerevisiae*. Additionally, in yeast, *RAD30*, which encodes polymerase η, appears to function independently from *REV1*, *REV3*, and *REV7* (126, 207), whereas in vertebrates, DNA polymerase η physically interacts with Rev1 (57, 188). Thus, the genetic interactions between TLS polymerases are not yet clear, although the prospects of clean genetic analyses in higher eukaryotes are promising in chicken DT40 cells using gene deletions to avoid potential artifacts caused by RNAi knock-downs (208).

The process of translesion synthesis seems to exclusively use the monoubiquitinated form of PCNA (64, 185). Monoubiquitination of PCNA may activate the catalytic activity of certain TLS polymerases, such as polymerase η and Rev1 to increase bypass of lesions (50), however other evidence contradicts this point (67). Additionally, the eukaryotic Y-family polymerases each have a higher affinity for the monoubiquitinated form of PCNA than the unmodified form (15, 17, 58, 89, 199). Thus, it is thought that, in addition to potential catalytic stimulation, monoubiquitinated PCNA activates TLS polymerases by recruiting them to stalled replication forks where they are needed (45, 89, 199). Indeed, the monoubiquitination of PCNA is currently thought to be the central switch mediating the polymerase handoff between replicative and TLS polymerases at stalled replication forks (45, 89, 199). The evidence for the importance of PCNA modification is strong, but it should be kept in mind that there are likely a number of other factors controlling not only the polymerase handoff but also TLS polymerase activity. Furthermore, the functions of DNA polymerase ζ and Rev1 have been found to be partially

independent of monoubiquitinated or SUMOylated PCNA after ionizing radiation (29). Thus, TLS may well not require modified PCNA under other circumstances as well.

1.3.6 Translesion polymerases

Translesion DNA polymerases are structurally optimized to bypass DNA lesions and bear a significant similarity to each other, yet are also specialized for particular classes of DNA lesions and exhibit a range of phenotypes *in vivo*. Thus, it is not surprising that most TLS polymerases are members of a superfamily of DNA polymerases known as the Y family, falling broadly into four different clusters with specific features within the Y family (148). For example, although all TLS polymerases have a low replication fidelity on undamaged DNA, only some are responsible for the majority of spontaneous and induced mutagenesis *in vivo*. Loss of function or overexpression phenotypes for DNA polymerases η, ι, and κ display modest or very specific alterations of cellular mutagenesis (10, 33, 92, 93, 95, 126, 157, 205, 209); in contrast, when mutated, UmuC, Rev1, and DNA polymerase ζ show profound defects in mutagenesis for a wide variety of DNA damaging agents and on a range of assay systems (47, 90, 102). Other major properties of each class of TLS polymerase are summarized below.

1.3.6.1 UmuC

UmuC is found ubiquitously throughout all prokaryotes and encodes a broad-specificity-range polymerase that preferentially bypasses UV photoproducts, abasic sites, and certain bulky adducts (47, 56). *In vivo*, it is responsible for much of spontaneous and damage-induced mutagenesis, as seen by the fact that loss of function alleles exhibit an *unmutable* phenotype (47, 90). In *E. coli*, UmuC is found in an operon with its accessory factor UmuD (47). The *umuDC* operon is under the control of the SOS response and is transcriptionally upregulated after DNA damage (47). Additionally, post-translational regulation plays a major role in UmuC function. Upon binding to the RecA-ssDNA nucleoprotein filament generated after DNA damage, the UmuD protein undergoes an autocleavage event which removes the N-terminal 24 amino acids to produce a shorter UmuD′ protein. These two products of the *umuD* gene perform different functions in the DNA damage response. The shorter UmuD′ stimulates translesion synthesis by

UmuC (167), whereas the full-length UmuD protein effects a primitive DNA damage checkpoint by inhibiting the resumption of DNA synthesis after DNA damage by the replicative polymerase III (151). Temporal regulation of the appearance of the post-translationally processed UmuD′ protein may provide a cellular mechanism to delay mutagenic translesion synthesis until more high-fidelity repair systems have had a chance to repair DNA lesions (151). UmuC was the fifth DNA polymerase identified in *E. coli*. Since UmuC exhibits virtually no activity in the absence of the UmuD′ dimer, the term polymerase V typically refers to the UmuD′$_2$C complex (47).

1.3.6.2 DinB (polymerase κ)

DinB is the most highly represented TLS polymerase, being found throughout eukaryotes, prokaryotes, and archaea (148). In eukaryotes, DinB is known as polymerase κ. Polymerase κ was lost from the ascomycete lineage that gave rise to *S. cerevisiae*, so a detailed genetic understanding of its activity is missing in eukaryotes. Additionally, polymerase κ appears to be missing from Drosophila and other insects, as well as protists (A. Hardin, L. Waters, unpublished observation). In contrast to UmuC, loss of *E. coli* DinB does not seem to affect mutagenesis profoundly, indicating that *in vivo* under normal regulation, DinB does not contribute to mutagenesis (95, 205). However, when misregulated by overexpression, DinB does promote -1 frameshift mutations, but this effect is sequence-specific and only modestly affects base substitutions (92). In mammalian cells, loss of polymerase κ increases mutagenesis induced by benzo[*a*]pyrene, indicating the polymerase κ bypasses benzo[*a*]pyrene N^2-dG adducts relatively accurately *in vivo* (3). Similarly to DNA polymerase η, DNA polymerase κ and its archaeal and prokaryotic homologs have been proposed to operate with high accuracy and efficiency opposite its cognate lesion of N^2-adducted dG residues (80). Additionally, polymerase κ appears to be specialized to extend mismatched primer termini and likely functions as a second "extender" polymerase when two TLS polymerases are required in concert to bypass a lesion (164). Cell biological studies have revealed that polymerase κ relocalizes from a diffuse nuclear pattern into foci upon DNA damage (13, 16, 143). Focus formation of polymerase κ requires both its PCNA-interaction motif and its ubiquitin-binding motifs (143). Interestingly, polymerase κ relocalizes in response to DNA damage differently from the other Y family

members, forming fewer spontaneous and damage-induced foci (16, 143). The reports disagree, however, on whether DNA polymerase κ forms foci during S-phase (16, 143). Also distinct from the other TLS polymerases, DNA polymerase κ also appears to contribute substantially to NER (145).

1.3.6.3 Rad30A (polymerase η) and Rad30B (polymerase ι)

The Rad30 subfamily is found only in eukaryotes and contains two paralogs: Rad30A, which encodes DNA polymerase η, and Rad30B, which encodes the highly related polymerase ι. Polymerase η is perhaps the most thoroughly characterized TLS polymerase since, in humans, loss of polymerase η activity results in a cancer-prone syndrome known as Xeroderma Pigmentosum Variant (XPV) (106, 120). *In vitro*, polymerase η has been shown to bypass *cis-syn* cyclobutane pyrimidine dimers (CPD) with high accuracy and efficiency (81) and *in vivo*, it is thought to be responsible for restarting stalled replication forks and allowing continuous DNA synthesis past sites of UV damage (106). In the absence of polymerase η, other DNA polymerases may substitute to bypass *cis-syn* TT dimers in a more error-prone manner, resulting in an increased frequency of cancer in XPV patients (106, 120). Interestingly, though polymerase η plays a major role in accurately bypassing one particular type of DNA lesion, on undamaged DNA it exhibits among the lowest fidelity of any DNA polymerase (121, 164). Yet, polymerase η mutants display no major reduction in spontaneous or induced mutagenesis with agents other than UV (126, 170). Intriguingly, overexpression also does not produce a profound alteration of levels of mutagenesis (93, 157), indicating that polymerase η is restricted from accessing undamaged DNA even when overexpressed. Certain polymerase η mutants which confer XPV are capable of translesion synthesis activity *in vitro*, implying that key regulatory motifs allowing polymerase η to be recruited to sites of DNA damage are compromised in these patients (21). These findings emphasize the importance of regulation of polymerase η to its function. The subcellular localization of polymerase η has been investigated in multiple reports. Polymerase η forms foci spontaneously in a small percentage of cells and focus formation is increased dramatically after exposure to many DNA damaging agents (1, 16, 86, 87). These foci are thought to form at sites of DNA damage since they colocalize with PCNA (1, 86, 87).

Additionally, polymerase η foci formation is stimulated by interaction with monoubiquitinated PCNA (15, 17, 89, 161, 199). Together with the fact that polymerase η mutants progress more slowly through S-phase after DNA damage (1, 16, 184), these findings linking polymerase η and PCNA have led to a model in which polymerase η rescues replication forks that have stalled at sites of DNA damage by bypassing the blocking lesion allowing the resumption of continuous DNA synthesis.

In contrast to the wealth of information about polymerase η, the role of its paralog, polymerase ι, is poorly understood. Although stated to be found only in higher eukaryotes, polymerase ι has been identified in scattered organisms throughout Eukaryota, including some yeasts (L. Waters, unpublished observation). However, since polymerase ι is lacking in *S. cerevisiae*, little is known about its genetic relationships to other DNA damage tolerance pathways. Biochemically, polymerase ι is one of the least accurate DNA polymerases, especially opposite pyrimidines (164). However, physiologically, loss of polymerase ι seems to have little consequence, as the 129/J strain of mice bears a nonsense mutation in the polymerase ι gene that prevents its expression, yet these mice have no observable phenotype (125). Additionally, alteration in the levels of polymerase ι does not lead to profound changes in the frequency of mutagenesis (33, 209). Polymerase ι interacts physically with polymerase η and this interaction is required for its localization into DNA damage induced foci (87). Additionally, the PCNA-interaction motif and ubiquitin binding motifs of polymerase ι are also required for localization into foci after DNA damage, indicating that recruitment to stalled replication forks by monoubiquitinated PCNA mediates the function of polymerase ι (17, 193). However, it should be noted that the biological relevance of the interaction with monoubiquitinated PCNA has not yet been tested for polymerase ι. Future work revealing the cognate lesion for polymerase ι *in vivo* will provide insight into the role of this less characterized TLS polymerase.

1.3.6.4 Rev1

Uniquely among Y family polymerases in eukaryotes, Rev1 actively promotes the generation of mutations from unicellular yeast to multicellular humans (54, 109). Like prokaryotic UmuC, *rev1* mutants display a drastic reduction in spontaneous and induced

mutagenesis by a wide variety of DNA damaging agents (47, 102). In fact, Rev1 was isolated from the first screen in any organism to identify genes specifically responsible for mutagenesis (109). Rev1 was named for its *reversionless* phenotype, reflecting the inability of the *rev1* mutant strain to revert an auxotropic marker to the wild-type allele after UV irradiation (109). In almost every genetic background tested, mutants of *REV1* abolish mutagenesis, indicating its fundamental importance to mutagenesis induced by all types of DNA lesions and genetic alterations (102). Indeed, Rev1 is responsible for ~95% of all mutagenesis (103). Although only marginally correlated with the onset of cancer (173), *REV1* in higher eukaryotes may contribute to cancer progression. Recently, *REV1* was shown to modulate the frequencies with which cisplatin resistant cells were generated from an ovarian carcinoma cell line: reduced *REV1* expression decreased the frequency of cisplatin resistance and correspondingly, increased *REV1* levels led to a higher frequency of cisplatin resistant cells (112, 150). Thus, understanding the regulation of Rev1 activity is crucial to a complete knowledge of mutagenesis in eukaryotes. Studies addressing the regulation of Rev1 are discussed further in Chapter Two.

Rev1 exhibits distinctive properties from the other Y family members. In contrast to other translesion polymerases, Rev1 has only a limited polymerase activity restricted to inserting primarily dCMP residues opposite template G's and certain DNA lesions (102, 140). Biochemically, Rev1 appears particularly suited to bypass abasic sites and adducted G residues (102, 197). To accomplish this specificity, Rev1 uses a novel catalytic mechanism that selects dCTP as the incoming nucleotide not through base pairing with the template, but rather through hydrogen bonds with a conserved arginine (137). Contacts are made with the template base to ensure its identity as a G, but the template base is flipped out of the active site, thus explaining Rev1's ability to bypass large bulky adducts on G residues (137). Rev1 was the first member of the Y superfamily to be shown to have polymerase activity (140), however since the activity was limited, the significance of this finding was not appreciated for several years until the discovery that other Y family members also were capable of polymerase activity. Intriguingly, a catalytically inactive mutant of Rev1 displays no reduction in levels of mutagenesis (65, 169), although a change in the mutation spectrum is observed (152, 168). Therefore, although Rev1's unique and highly specialized dCMP transferase catalytic activity is conserved from yeast to humans (111, 140), its activity does not seem to be required for *REV1* function from yeast to higher eukaryotes. Consistent with the importance of *REV1* to lesion bypass independent of its

catalytic activity, the presence of *REV1* is required for bypass of a 6-4 TT dimer *in vivo* even though *in vitro* Rev1 is unable to insert a nucleotide opposite to UV photoproducts (139, 152, 215).

Additionally, Rev1 contains an N-terminal BRCT domain not found in any other Y family polymerase. First characterized in the BRCA1 breast cancer susceptibility protein, BRCT (*BR*CA1 *C-t*erminus) domains are found predominantly in DNA metabolism genes where they mediate protein-protein or protein-DNA interactions (25, 55, 94, 202). Tandem BRCT domains have been shown to interact preferentially with phosphorylated targets, which are often components of the DNA damage checkpoint response (55). Rev1 only has a single BRCT, but nonetheless has also been implicated in phosphopeptide binding, however the biological significance of this is currently unknown (214). Unlike the other Y family polymerases, Rev1 does not have a PCNA-interacting motif. However, the BRCT domain interacts directly with PCNA (58), as well as with the Rev7 subunit of DNA polymerase ζ (40) and possibly also with DNA (94). As BRCT domains tend to interact with DNA at single-stranded regions or double strand breaks (94, 202), the BRCT of Rev1 may also be involved in localizing Rev1 to aberrant DNA structures. Interestingly, in contrast to the catalytic dead mutation, mutations affecting the BRCT domain inactivate Rev1 *in vivo*. In yeast, BRCT mutants exhibit a severe defect in survival and mutagenesis after DNA damage (109). In higher eukaryotes, mutations in the BRCT domain reduce *REV1* function, however the extent of the defect varies between studies (58, 79, 169). Thus, although the mechanism is not yet clear, the BRCT domain evidently contributes to *REV1* function *in vivo* by mediating interactions with PCNA, Rev7, and likely DNA and other proteins.

In addition to the BRCT domain, Rev1 also contains other protein-protein interaction modules which are critical for *REV1*-mediated mutagenesis and resistance to DNA damaging agents. The extreme C-terminal ~100 amino acids of mammalian Rev1 interact with all of the other TLS polymerases and also DNA polymerase λ (57, 135, 147, 188). Additionally, the C-terminus of Drosophila Rev1 was used in an affinity column as a purification for DNA polymerase ζ (187). Initially, the C-terminus of Rev1 did not seem to be conserved between higher eukaryotes and yeast (88, 135, 188), however extensive sequence alignment and functional studies have revealed that yeast Rev1 does likely interact with other TLS polymerases

through its C-terminus (40, 169). For further discussion of the role of the C-terminus in yeast, see Chapter Three.

Finally, Rev1 displays ubiquitin binding mediated by a non-canonical ubiquitin-binding motif (UBM) and this interaction with ubiquitin is necessary for its localization into DNA damage induced foci (59). Mutants in the UBM display increased chromosomal aberrations, decreased viability, and decreased mutagenesis after exposure to DNA damaging agents [(59); L. Waters, unpublished data, see Chapter Three].

As described above, multiple protein-protein interaction domains are critical to *REV1* function whereas the catalytic activity of Rev1 is dispensable for *REV1* function under most circumstances (65, 169). Taken together, these findings have led to a model in which Rev1 functions primarily as a scaffold for various post-replication repair proteins to localize mutagenic translesion complexes to sites of DNA damage and/or to modulate polymerase switching at the site of a DNA lesion (45, 106). Thus, Rev1 is thought to play a central role in translesion synthesis by regulating access of TLS polymerases to the primer terminus (45, 106).

Localization studies have reported that, like the other Y family polymerases, Rev1 forms foci after DNA damage (58, 133, 134, 188). However, in one report Rev1 foci were not observed either spontaneously or after DNA damage (169). The authors used a more physiological expression level and propose that lack of foci formation reflects the need of the cell for only one or a small number of molecules of Rev1 at sites of stalled replication (169). Colocalization of Rev1 foci with PCNA and polymerase η have indicated that Rev1 associates with replication forks to enable continuous DNA synthesis on templates containing DNA lesions (58, 133, 188). In conjunction with the implicit assumptions in the polymerase switching model, the localization studies have led to a model in which Rev1 is thought to act mainly during replication (45, 106). However, other studies have proposed that Rev1 functions predominantly outside of S-phase (134, 200). For further discussion of the timing of Rev1 function, see Chapter Two.

1.3.6.5 Rev3/Rev7 (polymerase ζ)

One non-Y family DNA polymerase is also considered a TLS polymerase: DNA polymerase ζ, which is a heterodimer of the Rev3 catalytic subunit and the Rev7 accessory

subunit (141). Interestingly, Rev3 is a member of the B family of normally highly accurate replicative DNA polymerases, including DNA polymerases δ, ε, and α (102, 132). In contrast to the other B family replicative polymerases, DNA polymerase ζ lacks 3′ to 5′ exonuclease activity (102). Although it can bypass certain lesions like a *cis-syn* TT dimer, polymerase ζ appears to be specialized to extend distorted base pairs, such as mismatches that might result from inaccurate base insertion by a TLS polymerase or an accurate base pair involving a bulky DNA lesion (102, 164). Despite no conserved PCNA interaction motifs, polymerase ζ exhibits increased lesion bypass activity in the presence of PCNA, however this stimulation is not observed with either monoubiquitinated PCNA or the alternative 9-1-1 processivity clamp (50, 51).

Not unexpectedly given its proficiency for extending mismatches, polymerase ζ contributes significantly to mutagenesis (102). In fact, *REV3* was isolated together with *REV1* from a screen for *reversionless* mutants of yeast (109) and *REV7* by a similar screen a few years later (104). Like *rev1*, *rev3* and *rev7* mutants are severely defective for spontaneous mutagenesis, as well as mutagenesis induced by a wide variety of DNA damaging agents and mutations in DNA repair and tolerance pathways (102). *REV1*, *REV3*, and *REV7* are considered to be in the same branch of the *RAD6* epistasis group based on phenotypic similarity and limited epistasis analysis (70, 102). Like *REV1*, DNA polymerase ζ also actively promotes mutagenesis from yeast to humans (32, 53, 109) and promotes cisplatin resistance in human cancer cells (113). Together, Rev1 and DNA polymerase ζ are thought to mediate the vast majority of the mutagenic DNA damage tolerance *in vivo* and, as such, are central to understanding diseases such as cancer.

Although a very large protein, Rev3 does not seem to contain any known protein-protein interaction modules or other regulatory motifs. Instead, its accessory factor may control its regulation since Rev7 contains a HORMA domain known to interact with chromatin (2). In yeast, Rev7 binds to the 9-1-1 alternative DNA processivity clamp and may recruit DNA polymerase ζ to sites of DNA damage (172). Additionally, Rev7 interacts with Rev1 (57, 135, 147, 187, 188), which may also allow localization of DNA polymerase ζ to DNA lesions. Moreover, in higher eukaryotes Rev7 has been shown to interact with the specificity factors Cdh1 and/or Cdc20 of the anaphase promoting complex/cyclosome (APC/C) as well as the

spindle checkpoint protein Mad2 (30, 136, 160). Interaction with Rev7 inhibits the ubiquitin ligase activity of the APC and prevents the onset of mitotic anaphase (30, 160).

Multiple mechanisms collaborate to keep polymerase ζ levels low (102), indicating that overexpression may be detrimental to cells. Yet loss of polymerase ζ causes embryonic lethality in mice (12, 41, 203), indicating that during rapid proliferation, mammalian cells require a function of polymerase ζ. The inability to study *rev3* mutant cell lines in mammalian systems has hampered understanding of polymerase ζ function. However, recent studies in the chicken DT40 line have provided insight into the role of polymerase ζ *in vivo*, in particular, the contribution of *REV1*, *REV3*, and *REV7* to chromosomal rearrangements during recombination and interstrand crosslink repair (149, 177). Additionally in yeast, *REV3* contributes to mutagenesis in the break-induced replication (BIR) subpathway of homologous recombination (74, 166). Interestingly, *REV1* appears to function independently of *REV3/7* in the generation of sister chromatid exchanges during the recombinational bypass mode of damage tolerance (149).

1.3.6.6 All polymerases capable of translesion synthesis

It is worth noting that there are other non-replicative DNA polymerases which have varying abilities to bypass DNA lesions and which synthesize DNA with a range of fidelities (47). The members of the X family of DNA polymerases in particular can insert nucleotides opposite to certain lesions, mainly abasic sites or bulged templates (18), and, after the Y family, display the next lowest replication fidelity of the six major DNA polymerase families (96). The X family polymerases are occasionally referred to as translesion polymerases and, indeed, can lay a claim to the name. Even the highly stringent replicative DNA polymerases have very weak abilities to replicate over certain lesions. However, in general these other polymerases have other primary physiological functions, such as participation in BER and NHEJ by the X family polymerases. Accordingly, the term TLS polymerases generally refers to the Y superfamily and DNA polymerase ζ, which have clearly specialized roles limited almost exclusively to lesion bypass (47, 102).

1.3.7 Evolutionary significance of mutagenic polymerases

Why do cells contain potentially mutagenic translesion polymerases? All organisms—prokaryotes, archaea, and eukaryotes—contain translesion polymerases that have been shown to promote both spontaneous and damage-induced mutagenesis. Though potentially dangerous due to their low fidelity of replication on undamaged DNA, TLS polymerases must serve a valuable purpose or they would not have been maintained throughout evolution. Translesion polymerases presumably confer advantages that outweigh the risk of potentially lethal mutations.

Firstly, TLS polymerases provide a measure of resistance to DNA damaging agents; cells lacking one or more TLS polymerases exhibit a slight to moderate increase in the frequency of cell death after DNA damage (47). This increase in survival after DNA damage has classically been thought to reflect the ability to continue replication and cell division in the face of replication blocking lesions. However, recent evidence showing that the frequency of chromosomal aberrations is significantly increased in cells lacking TLS polymerases, combined with evidence for polymerase repriming events downstream of lesions, indicates that restoration of replication *per se* may not be the primary advantage conferred by TLS polymerases. Rather, TLS polymerases may allow the completion of replication, which minimizes recombination due to ssDNA and restores an intact and stable double helix. Thus, the increase in base substitutions, or even frameshift mutations, generated by TLS polymerase activity may ultimately prove less of a negative consequence for cells than genomic instability. This may be especially true in higher organisms with large, repetitive genomes containing fewer coding regions for point mutations to manifest as nonfunctional proteins and more sites of limited homology where illegitimate recombination may act to produce translocations. Indeed, in mammals, it is estimated that ~50% of DNA damage tolerance events occur through translesion synthesis rather than the more error-free recombinational bypass pathways (3). Additionally, breakage of the linear chromosomes found in eukaryotes can produce aneuploidy or trisomy.

Secondly, the mutations generated by TLS polymerases produce variation for natural selection to act upon. In particular, the upregulation of mutagenesis during cell stress could provide a mechanism for adaptive evolution (43, 46). This hypothesis would predict that mutants defective in TLS function would be at a competitive disadvantage in times of limited resources. Indeed, *E. coli* lacking DinB or UmuC exhibit a reduced fitness when grown in

competition with the wild-type strain (213). The generation of mutations can more easily be seen to be beneficial towards rapid adaption for unicellular organisms than in multicellular organisms, where mutations accumulated in somatic tissues provide no reproductive advantage. For multicellular organisms, which must balance the value of the mutagenic machinery against the risk of malignancy, it is less clear how the power of adaptive mutations could be harnessed. Interestingly, higher eukaryotes contain five TLS polymerases, relative to prokaryotes which have only two. Given that TLS polymerases appear to be specialized to bypass a particular cognate lesion in a relatively error-free way, the increase in the number of TLS polymerases in eukaryotes may increase the number of substrate lesions able to be bypassed accurately and actually decrease the mutagenic potential of DNA damage (46).

Finally, it may occasionally be advantageous for cells to make use of "programmed mutagenesis" in situations where an increase in mutation frequency would provide beneficial variation. In unicellular organisms, this has been seen in adaptive mutagenesis (43, 46) and in higher eukaryotes in the process of somatic hypermutation (26).

The elaborate regulation of translesion synthesis may provide the final clue into why cells contain potentially mutagenic translesion polymerases. As described above in Section 1.3.3, extensive regulatory mechanisms control the production and activation of translesion polymerases. Much progress has been made into discovering how TLS polymerases function and how their activity is controlled. However, many pressing questions still remain. The coming years will provide many new exciting insights and it is with regret that the author takes leave of the fascinating field of translesion synthesis and mutagenesis.

1.4 Thesis Summary

I have focused my thesis research on understanding the regulation and mechanism of action of the translesion DNA polymerase Rev1 in *S. cerevisiae*. As *REV1* promotes mutagenesis from yeast to humans, this knowledge will provide insight into a fundamental cellular process important for adaption, evolution, and disease. In Chapter Two, I present experiments revealing a novel cell-cycle regulation of Rev1 that led to an expanded model for how translesion synthesis functions. In Chapter Three, I describe experiments designed to address the contributions of the various domains of Rev1 to its function in promoting survival

and mutagenesis after DNA damage. The data presented is not complete and further experiments outlined in the discussion will be undertaken in the following months with the intent of publication. In the Appendices, I present unpublished data showing preliminary work characterizing the timing of UV sensitivity of a *rev1Δ* strain during the cell cycle, preliminary work towards understanding the molecular mechanism of the cell cycle regulation of Rev1, attempted purifications of Rev1 and its interacting partners, and a fine timecourse of the cell-cycle expression of Rev1. It is my hope that these lines of research will be carried to completion in the coming years by other lab members.

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Chapter Two

The Critical Mutagenic Translesion DNA Polymerase Rev1 Is Highly Expressed During G2/M Rather Than S-Phase

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Abstract

The Rev1 protein lies at the root of mutagenesis in eukaryotes. Together with DNA polymerase ζ (Rev3/7), Rev1 function is required for the active introduction of the majority of mutations into the genomes of eukaryotes from yeast to humans. Rev1 and polymerase ζ are error-prone translesion DNA polymerases, but Rev1's DNA polymerase catalytic activity is not essential for mutagenesis. Rather, Rev1 is thought to contribute to mutagenesis principally by engaging in crucial protein-protein interactions that regulate the access of translesion DNA polymerases to the primer terminus. This inference is based on the requirement of the N-terminal BRCT domain of *Saccharomyces cerevisiae* Rev1 for mutagenesis and the interaction of the C-terminal region of mammalian Rev1 with several other translesion DNA polymerases. Here we report that *S. cerevisiae* Rev1 is subject to pronounced cell-cycle control in which the levels of Rev1 protein are approximately 50-fold higher in G2 and throughout mitosis than during G1 and much of S-phase. Differential survival of a *rev1Δ* strain after UV irradiation at various points in the cell cycle indicates that this unanticipated regulation is physiologically relevant. This unexpected finding has important implications for the regulation of mutagenesis and challenges current models of error-prone lesion bypass as a process involving polymerase-switching that operates mainly during S-phase to rescue stalled replication forks.

Introduction

The *REV1* and *REV3* genes of *S. cerevisiae* were among the first genes known to be required for mutagenesis. Identified in 1971 in a screen for *reversionless* yeast strains (35), these genes play a central role in promoting mutagenesis from yeast to humans (14, 15). *REV1* and *REV3*, together with *REV7* (33), function in the "error-prone" branch of the *RAD6* post-replication repair pathway (28). In contrast, *RAD30*, which shares homology with *REV1*, appears to function in parallel with *REV1/3/7* in a separate "error-free" branch of the *RAD6* epistasis group (28). After decades of genetic characterization, *REV1*, *REV3*/7, and *RAD30* were shown to encode translesion DNA polymerases (17, 24, 43, 44).

Rev1 possesses a unique enzymatic activity *in vitro*, displaying a marked preference for inserting only dCMP opposite a template G and several DNA lesions (32, 41, 43). The Rev3/7 heterodimer forms DNA polymerase ζ, which, although it is proposed to function mainly as an extender of mismatched primer termini (32), can also efficiently insert nucleotides across from lesions when stimulated by PCNA (12). Rad30 encodes DNA polymerase η which bypasses UV-induced lesions efficiently and accurately and, when mutated in humans, causes the cancer-prone syndrome Xeroderma Pigmentosum Variant (34). Intriguingly, although Rev1's highly specialized catalytic activity has an effect on the spectrum of mutations generated (48, 54), its dCMP transferase activity is not required for its functions in induced mutagenesis or resistance to DNA damage [(20, 55); L. Waters and G. C. Walker, unpublished data].

In contrast, Rev1's BRCT domain is required for mutagenesis and resistance to DNA damaging agents in yeast (35), although it may be less important in higher eukaryotes (23, 55). First characterized as the BRCA1 C-terminus, BRCT domains mediate protein-protein interactions in many cell-cycle and DNA repair proteins (16). Interestingly, the original loss-of-function *rev1-1* mutant (35) carries a point mutation affecting the BRCT domain (15, 30). Since the purified Rev1-1 protein retains translesion synthesis (TLS) activity *in vitro* (42) while the *rev1-1* mutant is non-mutable *in vivo*, the alteration of the BRCT domain is thought to disrupt key interactions.

In addition to the N-terminal BRCT domain and a central TLS polymerase domain, the Rev1 protein also contains a C-terminal region which, in mammalian cells, has been shown to interact with multiple other TLS polymerases (19, 40, 45, 63, 64). Rev1's C-terminal interaction

region is required for resistance to DNA damaging agents in vertebrates (55) and in yeast [(30); L. Waters, S D'Souza, G. C. Walker, unpublished data]. Additionally, the C-terminus, as well as BRCT and little finger domains, of yeast Rev1 were recently reported to interact with Rev7 (1, 8). Since Rev1's protein-protein interaction motifs are required for its function *in vivo* while its enzymatic activity is not, this enigmatic translesion polymerase is thought to play a predominantly structural role in assembling a TLS complex (19, 20, 64).

Polymerase switching during DNA replication has been proposed to be a fundamental mechanism by which cells control the action of TLS polymerases (10, 51, 52), all of which have low fidelity on undamaged DNA relative to replicative DNA polymerases (17, 27). Polymerase switching models suggest that when a replicative DNA polymerase stalls at a blocking lesion, a handoff allows one or more TLS polymerases access to the primer terminus, enabling lesion bypass and extension past the distortion. A further reciprocal switch would restore the highly processive and accurate replicative DNA polymerase to the primer terminus. Current models (10, 34) postulate that Rev1 plays a central role in the polymerase-switching mechanism during S-phase to facilitate error-prone bypass of DNA lesions either itself, using its limited polymerase activity, or by recruiting other TLS polymerases to bypass the lesion.

A recent report from Lopes *et al.* (37) shows that when yeast replication forks encounter a lesion, leading and lagging strand synthesis uncouple. Repriming events downstream of a DNA lesion then lead to persistent ssDNA gaps on both strands of the replication fork, which may remain throughout S-phase into G2. Interestingly, deletion of all of the TLS polymerases did not further affect uncoupling or replication fork speed over damaged DNA, but rather led to an increase in single-stranded DNA (ssDNA) gaps along replicated regions. These data strongly suggest that some component of TLS may occur behind replication forks and possibly postreplicatively outside of S-phase.

We report here that Rev1 is expressed in a cell-cycle dependent manner and is highly upregulated specifically during G2/M phases rather than during DNA replication in S-phase. Rev1's G2/M expression pattern does not significantly change after DNA damage. Moreover, *REV1* function is required for resistance to DNA damage differentially during the cell cycle. This finding suggests that Rev1-dependent TLS, and therefore much of mutagenesis, occurs to a significant extent, if not mostly, outside of S-phase during G2/M.

Results

Rev1 Protein and mRNA Are Cell-Cycle Regulated and Reach Maximal Levels After Most Replication Is Completed.

To facilitate analysis of *S. cerevisiae* Rev1 regulation, a chromosomally located C-terminally tagged Rev1 construct was expressed from the native *REV1* promoter. The tagged strain was indistinguishable from wild-type in its ability to survive DNA damage and to undergo mutagenesis (Fig. 1).

Our ability to visualize endogenous levels of Rev1 protein led to the unanticipated discovery that Rev1 is subject to pronounced cell cycle control (Fig. 2A). *S. cerevisiae* cells were arrested in G1 with α-factor, released, and allowed to proceed synchronously through the cell cycle. In α-factor arrested cells, Rev1 levels are almost undetectable. Surprisingly, Rev1 levels are very low in early S-phase and rise only modestly as cells transit through S-phase (also see Fig. 6). Substantial Rev1 accumulation occurs as most cells attain a G2 content of DNA (Fig. 2B) indicating that Rev1 levels do not peak as DNA is being synthesized, but rather after most replication is completed. Using anti-tubulin immunofluorescence to monitor spindle length reveals that Rev1 is present at high levels as the chromosomes align during metaphase (Fig. 3A) and is maintained at high levels even after most cells achieve fully-extended spindles and completely separate their DNA masses (Fig. 3B). This implies that Rev1 is highly expressed throughout mitosis and that maximal protein levels are maintained until cells re-enter G1. Levels of *REV1* mRNA exhibit a similar pattern of cell cycle regulation as the protein, peaking slightly before the Rev1 protein levels in G2/M (Fig. 1C).

Peak levels of Rev1 protein in G2/M cells are approximately 50-fold higher than the barely-detectable Rev1 signal in G1 arrested cells (Fig. 3C), whereas we found only a ~3-fold change between maximal and minimal levels of *REV1* transcript (Fig. 3D). Thus, the cell cycle control of Rev1 levels is primarily post-transcriptional. The observed cell cycle regulation is not an α-factor specific effect, as cells synchronized by elutriation exhibit a similar pattern of Rev1 expression (Fig. 4). An identically tagged Rad30 shows no change during the cell cycle (Fig. 2A), indicating that this type of cell cycle control is not a general property of all TLS polymerases.

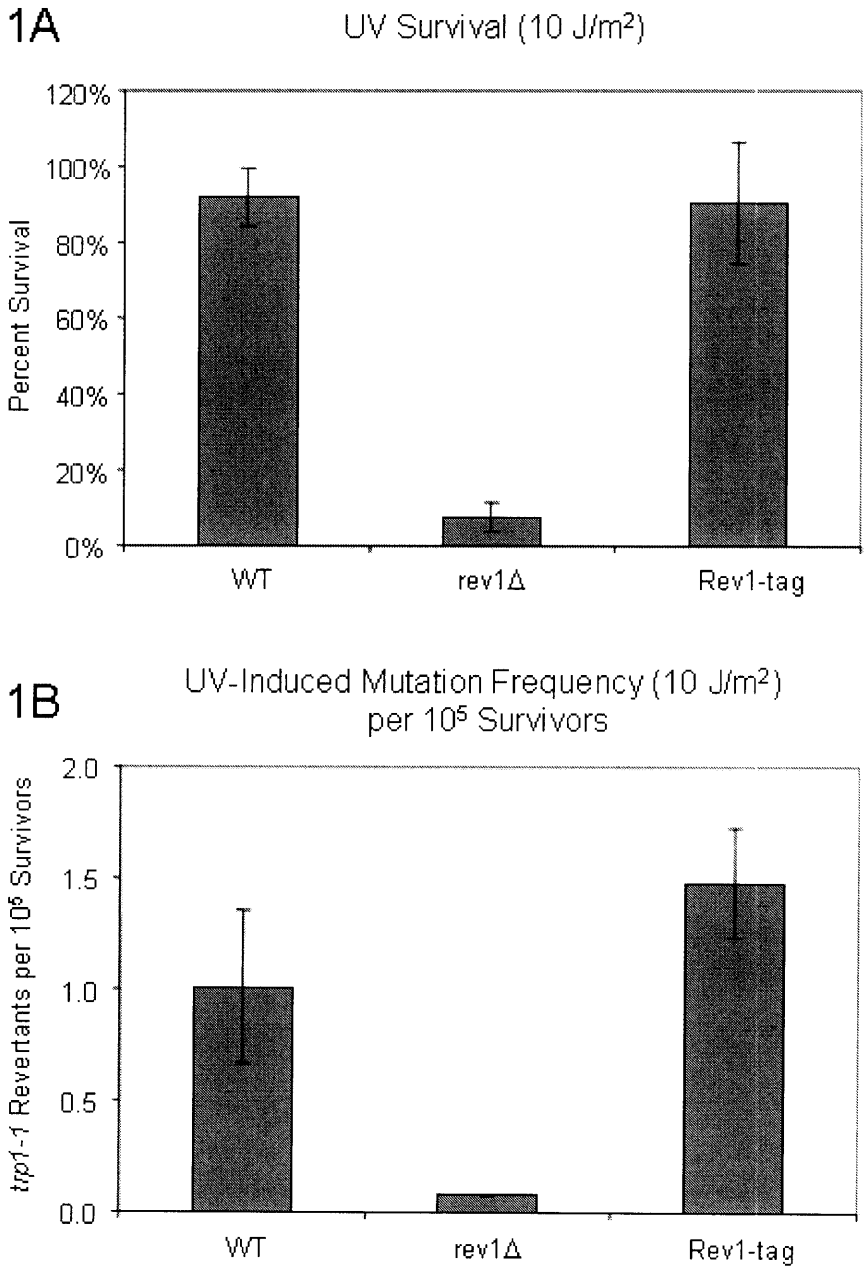

Fig. 1. The C-terminally tagged Rev1 construct retains full function. (*A*, *B*) Isogenic strains of the wild-type background, a *rev1Δ*, and the tagged Rev1 were compared for survival (*A*) and mutagenesis (*B*) after 10 J/m^2 UV irradiation. The data plotted for the *rev1Δ* reversion frequency is the limit of detection, since no revertants were recovered. Therefore, the *rev1Δ* reversion frequency is likely lower than indicated.

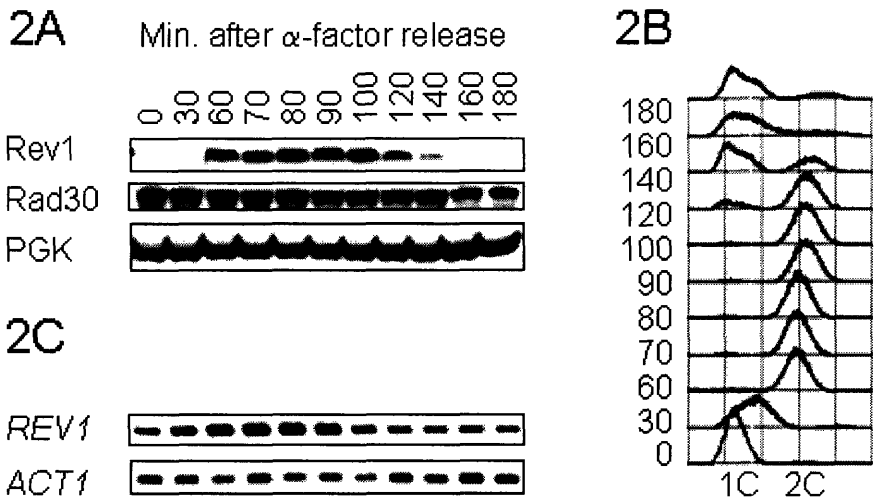

Fig. 2. Rev1 is cell-cycle regulated and expressed maximally at G2/M. (*A*) Immunoblot against the protein A epitope shows Rev1 and Rad30 protein levels at indicated timepoints after release from G1 α-factor arrest. PGK is the standard loading control phosphoglycerate kinase. (*B*) FACS analysis of the DNA content of cells. (*C*) RT-PCR showing *REV1* mRNA levels. *ACT1* was used as a loading control.

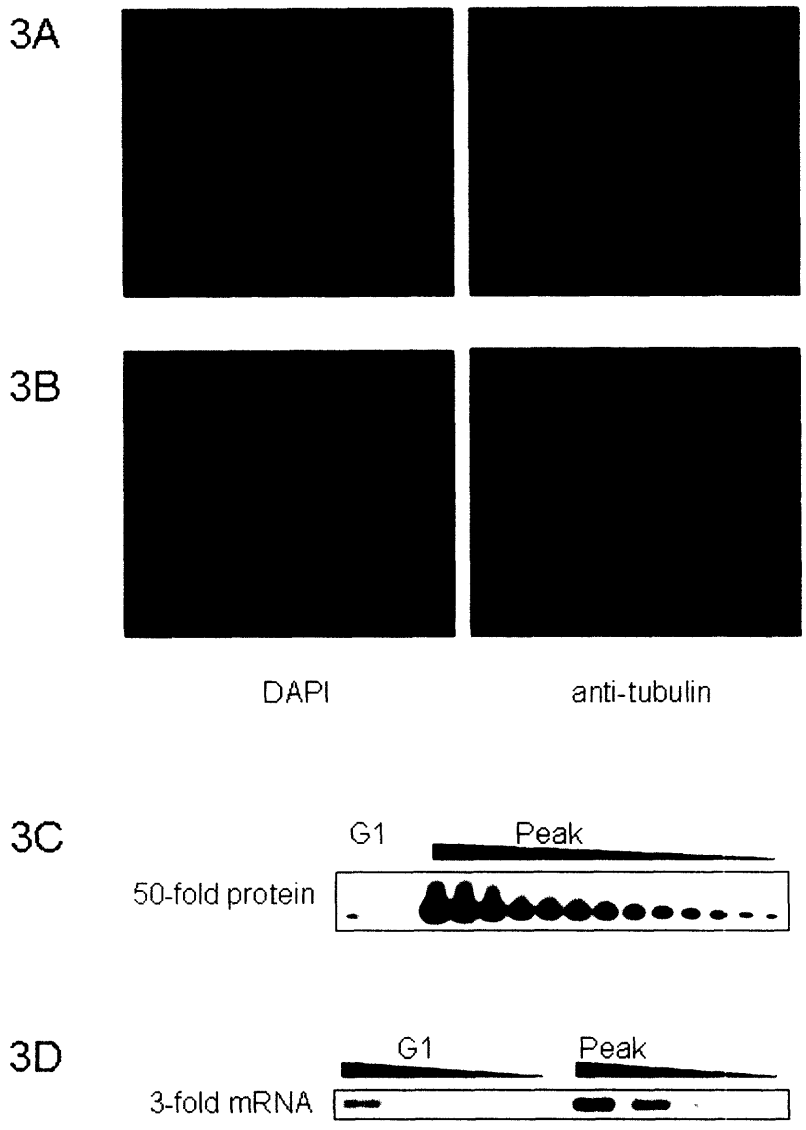

Fig. 3. Rev1 is high throughout mitosis and is post-transcriptionally regulated. (*A*, *B*) Cells from the 60 (*A*) and 90 (*B*) min timepoints stained with DAPI and anti-tubulin to visualize DNA or anaphase spindles, respectively. (*C*) Immunoblot quantitating the change in Rev1 protein levels between minimal and maximal points, comparison of an undiluted G1 sample to various dilutions (1 to 8, 10, 13, 15, 16, 18, 20, 23, 25, 32, 40, 45, and 50 fold) of a G2/M sample. (*D*) RT-PCR samples of minimal and maximal *REV1* mRNA levels were compared quantitatively using two-fold serial dilutions of input cDNA.

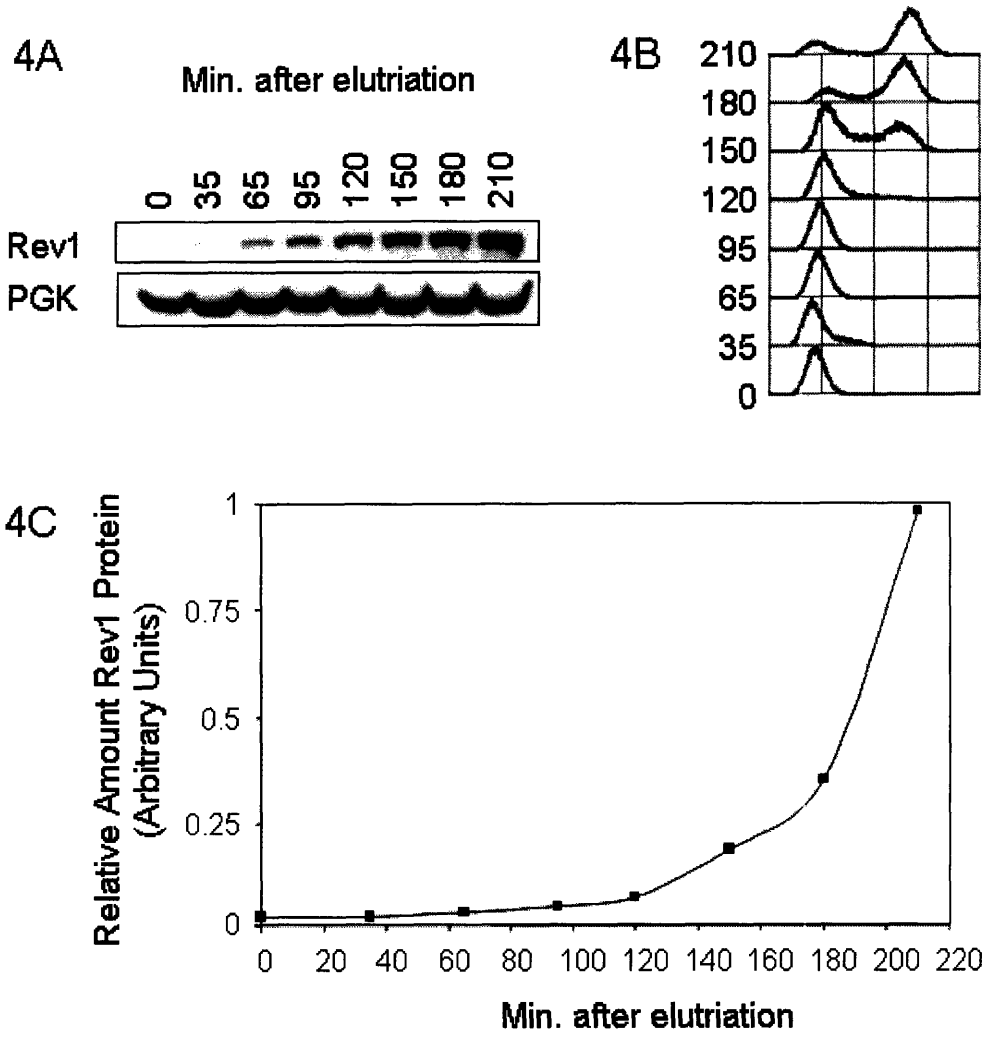

Fig. 4. Synchronization by elutriation shows Rev1 cell cycle expression. (*A*) Immunoblot against the protein A epitope tag of Rev1 and the loading control PGK. Small, unbudded cells were collected by centrifugal elutriation and samples taken at the indicated times after resuspension in rich media. (*B*) FACS analysis of the DNA content. (*C*) Plot showing quantitation of the immunoblot from (*A*) normalized in arbitrary units to a standard dilution curve.

Rev1 Protein Is Stably Present Throughout Mitosis

To analyze the timing of Rev1 accumulation more precisely, *cdc23-1* and *cdc15-2* temperature sensitive strains were used to arrest cells at the metaphase-to-anaphase transition and at telophase, respectively (2, 22). Pds1 (securin) was used as a marker for cell-cycle progression since it is synthesized during S-phase and is degraded at the metaphase-to-anaphase transition (67). Cells were synchronized in G1 with α-factor and then, upon removal of α-factor, shifted to the restrictive temperature to induce the second cell cycle arrest. We found that Rev1 levels do not rise until after Pds1 has accumulated during S-phase, again demonstrating that Rev1 levels are low during much of DNA replication. Furthermore, Rev1 is present at high levels in *cdc23-1* metaphase-arrested cells (Fig. 5A), indicating that Rev1 accumulation begins during G2 before metaphase. Even more interestingly, Rev1 is also stable in *cdc15-2* telophase-arrested cells (Fig. 5B). The *cdc15-2* allele produces a very late arrest in the cell cycle during exit-from-mitosis, just before re-entry into G1 (2). Following release from the *cdc15-2* telophase block, Rev1 levels decrease as cells re-enter G1 (Fig. 5C). Therefore, contrary to prevailing expectations for a TLS polymerase, we demonstrate that Rev1 is maximally present after the majority of DNA replication is finished, remains throughout all of mitosis, and is present even during exit-from-mitosis while cells reset for G1.

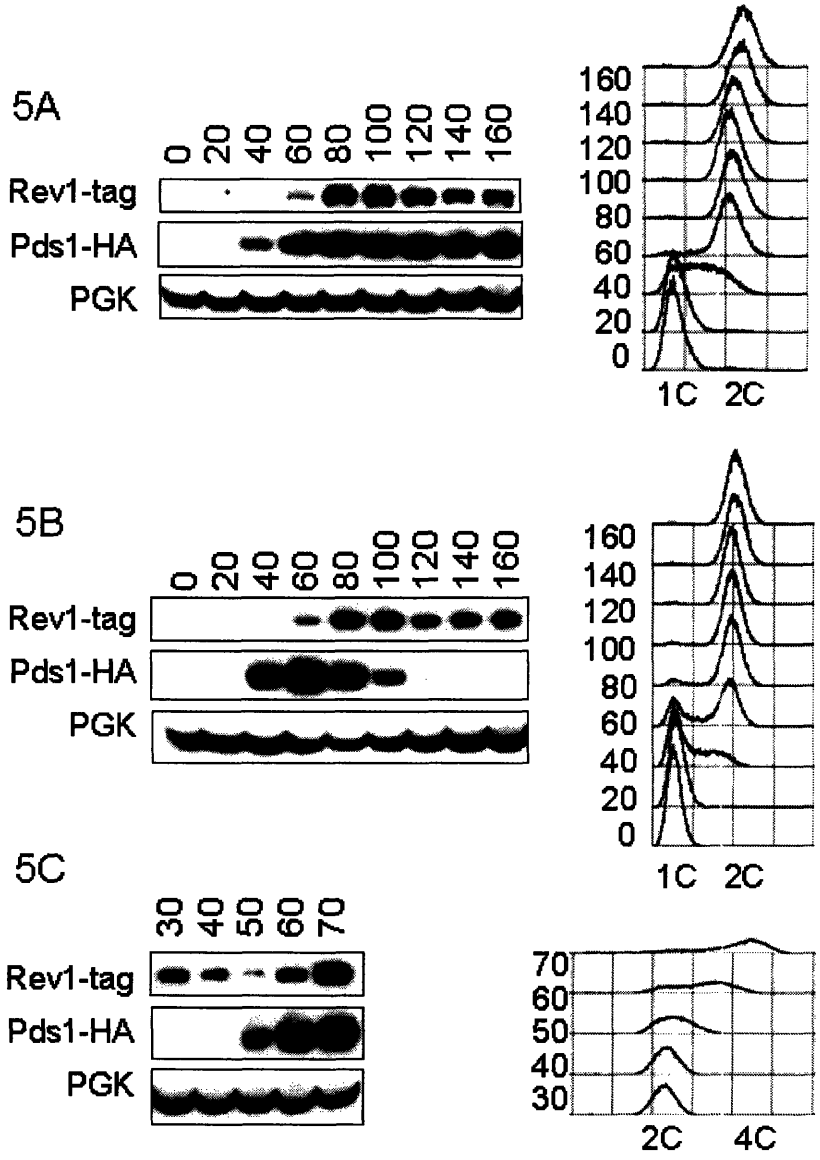

Fig. 5. Rev1 protein is stable in both metaphase and telophase arrested cells. (*A*, *B*) Immunoblot showing Rev1 and Pds1 in *cdc23-1* (*A*) or *cdc15-2* (*B*) arrested cells. Timepoints were taken every 20 min after release from α-factor and shift to the restrictive temperature. (*C*) Immunoblot showing Rev1 and Pds1 levels at the indicated times after release from the *cdc15-2* block. Cells released from a *cdc15-2* arrest fail to separate due to a cytokinesis defect and generate a 4C peak on FACS indicative of a second round of DNA replication.

DNA Damage Does Not Significantly Alter Rev1's Expression Pattern

Taken together, these observations suggest that, in undamaged cells, the major physiological role of Rev1 in spontaneous mutagenesis occurs predominantly in G2/M. We wondered, however, if exogenous DNA damage would significantly alter Rev1 expression so that it would accumulate mainly during S-phase when the replication machinery would be actively encountering lesions. Since Rev1 is required for bypass of the 6-4 photoproduct induced by UV (42), we irradiated cells arrested in G1 and followed Rev1 levels through the cell cycle after DNA damage. Doses of UV irradiation of 10 J/m^2 and 50 J/m^2 resulted in *ca.* 100%, and 60% survival of the tagged Rev1 strain and *ca.* 75%, and 1% survival of an isogenic *rev1Δ* strain. We found that DNA damage did not result in a radical alteration of the overall pattern of Rev1 expression (Fig. 6). Despite the fact that replication forks would have encountered UV-induced lesions from the beginning of S-phase, Rev1 levels were not dramatically increased early in S-phase relative to an unirradiated strain. As observed with undamaged cells, Rev1 accumulated slowly through S-phase, only reaching its peak when most of the cells were in G2.

Some changes in the timing of Rev1p accumulation, however, were discernable. After 10 J/m^2 of UV, low levels of Rev1 were still found in early S-phase but began increasing slightly earlier to achieve higher levels during late S than in the absence of UV damage (Fig. 6A). After 50 J/m^2 of UV, this shift in Rev1 accumulation became more pronounced (Fig. 6B). The cells proceeded more slowly though the cell cycle after significant amounts of DNA damage, so direct comparisons of timecourses by minutes after release do not reflect cell cycle stage. Despite this moderate shift in timing after substantial DNA damage, Rev1 protein is not present at high levels throughout S-phase, as would be expected for a replication protein or an S-phase repair protein. Instead, at high doses of UV, Rev1 accumulation appears to track slightly after the metaphase protein Pds1 (Fig. 6B). Additionally, as with undamaged cells, after UV irradiation Rev1 still appears to persist into G2/M as the cells complete replication and enter mitosis.

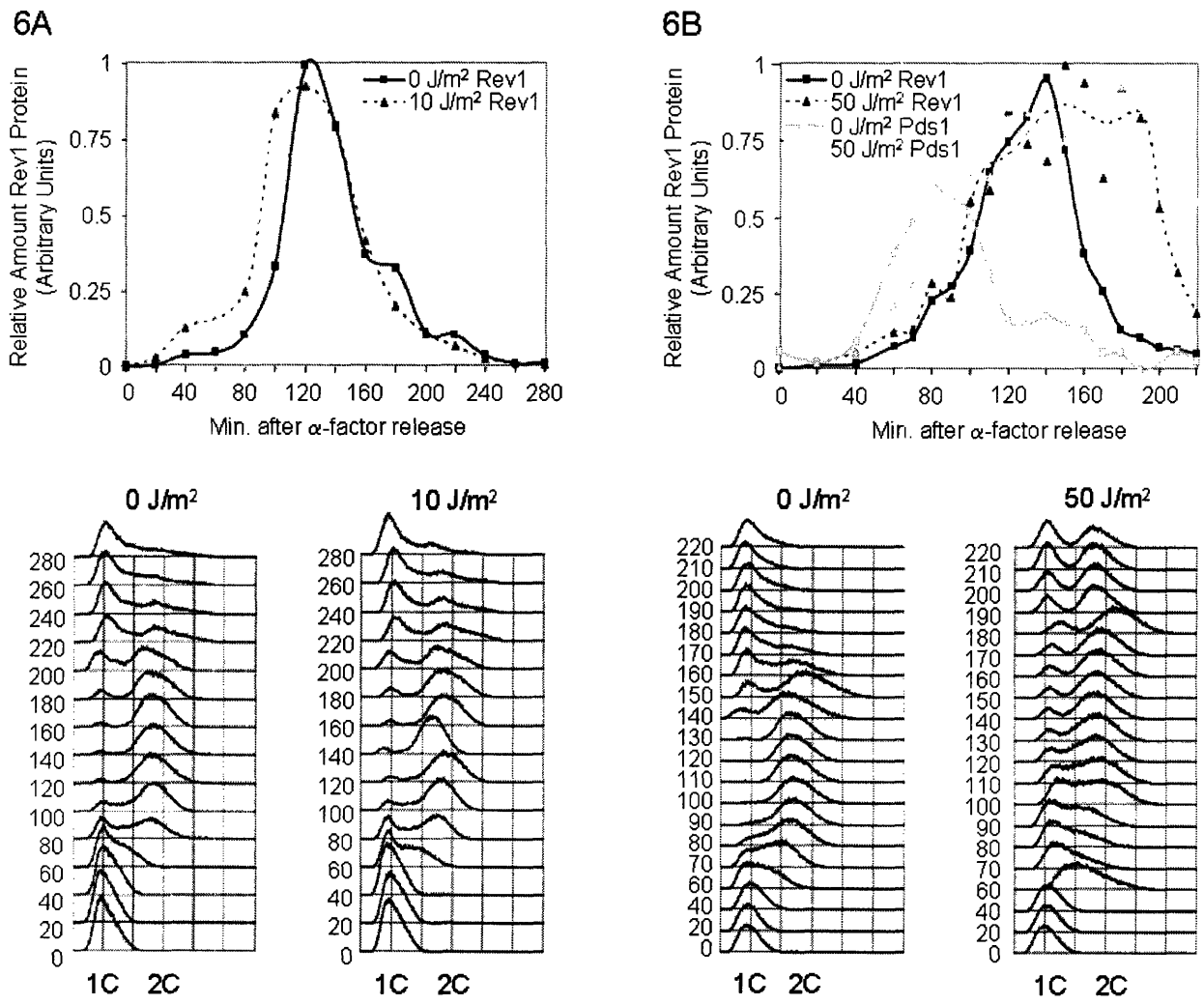

Fig. 6. Cell-cycle expression pattern of Rev1 is modestly altered after DNA damage. (*A*, *B*) Plot showing relative amount of Rev1 protein in arbitrary units as a function of cell cycle progression. G1-arrested cells were UV-irradiated with 10 J/m^2 (*A*) or 50 J/m^2 (*B*), released from α-factor block, and timepoints taken as indicated. Immunoblots were quantitated and normalized to a standard dilution curve of Rev1 to allow comparison between blots. FACS data monitors cell cycle progression.

REV1 Function Is Required Differentially During the Cell Cycle

To analyze the possible biological significance of the observed Rev1 cell-cycle regulation, we monitored survival after UV irradiation at different cell cycle stages. Cells were arrested in G1 with α-factor or in G2 with nocodazole, washed to remove the drugs, plated, and immediately UV irradiated. The WT strain was only slightly more sensitive to killing when UV irradiated just after release from G1 than when UV irradiated just after release from G2 (Fig. 7A), in agreement with previous reports (59). The mild sensitivity of a *rad30Δ* strain to killing by UV irradiation was likewise largely unaffected by the cell cycle stage during which the UV irradiation occurred (Fig. 7A), consistent with the observation that the Rad30 protein is constitutively expressed throughout the cell cycle (Fig. 1A).

In striking contrast, the *rev1Δ* strain was markedly more UV sensitive when irradiated after release from G1 than when irradiated after release from G2 (Fig. 7B), showing clearly that *REV1* function is required differentially throughout the cell cycle. Since Rev1 is largely absent in G1 and present in G2, a plausible explanation is that irradiation after release from G1 results in replication forks encountering DNA lesions before they can be completely repaired by nucleotide excision repair (NER), causing leading and lagging strand uncoupling and repriming events downstream that lead to the generation of ssDNA gaps at lesions (37). Such ssDNA gaps would require TLS. In contrast, irradiation in G2 would allow a more prolonged period for NER prior to DNA replication, thereby reducing ssDNA gap formation and hence the need for TLS. Consistent with this explanation, microscopic examination of the plates revealed that, when irradiated with 20 J/m^2 of UV after release from G1, *rev1Δ* cells arrest predominantly as budded cells (data not shown). This indicates that the lethal event after UV irradiation in *rev1Δ* cells occurs following replication, rather than in G1 or at a random point several generations later.

Interestingly, although the protein levels of Rev3 and Rev7 do not vary during the cell cycle (S. D'Souza and G. C. Walker, data not shown), the *rev3Δ* and *rev7Δ* strains display the same hypersensitivity to UV irradiation when irradiated after release from G1 as the *rev1Δ* strain (Fig. 7B). The striking similarity of the pattern of cell-cycle dependent UV sensitivity indicates that Rev1's cell-cycle regulation is used to control the activity of DNA polymerase ζ (Rev3/7) during the cell cycle.

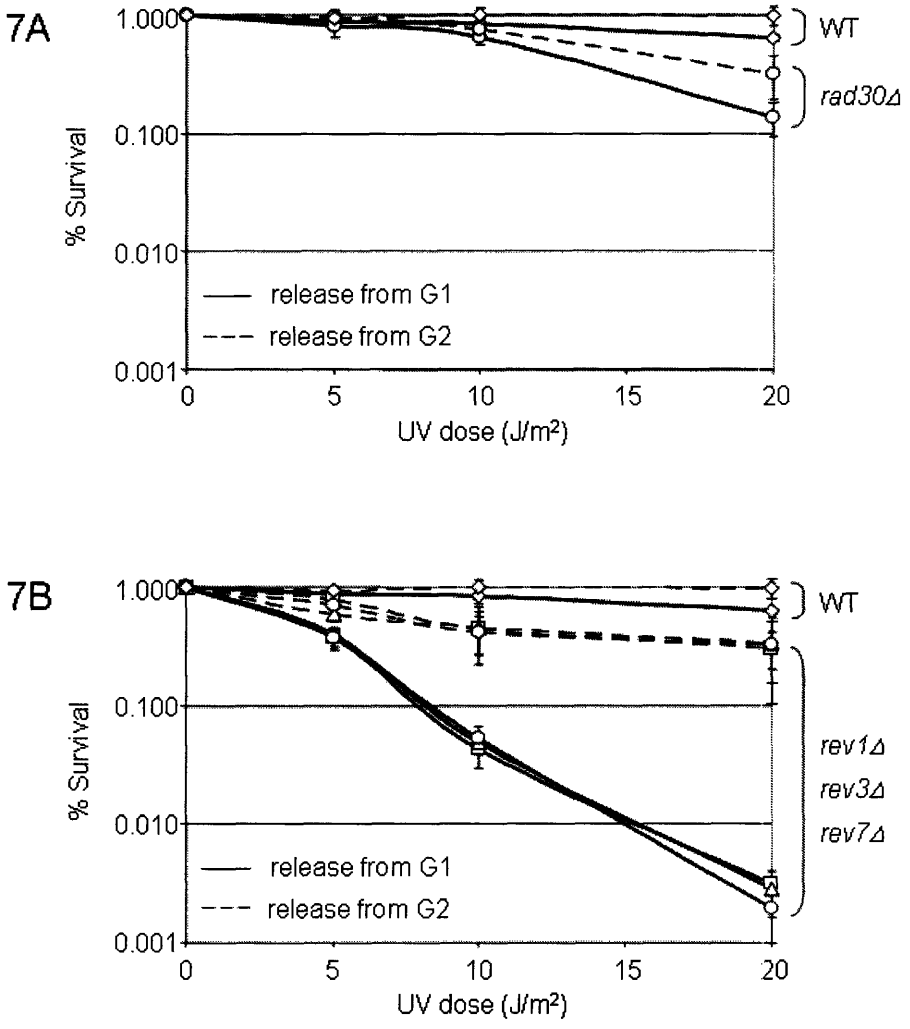

Fig. 7. *REV1*, *REV3*, and *REV7* are required differentially throughout the cell cycle for survival after UV irradiation. (*A*) Percent survival of the WT (diamonds) and *rad30Δ* strains (circles) after release from G2 (dotted line) or G1 (solid line) arrests. (*B*) Percent survival of the WT (diamonds), *rev1Δ* (squares), *rev3Δ* (triangles), and *rev7Δ* (circles) strains after release from G2 (dotted line) or G1 (solid line) arrests. Note that *rev1Δ*, *rev3Δ*, and *rev7Δ* strains exhibit such similar survival that the strains can hardly be distinguished from each other.

Discussion

We report here that in *S. cerevisiae*, Rev1 protein levels are dramatically cell-cycle regulated, being at least 50-fold higher in G2/M than in G1 and much of S-phase. The remarkable dependence of the UV sensitivity of a *rev1Δ* mutant, but not a *rad30Δ* mutant, to the cell cycle stage in which UV irradiation occurs indicates that the cell cycle regulation of Rev1 is of major biological significance. As Rev1 and polymerase ζ are required for ~95% of the mutagenic events in a cell (32), the cell cycle regulation of Rev1 has profound implications for when mutagenesis takes place during the cell cycle.

We show that the amount of Rev1 protein is extremely low during G1 and rises slowly throughout early and mid S-phase. Rev1 levels only begin to increase rapidly in late S-phase, reaching maximum levels in G2. The Rev1 protein is then maintained at a high intracellular concentration throughout mitosis until after telophase. DNA damage causes Rev1 to accumulate somewhat earlier in late S without significantly affecting the level reached in G2/M, but does not convert Rev1's expression pattern into that of a canonical replication protein, such as PCNA or a replicative DNA polymerase (9, 62). The observed pattern of cell-cycle dependent expression was initially surprising, given current models postulating that polymerase-switching allows translesion synthesis to restart stalled replication forks during S-phase (10, 34, 51, 52). In contrast, our unexpected finding that Rev1 is cell-cycle regulated with maximal expression during G2/M suggests that Rev1 acts predominantly in G2/M rather than during the active phase of DNA replication in S-phase. This is consistent with the report from Lopes *et al.* (37), which challenges the assumption that the polymerase switch event occurs solely at blocked replication forks in S-phase. We propose that Rev1 acts postreplicatively during G2, and even M, by carrying out its well established roles in mutagenic TLS. During this process, Rev1 could function both as a DNA polymerase and also recruit other TLS polymerases to fill the ssDNA gaps that are left behind as a consequence of replication forks encountering lesions.

A *rev1Δ* strain is differentially sensitive to UV irradiation during the cell cycle, demonstrating that *REV1* functions in a cell-cycle dependent manner. In yeast, DNA polymerase ζ (Rev3/7) (1, 32) does not display cell-cycle regulated protein levels (S. D'Souza and G. C. Walker, unpublished data), nor does the related Y family translesion DNA polymerase η (Rad30) (Fig. 1A). A *rad30Δ* strain showed no cell-cycle dependence in its sensitivity to UV

damage beyond that of the WT strain. However, the *rev3Δ* and *rev7Δ* strains were indistinguishable from the *rev1Δ* strain in their responses to UV damage after release from G1 or G2 arrests. Therefore, although the cell-cycle regulation exhibited by the Rev1 protein appears to be unique among TLS polymerases, it is likely used to control the Rev1/3/7-dependent error-prone mode of translesion synthesis. Additionally, as polymerase ζ dependent crosslink repair also shows cell-cycle dependence (58), Rev1's cell-cycle regulation may be used to coordinate the responses of other damage tolerance pathways as well.

Although this is the first direct evidence for cell-cycle regulation of Rev1, other recent results are consistent with Rev1 and its partners Rev3/7 acting late in the cell cycle. For example, Rev1 functions in preventing chromosomal breaks in mouse ES and transformed chicken DT40 cells in late S/G2 (23, 46, 60). Similiar to our observation with UV, *rev3Δ* cells progress through S-phase normally but arrest permanently in G2 after cisplatin treatment (18). Analogous results have been observed in mouse and chicken cells with *Rev1 BRCT*$^{-/-}$ and *Rev3*$^{-/-}$ deficient lines (23, 60, 68). Our discovery that Rev1 levels are highest in G2, after a sister chromatid has been generated, is also consistent with the growing evidence for *REV1/3/7* involvement in certain aspects of homologous recombination (HR) (46). Evidence consistent with Rev1 and Rev3/7 contributing to the processing of double-strand breaks during HR in meiosis includes the observations that, in yeast, each of the *REV* genes is upregulated during sporulation (4, 6, 61) and in mammals, *REV1* and all of the other TLS polymerases are at high levels in the testes (29). Further supporting an involvement in facets of HR repair, *REV3* is required for the break-repair-induced mutagenesis observed during DSB repair (21, 53). However our demonstration that Rev1 persists until well after anaphase and sister-chromatid separation suggests that, beyond any contribution to HR, Rev1 may play a role during mitosis after sister chromatids are physically separated and unable to synapse. These data, together with the observation that hREV7 (hMAD2B) inhibits the metaphase-to-anaphase transition through the spindle checkpoint in Xenopus extracts (5, 50), strongly indicate that Rev1/3/7 play a major role at the end of DNA replication and throughout mitosis.

Is it reasonable that the majority of Rev1-dependent mutagenic translesion synthesis could occur after most DNA replication is completed and extend throughout mitosis? The inhibition of many polymerases by DNA lesions in *in vitro* studies employing primed single-strand templates has contributed to a widespread impression that real replication forks can be

similarly stalled by a single lesion. However, *in vivo* in both eukaryotes and prokaryotes, replication forks uncouple leading and lagging strand synthesis when they encounter lesions and leave gaps in their wake (7, 37, 49, 56), which may persist as cells enter G2. The recent results of Lopes *et al.* (37) show that TLS defective *S. cevervisiae* cells do not further uncouple leading and lagging strands, but rather have an increase in ssDNA gaps, consistent with the idea that TLS may occur behind the replication fork and even after bulk replication has been completed. Interestingly, after DNA damage *E. coli* seems to delay mutagenic translesion synthesis by using the kinetics of the SOS-regulated UmuD → UmuD′ transition to impose a phase of largely accurate DNA repair and tolerance followed by a phase of error-prone lesion bypass (47). Restricting Rev1 to the latter part of the cell cycle may be a conceptually similar strategy to reserve *REV1/3/7*-dependent mutagenic TLS until after high-fidelity repair or damage-tolerance mechanisms have been attempted (Fig. 8A).

In our model, the major site of Rev1 action is at inappropriate primer termini remaining in G2/M at gaps caused by lesions (Fig. 8B). A persistent gap in G2 may be recognized by Rev1 by its ability as a polymerase to bind primer termini or using its BRCT domain, since some BRCT domains can bind DNA, particularly single- or double-stranded breaks (65, 66). Therefore, the *rev1-1* mutation might also inactivate Rev1's localization to aberrant DNA structures rather than exclusively disrupting a protein-protein complex. Additionally, since it is possible that modified forms of PCNA may persist at ssDNA gaps and serve as a marker for repair activities, Rev1 may recognize a ssDNA gap remaining in G2 by binding to a monoubiquitinylated PCNA through its UBM ubiquitin-interacting domains, in a manner analogous to DNA polymerase ι in mammalian cells (3). Interaction with monoubiquitinylated PCNA also stimulates Rev1's catalytic activity (11). Once at the lesion, Rev1 may facilitate tolerance and gap-filling either by using its own dCMP transferase activity or by recruiting other TLS polymerases through its C-terminal region (19, 40, 45, 63, 64). Rev1 may also interact with other DNA repair or damage checkpoint signaling factors, for example using its BRCT domain to form a complex with other BRCT-containing proteins or indirectly through PCNA (55) or the alternative clamp 9-1-1 (57). Once a gap has been filled and the lesion bypassed, excision repair machinery could then be recruited by Rev1 to remove the lesion prior to the start of the next cell cycle.

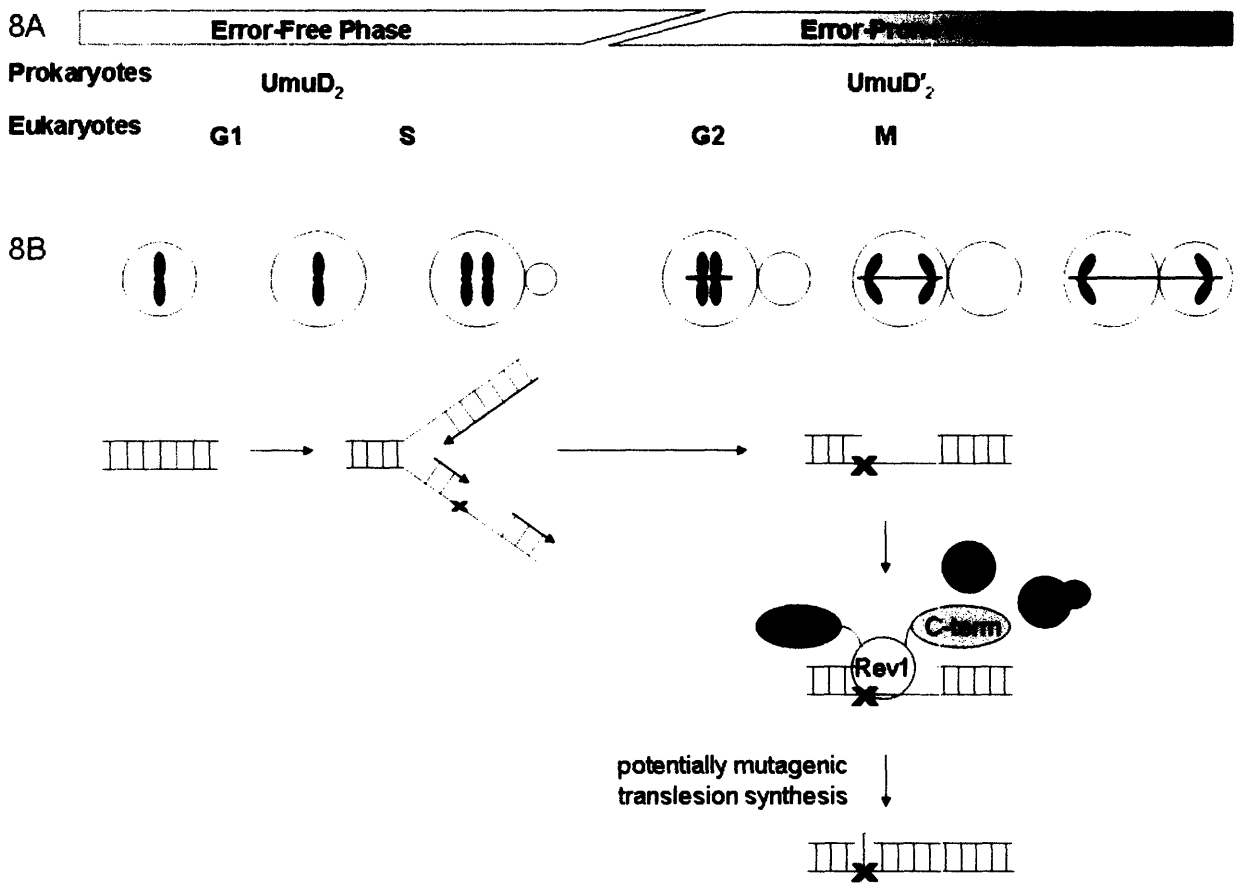

Fig. 8. Rev1 may function after replication has been completed to fill gaps remaining opposite to lesions. (*A*) Putative evolutionary conservation of delay in mutagenic DNA lesion processing. (*B*) Model showing generation of single-stranded gaps in S-phase by replication fork uncoupling at the site of a DNA lesion. Rev1 may recognize a persistent gap in G2 and carry out limited TLS itself and/or recruit other factors, such as the other TLS polymerases.

We cannot exclude some contribution of Rev1 during S-phase, since the low levels we observe during DNA replication may be sufficient for at least some TLS. However, the levels of Rev1 during S-phase are likely significantly lower than those of replicative DNA polymerases, perhaps 10-fold or more. Asynchronous yeast cultures contain only ~500 Rev1 molecules/cell, the majority of which is presumably due to the G2/M cells in the population, compared to ~2000 molecules/cell for Rad30 or the replicative polymerases (13). Furthermore, Rev1 and Rev3 are also thought to be present at a very low cellular concentration in higher eukaryotes (14, 15, 36, 55). This low level of Rev1, coupled with the cell-cycle regulation we have observed, suggests that caution should be used in interpreting studies in which Rev1 is overexpressed (38, 64). The finding that overexpressed Rev1 localizes to replication forks may provide a rationale for why cells keep the amount of Rev1 low during S-phase; if Rev1 were present at high levels, it might be recruited inappropriately to replication forks when not needed with mutagenic or lethal consequences. During S-phase, relatively accurate TLS at stalled replication forks may be accomplished by TLS polymerases such as polymerase η (Rad30/XPV) recruited by mono-ubiquitinylated PCNA (34). In contrast, we suggest that Rev1 acts mostly outside of outside of S-phase coordinating a more mutagenic usage of TLS polymerases later in the cell cycle.

While this paper was under revision, a report appeared showing ectopically overexpressed hRev1 formed foci in S-phase as well as in G1 (39). Although focus formation is frequently interpreted as indicating the site of a protein's major function, in this case the most biologically significant action of Rev1 might not manifest itself as a focus. Whereas recruitment of many molecules of Rev1 to a replication factory or repair center would likely generate a focus, it is not clear that recruitment of Rev1 to multiple ssDNA gaps spaced out along replicated DNA would result in a high local concentration of Rev1.

Since most aspects of cell cycle control are shared between yeast and mammals, Rev1's cell cycle regulation may have general implications for TLS-dependent mutagenesis. Our results suggest that cells delay potentially mutagenic TLS until later in the cell cycle as a strategy for minimizing the mutagenic effects of DNA damage. In the environment, *S. cerevisiae* and other microorganisms likely spend most of their life in a quiescent, nonproliferating state. Most cells in higher eukaryotes are terminally differentiated and have withdrawn from the cell cycle. Thus, restricting Rev1 protein expression to G2/M may reflect a cellular mechanism for reducing mutagenesis in resting cells.

Materials and Methods

Strains. Strains used were derivatives of W1588-4C, a W303 strain corrected for *RAD5* (69). pYM10 was used to generate a C-terminal –TEV-ProA-7His tag (26). Strain information is shown in Table 1 and primer sequences in Table 2.

Immunoblots. Whole cell extracts were prepared by TCA precipitation (26). Antibodies used were rabbit PAP antibody (Sigma) against the protein A tag, anti-HA.11 (Covance), and anti-phosphoglycerate kinase (Molecular Probes). Quantitation was performed using the Typhoon 9400 (GE Healthcare) and ImageQuant software. Plots were generated by averaging two to four replicate immunoblots.

Flow Cytometry. Cells were prepared essentially as described (31) and analyzed on a Becton Dickson FACSCalibur flow cytometer.

Cell Synchronization. Logarithmically growing *bar1Δ* yeast were arrested with 50 ng/mL α-factor for 4 hours at 25 °C and washed to remove α-factor. In Fig. 1, cells were resuspended in 25 °C media and α-factor was added back after 75 min. In Fig. 2, cells were resuspended in media pre-warmed to 37 °C. After 3 hours at 37 °C, cells were released from the *cdc15-2* arrest by harvesting and resuspending cells in 25 °C media. In Fig. 3, cells were resuspended in 20 mL water, transferred to a 150 x 15 mm petri plate, and irradiated . Aliquots were assayed for viability. Cells were diluted in 20 mL 2x media to start the timecourse. In Fig. 3A, α-factor was added back after 90 min, in Fig. 3B after 80 min (0 J/m^2) or 100 min (50 J/m^2 UV).

Cell Cycle UV Survival Assay. At least 3 independent cultures of each strain were arrested with 50 ng/mL α-factor or 15 μg/mL nocodazole for 3 hours at 30 °C and washed with water or 1% DMSO in YEP media to remove α-factor or nocodazole respectively. Microscopic analysis of cells confirmed arrest. Cells were diluted appropriately in water, plated on SC media, immediately UV irradiated at 1 J/m^2/sec using a G15T8 UV lamp (General Electric) at 254 nm, and incubated for 3 days at 30 °C in the dark.

Immunofluorescence. Immunofluorescence was performed using anti-tubulin (Oxford Biotechnology) and anti-rat-FITC (Jackson ImmunoResearch) (25).

RT-PCR. RNA was isolated using RNeasy Mini Kit (Qiagen) according to manufacturer's protocol. To generate cDNA, 0.5 μg RNA was used in 20 μL reactions with 20 pmol oligo dT primer, and 25 U Stratascript reverse transcriptase (Stratagene). All PCRs were performed for 26 cycles and different amounts of input cDNA were used to analyze relative levels of *REV1* or *ACT1* mRNA in each sample.

Survival and Mutagenesis Assays. Three independent cultures of each strain were grown to saturation and appropriately diluted aliquots were plated on SC media to monitor survival. Mutation frequencies were analyzed by plating undiluted aliquots on SC-Trp media to score for reversion of the *trp1-1* allele. Plates were irradiated at 1 $J/m^2/sec$ using a G15T8 UV lamp (General Electric) at 254 nm and grown for 3 or 6 days at 30 °C for survival and mutagenesis assays respectively.

Elutriation. Cells were collected by elutriation as described (2) in a JE-5.0 Elutriator (Beckman Coulter) and resuspended in YPD media to initiate the timecourse.

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Table 1: Strains used in this study

Strain	Genotype	Source
W1588-4C	*MATa ade2-1, ura3-1, his3-11,15, trp1-1, leu2-3,112, can1-100, RAD5*	Rothstein lab (66)
JAy19	*REV1-TEV-ProA-7His:HIS3MX6, RAD30-TEV-ProA-7His:HIS3MX, bar1::LEU2*	R. Woodruff
A2	*REV1-TEV-ProA-7His:HIS3MX6, RAD30-TEV-ProA-7His:HIS3MX6, bar1::LEU2*	this study
E	*REV1-TEV-ProA-7His:HIS3MX6, bar1::URA3, cdc23-1, PDS1-HA:LEU2*	this study
F	*REV1-TEV-ProA-7His:HIS3MX6, bar1::URA3, cdc15-2, PDS1-HA:LEU2, SCC1-MYC:TRP1*	this study
W1588-4C *bar1Δ*	*bar1::LEU2*	this study
rev1Δ bar1Δ	*rev1::kanMX, bar1::LEU2*	this study
rev3Δ	*rev3::kanMX*	S. D'Souza
rev3Δ bar1Δ	*rev3::kanMX, bar1::LEU2*	this study
rev7Δ bar1Δ	*rev7::kanMX, bar1::LEU2*	this study
rad30Δ	*rad30::kanMX*	R. Woodruff
rad30Δ bar1Δ	*rad30::kanMX, bar1::LEU2*	this study

Table 2: Primers used in this study

Primer Name	Primer Sequence
Rev1-tag-fwd	CATACTTACCAGACTGTGCGTAAACTTGACATGGACTTTGAA GTTcgtacgctgcaggtcgac
Rev1-tag-rev	CGCAAACTGCGTGTTTACTGTATGCTGAAATGTTTTTTTTTTT TTAATatcgatgaattcgagctcg
Rev1D	GTGAAACAATGGGTTGCCGAAACTTTAGGTGATGG
Rev1E	GGCGAGGTCTTTCGGAATGGTGG
oligo-dT	TTTTTTTTTTTTTTTTTTTC
ACT1SEQA	CAAGAAATGCAAACCGCTGC
ACT1SEQB	GGTCAATACCGGCAGATTCC
pRS-Rev1-fwd	GCTTTGAGTTGGGGTAGATTATCGC
pRS-Rev1-rev	GTGTTGGTACCAAAGGAGGAGTCGGCCATTCC
REV7DELAFWD	AGTATGTATTTCTTTTCCCCTTGCT
REV7DELDBKWD	CGCCACTTACAAAATATTCAAGACT

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Chapter Three

Characterization of Novel Motifs Required for *REV1* Function

Abstract

Identified in 1971 in a screen for *reversionless* mutants, the *REV1* gene underlies the majority of spontaneous and damage-induced mutagenesis in eukaryotes from yeast to humans (11, 33). Although a member of the Y family of translesion DNA polymerases (44), Rev1 has unique properties not shared by the other Y family members. Rev1 possesses limited polymerase activity specialized to insert primarily dCMP nucleotides opposite to template G's and certain DNA lesions (29, 42, 62). Additionally, Rev1 contains domains not present in other Y family members, including an N-terminal BRCT domain (3) and a C-terminal interaction region (14, 39, 43, 54, 55). Intriguingly, the catalytic activity of Rev1 is not essential for its function in survival and mutagenesis *in vivo* (18, 51) whereas a role provided by the BRCT domain and the C-terminal region is required for *REV1* activity (15, 28, 33, 51). Differences have been observed in the contributions of the various domains of Rev1 to its function in the several systems used to study *REV1* activity (15, 19, 50, 51). In particular, the C-terminal interaction region of Rev1 has been reported to be lacking or very degenerate in yeast (6, 22, 39, 51, 55). To clarify the roles of each domain in Rev1 function, as well as to address the conservation of the C-terminal interaction region in yeast, I have undertaken a structure-function study to probe the molecular mechanisms behind Rev1's enigmatic role in mutagenesis *in vivo*. I have generated extensive protein sequence alignments which reveal conserved motifs among Rev1 proteins from various organisms, in particular in the C-terminal interaction region. Site-directed mutants in these motifs showed a functional relevance of these domains to *REV1* function. Additionally, mutants affecting the catalytic domain revealed further details of *REV1* activity. Future work to characterize the molecular nature of the defects uncovered will provide a robust understanding of the mechanisms controlling Rev1's unique role in DNA damage tolerance *in vivo*.

Introduction

Cells are constantly exposed to DNA damage from endogenous, as well as exogenous, sources. For example, it has been estimated that mammalian cells suffer ~30,000 spontaneous DNA lesions per day (34). To respond to and repair this extensive DNA damage, cells have developed a battery of DNA repair and damage tolerance pathways (8). The majority of such responses function in an error-free manner to restore the correct DNA sequence and maintain genomic stability. However, one DNA damage tolerance pathway, known as translesion synthesis (TLS), is responsible for the active generation of mutations into the genomes of all organisms (8, 29). Translesion synthesis is the process by which a nucleotide is inserted opposite to a DNA lesion and is mediated by a specialized class of DNA polymerases known as translesion polymerases (7, 13, 48).

Translesion polymerases are thought to rescue stalled replication forks and enable continuous DNA synthesis on damaged templates during replication (6, 31). In addition, TLS polymerases may promote post-replicative gap filling at regions of ssDNA produced by replication fork repriming downstream of a lesion (32, 35, 59). In either situation, TLS polymerases mediate their function by providing lesion bypass capabilities to cells. The ability to use an increased structural diversity of DNA bases as a template is paid for by a decreased fidelity of replication (13, 26, 48). On undamaged DNA, TLS polymerases display an increased error rate of up to five orders of magnitude relative to replicative DNA polymerases (7, 26). However, it is now thought that each TLS polymerase may be specialized to bypass a particular class of DNA lesions (7). For two TLS polymerases, the efficiency and fidelity of translesion synthesis opposite a cognate lesion has been shown to exceed that of polymerization on undamaged DNA templates (20, 27, 36, 37). Nevertheless, TLS polymerases are presumably tightly regulated inside the cell to avoid inappropriate synthesis on non-cognate lesions or undamaged DNA, uninhibited replication of which would lead to a high level of mutations. A major mode of regulation of TLS polymerases is accomplished at the level of protein-protein interactions which activate TLS both by recruitment to sites of DNA damage and by direct biochemical stimulation of lesion bypass (6, 8, 47, 49, 56). Additionally, protein-protein interactions leading to post-translational modifications of TLS polymerases control the activity

of prokaryotic TLS polymerases (8) and are likely to exist as well in eukaryotes [(2); L. Waters and M.E. Wiltrout, unpublished data. See Appendix B).

In 1971, a screen for *reversionless* mutants of yeast defective in UV-induced mutagenesis uncovered the *REV1* gene, which is required for ~95% of cellular mutagenesis (30, 33). Rev1 activity promotes mutagenesis from yeast to humans (11, 33). When cloned and sequenced, the Rev1 protein was shown to have homology with *E. coli* UmuC (28), isolated in a similar screen for *unmutable* bacteria strains (23). However, the significance of this finding was not appreciated for another decade until the recognition that Rev1 and UmuC are members of a novel family of DNA polymerases able to replicate over DNA lesions that would otherwise block replication (8, 13, 44, 48).

In addition to its central TLS polymerase domain, Rev1 has three other characterized regions: an N-terminal BRCT domain (3), two repeats of a ubiquitin binding motif (UBM) (2), and a C-terminal polymerase interaction region (14, 39, 43, 54, 55). Interestingly, mutations in all three of the latter regions have been reported to severely affect Rev1's ability to promote survival and mutagenesis after DNA damage (15, 16, 19, 28, 33, 51). In contrast, mutations perturbing catalytic function do not seem to alter overall levels of survival and mutagenesis (18, 51), although they do affect the spectrum of mutations generated after DNA damage (45, 50). Taken together, these findings have led to a model in which Rev1 functions primarily to assemble a mutagenic translesion complex rather than to directly bypass lesions itself (6, 14, 18, 31, 55). In support of a principally non-catalytic role for Rev1, it has been observed that *REV1* is required for bypass of 6-4 TT dimers *in vivo*, although *in vitro* the specialized dCMP transferase activity of Rev1 is unable to insert a nucleotide opposite to UV photoproducts (41, 45, 62).

First characterized as the *BR*CA1 *C-t*erminus, BRCT domains from a variety of proteins mediate protein-protein interactions, often in a phospho-specific manner (3, 12). Additionally, a limited subset of BRCT domains interact with DNA, often at regions of ssDNA or DSBs (25, 60). The original *rev1-1* mutation isolated in 1971 was determined to result from a G193R point mutation in the BRCT domain of Rev1 (11, 28). In yeast, this mutant severely disrupts *REV1* function, displaying at most ~10% activity [(5, 28, 33); L. Waters, unpublished data, see Fig. 5B, F]. The relative importance of the BRCT domain to *REV1* activity is less clear in higher eukaryotic systems. It has been reported that chicken DT40 cells expressing BRCT-defective versions of human *REV1* show no sensitization to DNA damaging agents (51). In contrast, other

groups have shown that DT40 cells expressing mouse *REV1* require an intact BRCT domain for *REV1* function *in vivo* (15) and mouse ES cells bearing a *REV1* allele lacking the BRCT domain exhibit a mild phenotype (19). The reason for the discrepancies between these results is not clear, however the report which found no contribution of the BRCT domain to *REV1* function only examined sensitivity to DNA damaging agents (51), while the latter two studies showed the importance of the BRCT domain in several assays, including subcellular localization (15), interactions with PCNA (15), cell cycle progression (19), UV-induced mutagenesis (19), chromosomal rearrangements (15, 19), and to DNA damaging agents (15, 19). Therefore it seems very likely the BRCT domain of Rev1 plays a major role in *REV1* function from yeast to humans. Importantly, the BRCT domain of both yeast and mouse Rev1 mediates an interaction with PCNA and this interaction is abolished in *rev1-1* mutants (15, 29). The Rev1-1 protein, when purified, retains its lesion bypass ability (41), consistent with the idea that the disruption of *REV1* function by mutation of the BRCT domain specifically affects a second, non-catalytic function of Rev1 mediated by key protein-protein interactions.

All the eukaryotic Y family polymerases have motifs shown to confer the ability to interact with ubiquitin: polymerase ι and Rev1 share a similar ubiquitin binding motif known as a UBM, while polymerase κ and polymerase η have a distinct motif composed of a zinc-finger, termed the UBZ (2). These ubiquitin binding motifs promote interaction with monoubiquitinated PCNA to localize the translesion polymerases into DNA damage induced foci at sites of stalled replication (2, 46). Very recently, it has been shown that the UBMs of mouse Rev1 mediate these same functions (16). I identified these motifs independently and have characterized their importance to Rev1 function in yeast.

Mammalian Rev1 protein interacts with all of the other translesion polymerases—polymerase η, polymerase ι, polymerase κ, and the Rev7 subunit of polymerase ζ, as well as the X-family DNA polymerase λ—through its C-terminal ~100 amino acids (14, 39, 43, 55). This interaction appears to be functionally important since cells expressing Rev1 lacking the C-terminus displays a severe loss of viability after DNA damage, comparable to a deletion mutant (28, 51). The presence of a conserved C-terminal interaction region in yeast has been debated since the region was originally reported to be absent from yeast Rev1 and is now thought to be quite degenerate (6, 39, 51). Moreover, its function in interacting with other TLS polymerases appears less complex in *S. cerevisiae,* which only contains two other TLS polymerases. In

contrast to its role in mammalian cells, DNA polymerase η appears to function independently of Rev1 genetically and biochemically in yeast [(6, 38); R. Woodruff, unpublished data]. Additionally, in yeast DNA polymerase ζ interacts with Rev1 outside of the C-terminal ~100 amino acids (1, 5), so the polymerase interaction motif may not be required or necessary. Thus, Rev1 from higher eukaryotes has been proposed to have developed additional functions not present in lower eukaryotes (6, 22, 39, 55). It should be noted, however, that full-length yeast Rev1, but not a version of Rev1 lacking the C-terminus, is able to stimulate the lesion bypass activity of polymerase ζ, indicating that the C-terminal region of yeast Rev1 interacts with polymerase ζ *in vitro* (17). Thus, the role of the C-terminus of Rev1 in yeast has remained unclear.

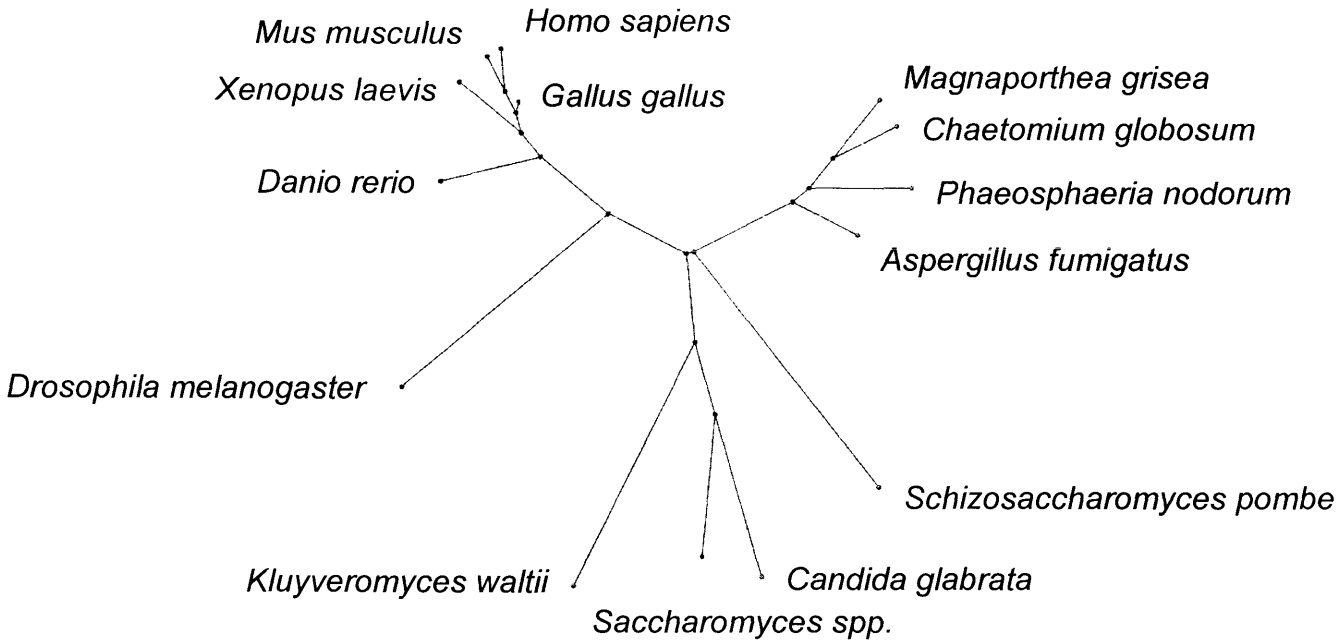
In this chapter, I show that the C-terminus of Rev1 does exhibit reasonable homology among most eukaryotes, with a major exception of worms and plants. Additionally, I show that conserved motifs in the C-terminus mediate a critical function for *REV1* activity. The regulation of Rev1 by ubiquitin binding was probed through mutations in the UBMs. I present data confirming that the catalytic activity of Rev1 is dispensable for survival and mutagenesis *in vivo*. In contrast, I demonstrate that mutations affecting non-catalytic function of the polymerase domain of Rev1 disrupt Rev1's function by showing that the DNA-binding ability of Rev1 is crucial for its function. Future experiments described in the Discussion will determine the molecular nature of the defects in the mutants and allow characterization of the mechanisms by which the various domains of Rev1 mediate its function in DNA damage resistance and mutagenesis.

Results

Alignments of Rev1 from multiple species reveal conserved motifs in the C-terminus which are critical for function.

To address the question of the conservation of the C-terminal ~100 amino acids of Rev1, which mediate contacts with multiple other polymerases in mammalian cells, I obtained and aligned Rev1 sequences from a variety of organisms, including multiple yeast species (Fig. 1A). Throughout this chapter, the term yeast will be used to refer specifically to *S. cerevisiae* unless indicated otherwise. It has been reported that yeast Rev1 lacks the C-terminal interaction region (39), however extensive inspection and hand alignment identified a reasonable level of homology (Fig. 1B). In the C-terminal ~100 amino acids, several motifs could be observed. Four amino acids were absolutely conserved throughout over 50 organisms: a tryptophan set into a putative α-helix, a proline flanked by small residues characteristic of a β-turn motif and followed by a highly conserved charged motif including an invariant aspartate, and a tryptophan in a less conserved region (Fig. 1B). To test whether these areas of limited homology mediated a conserved function between yeast and mammalian Rev1, I created site-directed alanine-patch mutations (Fig. 1B) and assayed for survival and mutagenesis after UV irradiation. Four of the five mutations (*rev1-108*, *rev1-109*, *rev1-110*, *rev-111*) completely abolished *REV1*-mediated survival and the fifth mutation (*rev1-112*) reduced survival efficiency by half (Fig. 2A, B). Preliminary mutagenesis results were consistent with the survival data, however were not statistically robust enough to include and will be repeated. Additionally, four of the five predicted polymerase interaction mutants (*rev1-108*, *rev1-109*, *rev1-110*, *rev-111*) displayed pronounced sensitivity to MMS, comparable to the *rev1Δ* mutation (Fig. 2C). Thus, the Rev1 C-terminus appears to be conserved between yeast and humans at the sequence and functional levels, mediating a critical function of *REV1* activity both in yeast and humans.

1A


Mus musculus
Homo sapiens
Xenopus laevis
Gallus gallus
Magnaporthea grisea
Chaetomium globosum
Danio rerio
Phaeosphaeria nodorum
Aspergillus fumigatus
Drosophila melanogaster
Schizosaccharomyces pombe
Kluyveromyces waltii
Candida glabrata
Saccharomyces spp.

1B

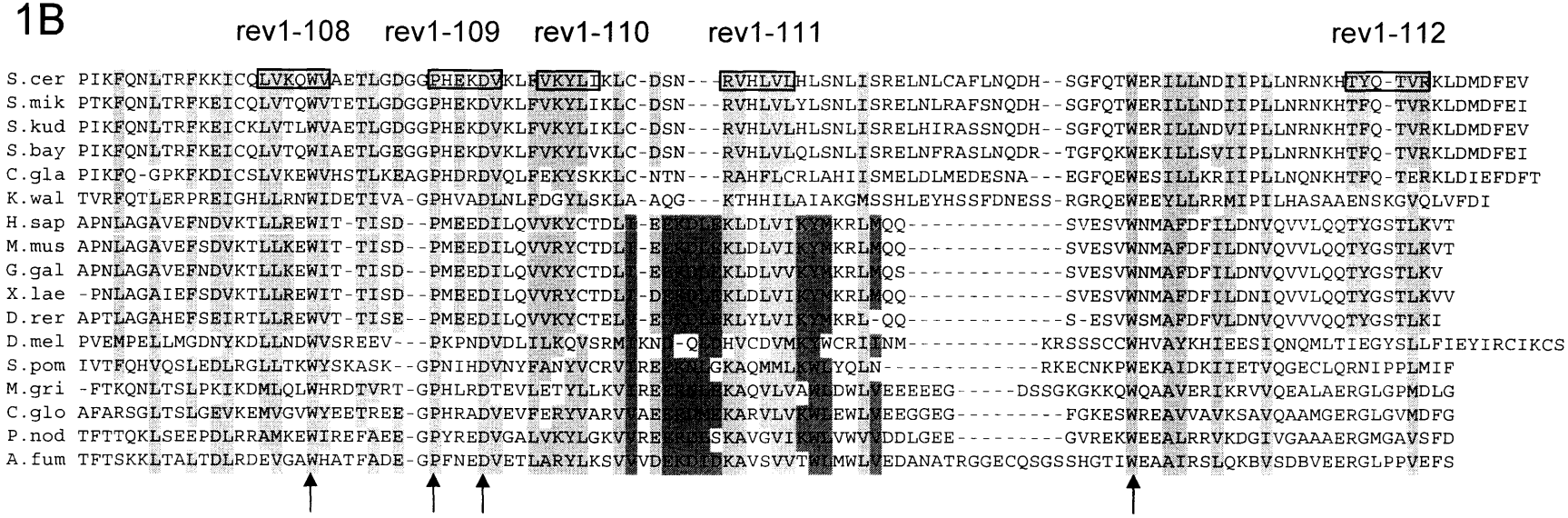

rev1-108
rev1-109
rev1-110
rev1-111
rev1-112
S.cer PIKFQNLTRFKKICQLVKQWVAETLGDGGPHEKDVKLHVKYLIKLC-DSN---RVHLVLHLSNLISRELNLCAFLNQDH--SGFQTWERILLNDIIPLLNRNKHTYQ-TVRKLDMDFEV
S.mik PTKFQNLTRFKEICQLVTQWVTETLGDGGPHEKDVKLFVKYLIKLC-DSN---RVHLVLYLSNLISRELNLRAFSNQDH--SGFQTWERILLNDIIPLLNRNKHTFQ-TVRKLDMDFEI
S.kud PIKFQNLTRFKEICKLVTLWVAETLGDGGPHEKDVKLFVKYLIKLC-DSN---RVHLVLHLSNLISRELHIRASSNQDH--SGFQTWERILLNDVIPLLNRNKHTFQ-TVRKLDMDFEV
S.bay PIKFQNLTRFKEICQLVTQWIAETLGEGGPHEKDVKLFVKYLVKLC-DSN---RVHLVLQLSNLISRELNFRASLNQDR--TGFQKWEKILLSVIIPLLNRNKHTFQ-TVRKLDMDFEI
C.gla PIKFQ-GPKFKDICSLVKEWVHSTLKEAGPHDRDVQLFEKYSKKLC-NTN---RAHFLCRLAHIISMELDLMEDESNA---EGFQEWESILLKRIIPLLNQNKHTFQ-TERKLDIEFDFT
K.wal TVRFQTLERPREIGHLLRNWIDETIVA-GPHVADLNLFDGYLSKLA-AQG---KTHHILAIAKGMSSHLEYHSSFDNESS-RGRQEWEEYLLRRMIPILHASAAENSKGVQLVFDI
H.sap APNLAGAVEFNDVKTLLREWIT-TISD--PMEEDILQVVKYCTDLE-EKYFKKLDLVIKYVKRLVQQ----------SVESVWNMAFDFILDNVQVVLQQTYGSTLKVT
M.mus APNLAGAVEFSDVKTLLKEWIT-TISD--PMEEDILQVVRYCTDLE-EKYFKKLDLVIKYVKRLVQQ----------SVESVWNMAFDFILDNVQVVLQQTYGSTLKVT
G.gal APNLAGAVEFNDVKTLLKEWIT-TISD--PMEEDILQVVKYCTDLE-EKYFKKLDLVVKYVKRLVQS----------SVESVWNMAFDFILDNVQVVLQQTYGSTLKV
X.lae -PNLAGAIEFSDVKTLLREWIT-TISD--PMEEDILQVVRYCTDLE-DKYFKKLDLVIKYVKRLVQQ----------SVESVWNMAFDFILDNIQVVLQQTYGSTLKVV
D.rer APTLAGAHEFSEIRTLLREWVT-TISE--PMEEDILQVVKYCTELE-EKYFKKLYLVIKYVKRL-QQ----------S-ESVWSMAFDFVLDNVQVVVQQTYGSTLKI
D.mel PVEMPELLMGDNYKDLLNDWVSREEV---PKPNDVDLILKQVSRMEKNLEQLGHVCDVMKYWCRIENM----------KRSSSCCWHVAYKHIEESIQNQMLTIEGYSLLFIEYIRCIKCS
S.pom IVTFQHVQSLEDLRGLLTKWYSKASK--GPNIHDVNYFANYVCRVERDKQLGKAQMMLKRLYQLN----------RKECNKPWEKAIDKIIETVQGECLQRNIPPLMIF
M.gri -FTKQNLTSLPKIKDMLQLWHRDTVRT-GPHLRDTEVLETYLLKVERESKLKAQVLVAKLDWLVEEEEEG-----DSSGKGKKQWQAAVERIKRVVQEALAERGLGPMDLG
C.glo AFARSGLTSLGEVKEMVGVWYEETREE-GPHRADVEVFERYVARVEAESKLKEKARVLVKKLEWLVEEGGEG---------FGKESWREAVVAVKSAVQAAMGERGLGVMDFG
P.nod TFTTQKLSEEPDLRRAMKEWIREFAEE-GPYREDVGALVKYLGKVERESKLESKAVGVIKKLVWVVDDLGEE---------GVREKWEEALRRVKDGIVGAAAERGMGAVSFD
A.fum TFTSKKLTALTDLRDEVGAWHATFADE-GPFNEDVETLARYLKSVVVDEKIDKAVSVVTWLMWLVEDANATRGGECQSGSSHGTIWEAAIRSLQKBVSDBVEERGLPPVEFS

Fig. 1 The C-terminal ~100 amino acids of Rev1 is conserved across a wide range of organisms. (*A*) An unrooted phylogenetic tree showing the diversity of species used to generate the alignment in part B. (*B*) Multiple sequence alignment of Rev1 sequences from selected species. Boxed residues and the text above indicate the alanine patch mutations. (See Table 3 for more details.) Amino acids highlighted in light grey show conservation across all species, while amino acids highlighted in dark grey indicate a conserved region not found in *S. cerevisiae* and closely related yeasts. Arrows indicate absolutely conserved amino acids. Abbreviations are as follows *S. cerevisiae* (S. cer), *S. mikatae* (S. mik), *S. kudriavzevii* (S. kud), and *S. bayanus* (S. bay). The remainder are as indicated in part A. Rev1 sequences from over 50 organisms were used to generate the alignment, but only 17 are shown due to space considerations.

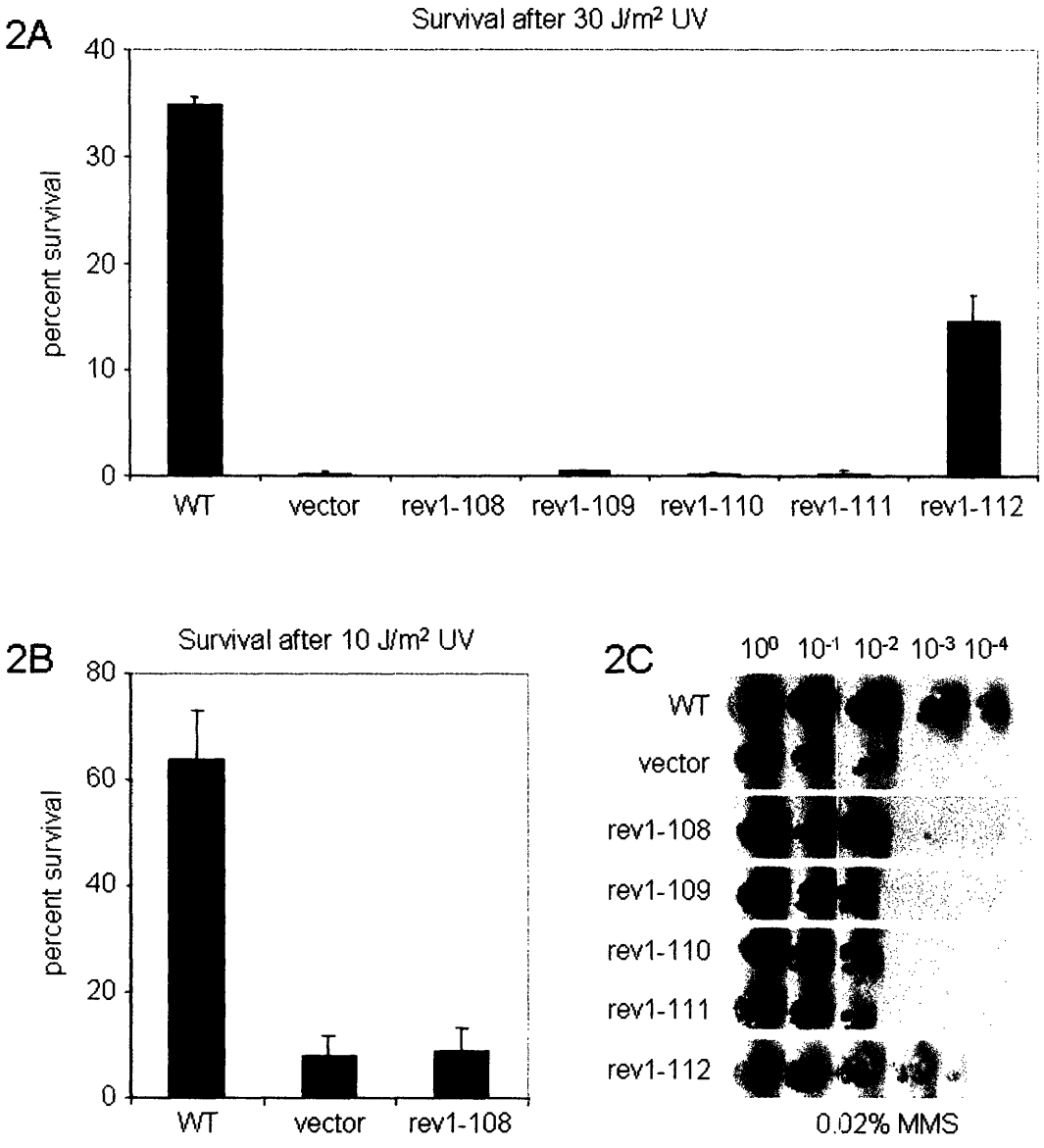
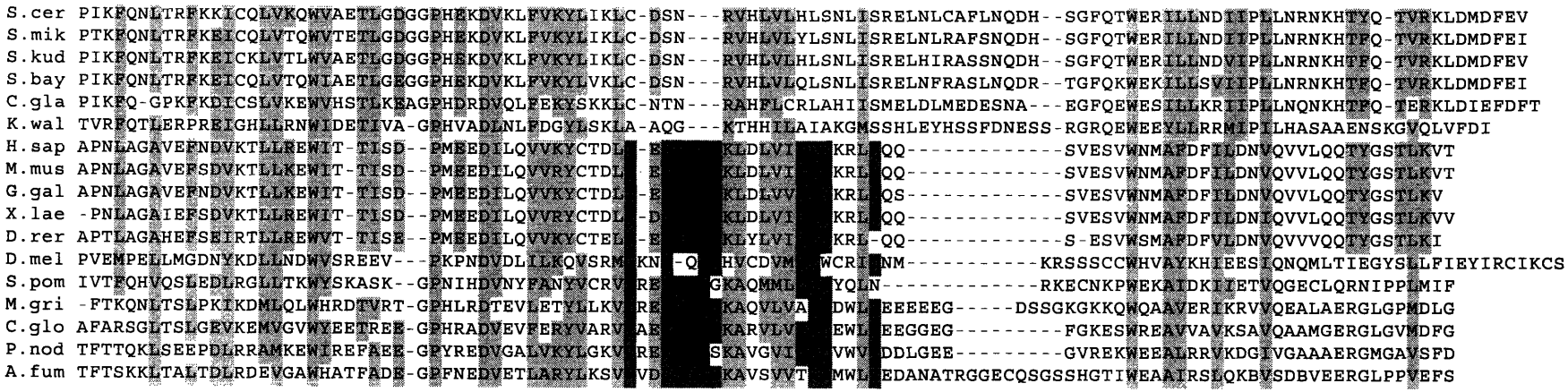


Fig. 2 Mutations in predicted polymerase interaction motifs disrupt *REV1*-mediated survival. (*A*) Survival of the indicated mutants after a dose of 30 J/m^2 UV irradiation. Note that no colonies were recovered for *rev1-108*. (*B*) Survival of *rev1-108* after a dose of 10 J/m^2 UV irradiation. (*C*) Sensitivity of the mutants to chronic exposure to 0.02 % MMS.

Further bioinformatic analysis of the Rev1 C-terminus revealed that the region may fold into an α-helical bundle (Fig. 3A, B). The first half of the C-terminal ~100 amino acids is predicted to have a highly conserved secondary structure consisting of two putative α-helices. The pattern of hydrophobic residues indicated that the predicted α-helical stretches, in particular the first helix, may form amphipathic helices (Fig. 3C). The charged/polar face may contain species-specific binding interface since it does not exhibit a conserved pattern of positive, negative, and polar residues. Interestingly, a particularly conserved motif starting just after the second predicted helix (highlighted in dark gray in Fig. 3A) is present only in organisms more distant from *Saccharomyces* and its relatives. This motif may represent a particular interaction site not found in *S. cerevisiae* Rev1. Like the first part of the C-terminus, the final ~50 amino acids appear to be primarily α-helical, however the location of the predicted helices is not as conserved throughout Rev1 sequences from various species. The reduced homology appears to correlate with a decreased importance of this region for *REV1* function in yeast (see Fig. 2A and 2C, *rev1-112*).

3A


S.cer PIKFQNLTRFKKICQLVKQWVAETLGDGGPHEKDVKLFVKYLIKLC-DSN---RVHLVLHLSNLISRELNLCAFLNQDH--SGFQTWERILLNDIIPLLNRNKHTYQ-TVRKLDMDFEV
S.mik PTKFQNLTRFKEICQLVTQWVTETLGDGGPHEKDVKLFVKYLIKLC-DSN---RVHLVLYLSNLISRELNLRAFSNQDH--SGFQTWERILLNDIIPLLNRNKHTFQ-TVRKLDMDFEI
S.kud PIKFQNLTRFKEICKLVTLWVAETLGDGGPHEKDVKLFVKYLIKLC-DSN---RVHLVLHLSNLISRELHIRASSNQDH--SGFQTWERILLNDVIPLLNRNKHTFQ-TVRKLDMDFEV
S.bay PIKFQNLTRFKEICQLVTQWIAETLGEGGPHEKDVKLFVKYLVKLC-DSN---RVHLVLQLSNLISRELNFRASLNQDR--TGFQKWEKILLSVIIPLLNRNKHTFQ-TVRKLDMDFEI
C.gla PIKFQ-GPKFKDICSLVKEWVHSTLKEAGPHDRDVQLFEKYSKKLC-NTN---RAHFLCRLAHIISMELDLMEDESNA---EGFQEWESILLKRIIPLLNQNKHTPQ-TERKLDIEFDFT
K.wal TVRFQTLERPREIGHLLRNWIDETIVA-GPHVADLNLFDGYLSKLA-AQG---KTHHILAIAKGMSSHLEYHSSFDNESS-RGRQEWEEYLLRRMIPILHASAAENSKGVQLVFDI
H.sap APNLAGAVEFNDVKTLLREWIT-TISD--PMEEDILQVVKYCTDL-E KLDLVI KRL QQ----------SVESVWNMAFDFILDNVQVVLQQTYGSTLKVT
M.mus APNLAGAVEFSDVKTLLKEWIT-TISD--PMEEDILQVVRYCTDL-E KLDLVI KRL QQ----------SVESVWNMAFDFILDNVQVVLQQTYGSTLKVT
G.gal APNLAGAVEFNDVKTLLKEWIT-TISD--PMEEDILQVVKYCTDL-E KLDLVV KRL QS----------SVESVWNMAFDFILDNVQVVLQQTYGSTLKV
X.lae -PNLAGAIEFSDVKTLLREWIT-TISD--PMEEDILQVVRYCTDL-D KLDLVI KRL QQ----------SVESVWNMAFDFILDNIQVVLQQTYGSTLKVV
D.rer APTLAGAHEFSEIRTLLREWVT-TISE--PMEEDILQVVKYCTEL-E KLYLVI KRL -QQ----------S-ESVWSMAFDFVLDNVQVVVQQTYGSTLKI
D.mel PVEMPELLMGDNYKDLLNDWVSREEV---PKPNDVDLILKQVSRM KN -Q HVCDVM WCRI NM----------KRSSSCCWHVAYKHIEESIQNQMLTIEGYSLLFIEYIRCIKCS
S.pom IVTFQHVQSLEDLRGLLTKWYSKASK--GPNIHDVNYFANYVCRV RE GKAQMML YQLN----------RKECNKPWEKAIDKIIETVQGECLQRNIPPLMIF
M.gri -FTKQNLTSLPKIKDMLQLWHRDTVRT-GPHLRDTEVLETYLLKV RE KAQVLVA DWL EEEEEG-----DSSGKGKKQWQAAVERIKRVVQEALAERGLGPMDLG
C.glo AFARSGLTSLGEVKEMVGVWYEETREE-GPHRADVEVFERYVARV AE KARVLV EWL EEGGEG---------FGKESWREAVVAVKSAVQAAMGERGLGVMDFG
P.nod TFTTQKLSEEPDLRRAMKEWIREFAEE-GPYREDVGALVKYLGKV RE SKAVGVI VWV DDLGEE---------GVREKWEEALRRVKDGIVGAAAERGMGAVSFD
A.fum TFTSKKLTALTDLRDEVGAWHATFADE-GPFNEDVETLARYLKSV VD KAVSVVT MWL EDANATRGGECQSGSSHGTIWEAAIRSLQKBVSDBVEERGLPPVEFS

3B


S.cer PIKFQNLTRFKKICQLVKQWVAETLGDGGPHEKDVKLFVKYLIKLC-DSN---RVHLVLHLSNLISRELNLCAFLNQDH--SGFQTWERILLNDIIPLLNRNKHTYQ-TVRKLDMDFEV
H.sap APNLAGAVEFNDVKTLLREWIT-TISD--PMEEDILQVVKYCTDL-E KLDLVI KRL QQ----------SVESVWNMAFDFILDNVQVVLQQTYGSTLKVT

3C

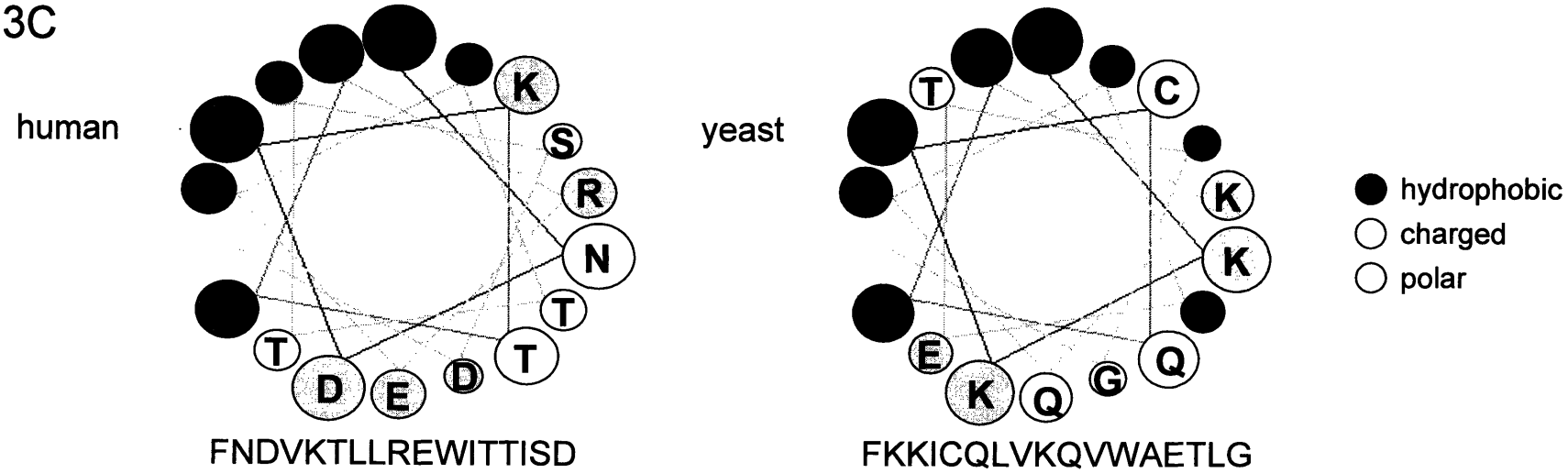
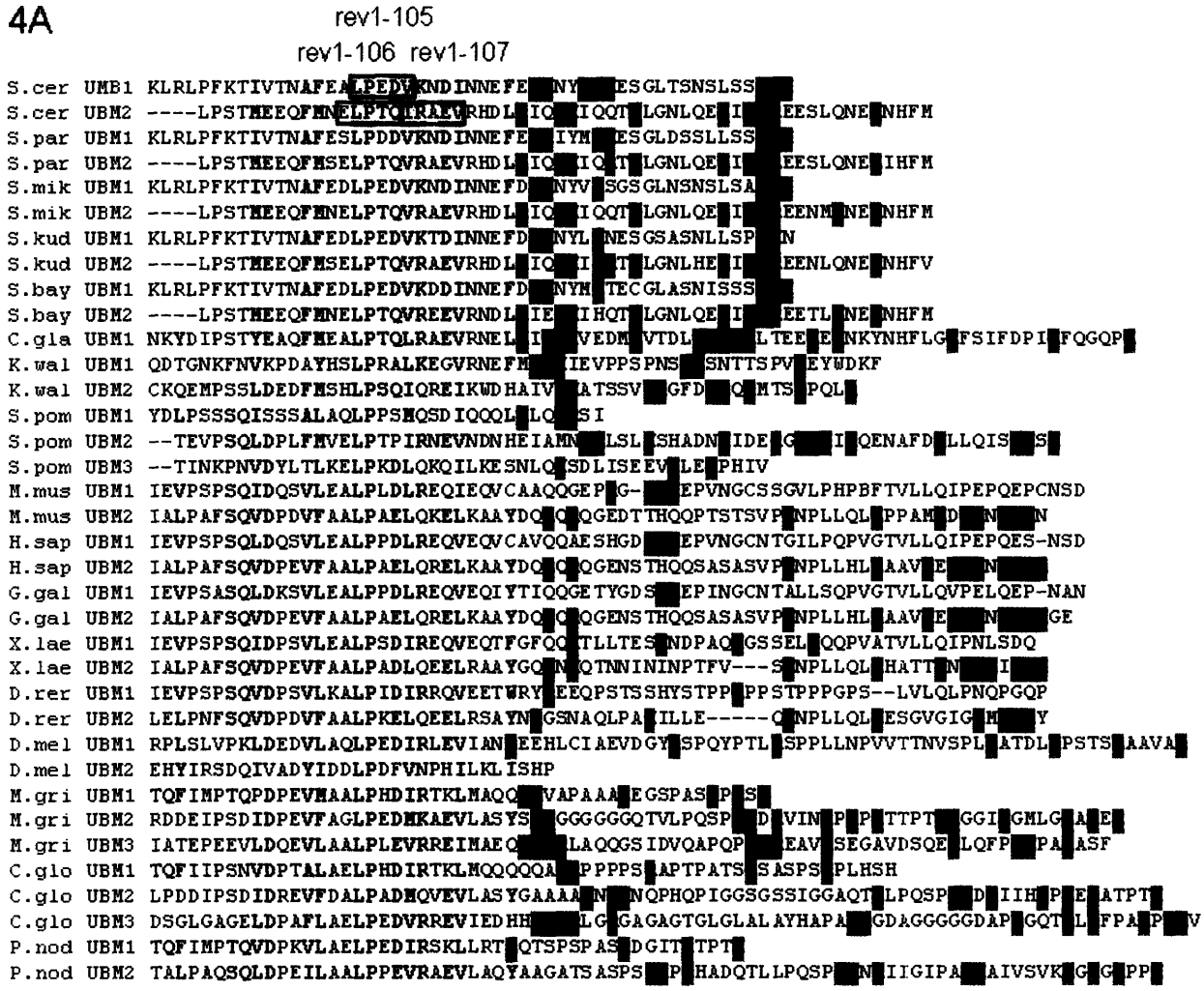

human
K
S
R
N
T
T
D
E
D
T
FNDVKTLLREWITTISD
yeast
T
C
K
K
E
K
Q
G
Q
FKKICQLVKQVWAETLG
hydrophobic
charged
polar

Fig. 3 Predicted secondary structure of the C-terminus of Rev1. (*A*) Multiple sequence alignment of Rev1 sequences from selected species. The dark line shows the location of the amino acids shown in part C. Amino acids highlighted in light grey show conservation across all species, while amino acids highlighted in dark grey indicate a conserved region not found in *S. cerevisiae* and closely related yeasts. (*B*) Alignment of yeast and human Rev1 excerpted from part A for clarity with the predicted helices for each sequence indicated by wavy lines. The dots connecting the last two helices of human Rev1 indicate that this helix is continuous. (*C*) Helical wheel projections of the first predicted helix. Dark grey indicates hydrophobic residues, light grey indicates charged residues, and white indicates polar residues and glycine. Size represents the position along the helix, the larger circles indicate more N-terminal residues.

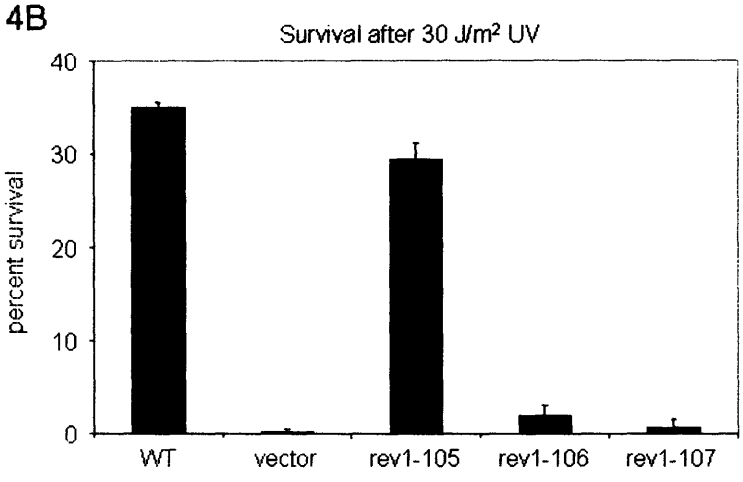
Identification of an additional conserved motif required for REV1 function.

In the intervening region between the end of the polymerase domain and the C-terminal ~100 amino acids of Rev1, very little homology was observed among Rev1 sequences from all organisms. This region was of variable lengths (~136 amino acids in yeast, ~326 amino acids in humans) and difficult to align. However, one motif was identified which was present in zero, one, two, or three repeats (Fig. 4A). This motif was approximately 20 amino acids in length and characterized by an absolutely conserved leucine-proline flanked by a fairly degenerate sequence (Fig. 4A). Interestingly, the motif was also found in the Tom1 protein, a ubiquitin ligase that functions in multiple processes including rRNA maturation and export, transcription, sporulation, stress responses, and cell cycle progression (4, 52, 53, 57). Recently, the "Tom1" motif in Rev1 was shown to be an ubiquitin binding motif (UBM) present in multiple TLS polymerases (2). To address the contribution of these motifs to Rev1 function, site-directed mutants consisting of alanine patches were generated and assayed for survival and mutagenesis after UV irradiation. Although UBM1 appears to be dispensable for *REV1* function, mutants in UBM2 displayed a severe defect in survival after UV irradiation and exposure to MMS (Fig. 4B, C). Preliminary mutagenesis experiments confirmed the findings. The lack of phenotype in the UBM1 mutant may not be surprising since UBM1 is less well conserved in yeasts. Interestingly, however, both UBM1 and UBM2 contribute equally to *REV1* function in mammalian cells (16).

4A


rev1-105
rev1-106 rev1-107
S.cer UMB1
S.cer UBM2
S.par UBM1
S.par UBM2
S.mik UBM1
S.mik UBM2
S.kud UBM1
S.kud UBM2
S.bay UBM1
S.bay UBM2
C.gla UBM1
K.wal UBM1
K.wal UBM2
S.pom UBM1
S.pom UBM2
S.pom UBM3
M.mus UBM1
M.mus UBM2
H.sap UBM1
H.sap UBM2
G.gal UBM1
G.gal UBM2
X.lae UBM1
X.lae UBM2
D.rer UBM1
D.rer UBM2
D.mel UBM1
D.mel UBM2
M.gri UBM1
M.gri UBM2
M.gri UBM3
C.glo UBM1
C.glo UBM2
C.glo UBM3
P.nod UBM1
P.nod UBM2

4B


Survival after 30 J/m² UV
percent survival
40
30
20
10
0
WT
vector
rev1-105
rev1-106
rev1-107

4C

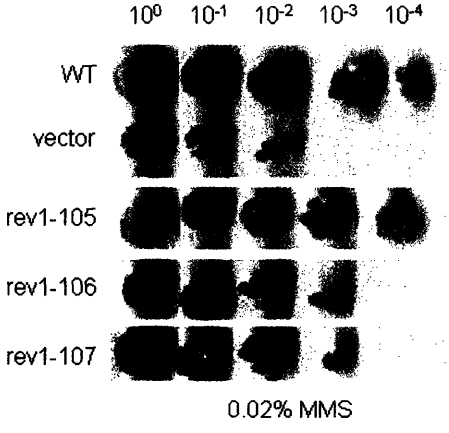

10⁰
10⁻¹
10⁻²
10⁻³
10⁻⁴
WT
vector
rev1-105
rev1-106
rev1-107
0.02% MMS

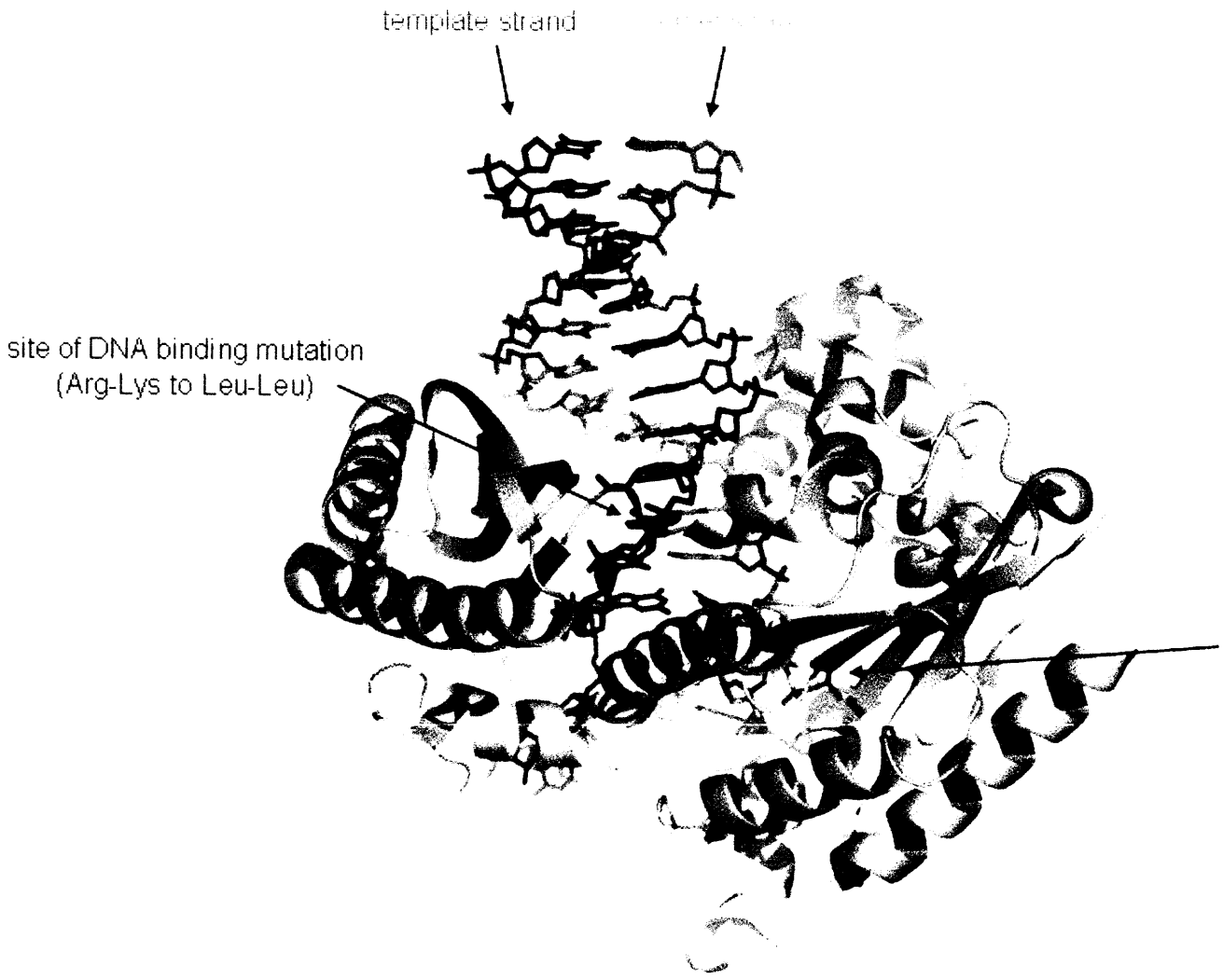
Fig. 4 Mutations in UBM2, but not UBM1, abolish *REV1*-mediated survival and mutagenesis. (*A*) Multiple sequence alignment of the Rev1 UBMs. Species and abbreviations are as in Fig. 1. Amino acids highlighted in light grey indicate conserved residues, amino acids highlighted in dark grey show positively charged Lys and Arg residues. Amino acids shown in yellow correspond to the SQ/TQ ATM/ATR consensus phosphyorylation sequence. (*B*) Survival of the indicated strains after a dose of 30 J/m^2 UV irradiation. (*C*) Sensitivity of the mutants to 0.02 % MMS.

The DNA binding activity of Rev1 appears critical for function while the catalytic activity is dispensable.

Next, I examined the importance of the catalytic domain of Rev1. Interestingly, mutations inactivating the catalytic activity of Rev1 do not reduce survival or affect the levels of lesion bypass and mutagenesis after exposure to most DNA damaging agents (18, 45, 50, 51). However, although not required *in vivo* for survival or mutagenesis, the catalytic activity of Rev1 is indeed employed during translesion synthesis. Catalytic dead variants of Rev1 exhibit an alteration in the spectrum of mutations generated, with a reduced frequency of C's inserted opposite to abasic sites (45, 50). Additionally, preliminary results from our lab suggest that the catalytic activity of Rev1 provides some resistance to DNA damage opposite certain lesions. In particular, a strain bearing a catalytic dead Rev1 shows increased frequency of cell death relative to wild-type after exposure to 4-nitroquinoline oxide (4-NQO) (M.E. Wiltrout, unpublished data). As the major products of 4-NQO include N^2-dG adducts which Rev1 is capable of bypassing *in vitro* and *in vivo* (58, 62, 63), this observation may indicate that the cognate lesion for Rev1's highly specialized catalytic activity is a N^2-dG adducted lesion. Ongoing studies are directed at clarifying this point (M.E. Wiltrout, unpublished data).

To investigate whether other activities of the catalytic domain besides the dCMP transferase activity were required for its function, a site-directed mutant was constructed with the goal of perturbing Rev1's ability to bind DNA. Based on the crystal structure of Rev1 (40), an arginine-lysine dyad that appeared to make specific contacts with the phosphodiester backbone were mutated to two leucines. These residues were chosen since they are far from the active site in an attempt to minimize effects on the catalytic activity of Rev1 *per se* (Fig 5A). Preliminary data from a chromatin spin-down assay indicates that this mutant exhibits greatly reduced binding to DNA relative to the wild-type Rev1 protein, however due to poor yield, the Western blot had to be overexposed to observe the bands and could not be reproduced in this thesis. Interestingly, the predicted DNA binding mutant showed a severe defect in survival and mutagenesis after UV irradiation (Fig 5B-E). In contrast, the catalytic dead mutant displayed a near wild-type level of survival and mutagenesis (Fig 5B-E), consistent with previous reports (18, 45, 51). These results suggest that although the insertion of dCMP opposite to lesions is dispensable for *REV1* function in survival and mutagenesis, the ability to bind DNA mediates a critical *REV1* activity.

5A


template strand
site of DNA binding mutation
(Arg-Lys to Leu-Leu)

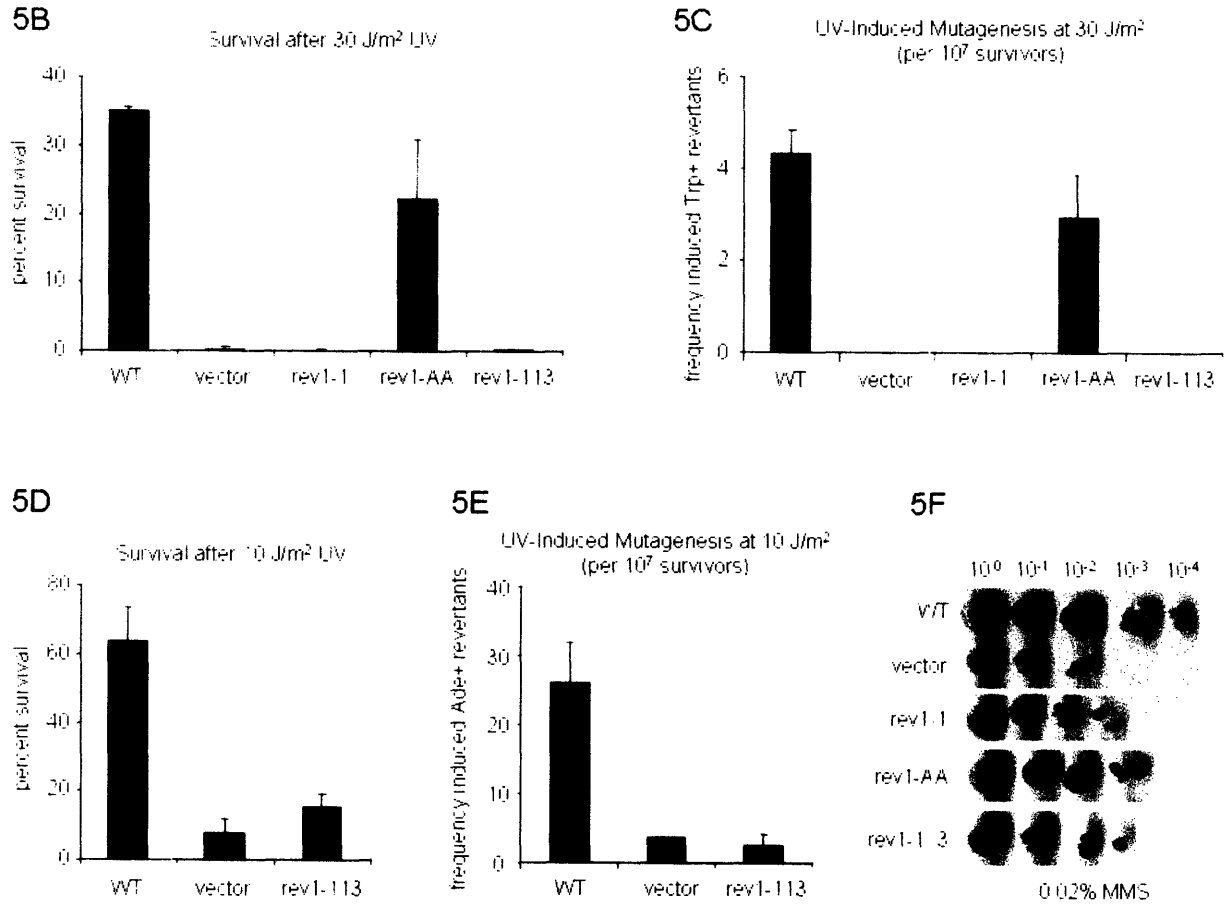


Fig. 5 The presumed DNA binding ability, but not the catalytic activity, of Rev1 is required for its function in survival and mutagenesis after UV irradiation or exposure to MMS. (*A*) X-ray crystal structure of Rev1 (40) showing the amino acids mutated in the predicted DNA binding mutant. The active site is indicated by the location of the incoming nucleotide. (*B, D*) Survival of the mutants after (*B*) 30 J/m^2 UV irradiation or (*D*) 10 J/m^2 UV irradiation. The *rev1-1* BRCT mutant was used as an additional negative control. (*C*) Reversion of the *trp1-1* allele after a dose of 30 J/m^2 UV irradiation was monitored to determine the effect of the mutants on *REV1*-mediated mutagenesis. Note that no colonies were recovered for the strains bearing the empty vector or the *rev1-1* and *rev1-113* plasmids. (*E*) Reversion of the *ade2-1* allele after 10 J/m^2 UV irradiation. (*D*) Sensitivity of the mutants to 0.02 % MMS.

Identification of additional homology preceding the BRCT.

The extensive alignments necessary to observe conservation of the Rev1 C-terminus also revealed a motif of approximately 40 amino acids just prior to the BRCT domain (Fig. 6). This extended BRCT motif is characterized by three highly conserved hydrophobic residues and an invariant lysine followed by a highly charged and polar glutamine-rich region, which could make sequence-independent contacts to the DNA backbone. This region has been identified independently in another lab by secondary structure predictions, although they could not identify any primary consensus sequence (25). Interestingly, the extended BRCT motif is characteristic of those BRCT domains that have the capability to bind to DNA, particularly DSBs (25). Thus, the BRCT domain of Rev1 may mediate protein-DNA interactions as well as protein-protein interactions. The BRCT may have a dual role in localizing Rev1 to sites of DNA damage either directly by binding to aberrant DNA structures or indirectly by interacting with monoubiquitinated PCNA marking sites of DNA damage (15).

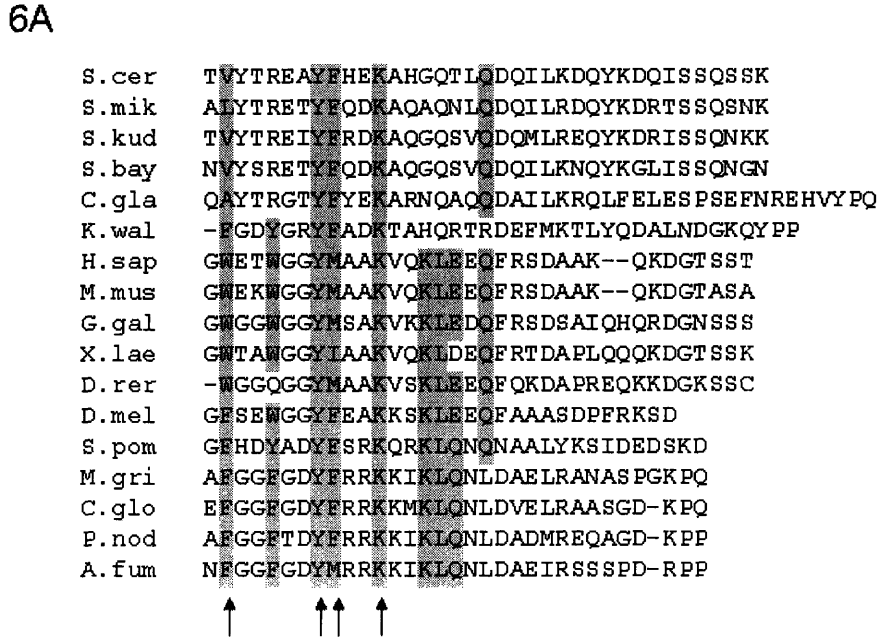


Fig. 6 Identification of an extended BRCT motif. (*A*) Multiple sequence alignment of Rev1 sequences just prior to the BRCT domain reveals a region of homology. Arrows indicate absolutely conserved amino acids and residues highlighted in light grey show similar amino acids.

Discussion

Summary of results

In this chapter, I present alignments that demonstrate homology between the polymerase interaction regions of yeast and mammalian Rev1. Importantly, I demonstrate that mutations in conserved motifs in the C-terminus of Rev1 abolish the function of Rev1 *in vivo*, supporting the idea that the C-terminus of Rev1 and its interactions with other TLS polymerases are conserved both in structure and in function. I also show that the second ubiquitin binding motif, UBM2, of Rev1 is required for its function, while UBM1 appears to be dispensable. Finally, I provide evidence that, although the catalytic activity of Rev1 is not necessary to provide resistance to UV irradiation or to facilitate mutagenesis after UV irradiation, the DNA binding ability of Rev1 may be required for these functions.

Bioinformatic analysis

C-terminal motifs and their relevance to polymerase interactions

In eukaryotes, there are five distinct TLS polymerase families: Rev1, polymerase ζ, polymerase η, polymerase ι, and polymerase κ. DNA polymerase η and polymerase ζ are conserved throughout all eukaryotes. It is thought that polymerase κ is also found in all eukaryotes and that polymerase ι is restricted to higher eukaryotes. However, both polymerase κ and polymerase ι appear to be scattered throughout Eukaryota in an ill-defined pattern (L. Waters, unpublished observation). For example, both polymerase κ and polymerase ι are found in some yeasts lineages like *Aspergillus* spp (L. Waters, unpublished observation). However, *S. cerevisiae* lacks both of these enzymes. It is currently unknown why these polymerases are found in some lineages and not in others.

Given that Rev1 from mammalian cells interacts with all of the other TLS polymerases through its C-terminal ~100 amino acids, it would be interesting to correlate the consensus sequences of the putative helices in the C-terminus of Rev1 (Fig. 3A, B) with the species in which polymerase κ and polymerase ι are found. For example, the first putative helix exhibits a

well conserved amphipathic pattern found in all species (Fig. 3C)—perhaps this helix mediates a conserved interaction with the Rev7 subunit of polymerase ζ in diverse organisms. In contrast, a region after the second predicted helix and continuing into the third helix displays a much higher level of conservation in vertebrates, flies, and Pezizomycotinate yeasts. Interestingly, the presence of this conserved motif correlates with those organisms which contain polymerase κ throughout all 50+ species investigated, suggesting that this motif may mediate a specific interaction with polymerase κ. Correlation of motifs with polymerase ι has not yet been addressed, but will be undertaken in the near future. Further such structure-function studies will provide vital information as to how Rev1 coordinates lesion bypass.

Sequence analysis of the C-terminus of Rev1 has complicated an already confusing relationship between Rev1 and polymerase η. In yeast, *REV1* and *RAD30*—the gene encoding polymerase η—exhibit additive sensitivities to DNA damaging agents, indicating that they act in parallel pathways (38). Moreover, despite intensive effort a physical interaction between them has not been identified to date [(6); R. Woodruff, unpublished data]. However, in mammalian cells, Rev1 and polymerase η interact robustly via the C-terminus of Rev1 (14, 43, 55). This has lent support to the idea that Rev1 from higher eukaryotes mediates additional functions through extra domains not found in lower eukaryotes (6, 22, 39, 55). However, I provide evidence that the C-terminal interaction region of mammalian Rev1 is conserved in yeast, both at a primary and secondary structure level, as well as functionally. These data may indicate that yeast Rev1 and Rad30 do interact in a very transient manner or under specific conditions not yet identified. Alternatively, yeast Rev1 and Rad30 may perform entirely separate roles in lesion bypass. In this case, the sequence of yeast Rev1 may reflect the lack of an interaction between the two translesion polymerases. Ideally, I would like to determine a Rad30 interaction motif by comparing those organisms in which Rev1 and polymerase η functionally interact or fail to interact, however that information is currently not known.

The C-terminus of mammalian Rev1 also interacts with the X family polymerase λ, but not the related polymerase β or polymerase μ (6). Interestingly, polymerase λ, like many enzymes which participate in BER and NHEJ, contains a BRCT domain (9). Perhaps the interaction with polymerase λ indicates that Rev1 plays a role in these repair systems, possibly by targeting BER factors to sites of unrepaired damage remaining after gap filling of ssDNA

regions opposite to DNA lesions. The interaction with polymerase λ adds another layer of complexity onto the distinct interactions the C-terminus must mediate.

Interestingly, consistent with another report (51), the C-terminal ~100 amino acids appear to be missing in Rev1 sequences from plants and extremely degenerate in worms, only being alignable in the first ~30 amino acids of the putative first α-helix. The functional significance of this is unknown as only very limited studies have been done on Rev1, or any translesion polymerases, in these organisms. As Rev1 from plants also has only one UBM which is quite degenerate and worms have no UBM repeats, this may point to either a different role for Rev1 in these organisms or a separation of function of various Rev1 activities into two distinct proteins.

Variability in UBM repeat number and sequence

I have observed that Rev1 from the Pezizomycotinate yeasts contains three UBMs, while Rev1 from plants has only one UBM. I have demonstrated that only one UBM is necessary in yeast (Fig. 4B, C), but both UBMs have been shown to contribute to Rev1 function in mouse cells (16). It would be interesting to determine the basis for this difference and to address why various organisms contain different numbers of UBM repeats. Intriguingly, Rev1 sequences from worms have no copies of the UBM repeat. Since I and others have shown that the UBMs mediate a critical function of *REV1* in yeast and mouse cells, the lack of a UBM in worm Rev1 poses the question as to whether *REV1*, and possibly other aspects of the DNA damage tolerance response, function in a different manner in worms than in other eukaryotes.

The Rev1 UBMs from most organisms contain a conserved SQ/TQ preceding the motif which represents the consensus site for ATM phosphorylation (Fig. 4A). *Saccharomyces* spp. and related yeasts lack this site before the UBM but do contain a TQ motif just after the absolutely conserved leucine-proline in UBM2. Interestingly, I have shown that UBM2 is essential for *REV1* function whereas UBM1, which lacks any SQ/TQ sites, is dispensable (Fig. 4B, C). Additionally, the UBMs are followed by basic regions rich in lysine and arginine residues (Fig. 4A). Such basic regions often represent nuclear import signals. Alternatively, they may mediate binding to negatively charged targets, such as DNA. The lysine residues may also be covalently modified with ubiquitin or SUMO moieties. It is known that the TLS polymerases become monoubiquitinated in a UBM-dependent manner, although it is currently

unclear what the biological relevance of this modification may be (2, 16). Therefore, besides mediating interactions with ubiquitin, phosphorylation or other modifications of the UBM may alter nuclear import, substrate binding, and/or protein stability of Rev1.

Additional and missing motifs an indicator of different REV1 functions?

In addition to the degeneracy of the sequences, the variation in the length between regions of homology led to difficulty in determining an alignment for Rev1 after the polymerase domain. In yeast, the length between the end of the little finger domain and the C-terminal region is ~138 amino acids, whereas in humans it is ~326 amino acids. In yeast, the entire ~138 amino acids seem to comprise only the two UBMs and short linker regions between them. Humans also have two UBMs, yet this region is more than twice as long. Although the significance of these additional sequences is not known, they may provide more binding surfaces, potentially for interactors specific to higher eukaryotes. Yeast Rev1 has ~120 amino acids preceding the N-terminal BRCT domain which are not found in human Rev1. This region may represent an alternative binding surface to the extra sequences flanking the human UBMs or it may mediate a yeast-specific *REV1* function. Rev1 sequences from other organisms also contain insertions or deletions relative to yeast and human Rev1. In the future, it would be interesting to delineate the differences, if any, between *REV1* functions throughout eukaryotes by determining whether these regions mediate novel functions or simply are alternate mechanisms of achieving the same end.

Future Directions

The data shown in this chapter provides evidence for the importance of various motifs to *REV1* function in survival and mutagenesis. However, the molecular nature of the defects in the mutants and their physiological relevance to *REV1* activity are currently unknown. Further investigations addressing these points are underway and are described below. First, new strains have been constructed to facilitate the analysis. The experiments presented here were performed with site-directed mutants created in a low-copy vector under the native *REV1* promoter and containing the native 3′ UTR. Epitope tagged versions of the plasmids were created, but

contained a mutation in the tag that inactivated it. Therefore, it is not known whether the UBM and polymerase interaction mutants are expressed at the native level. The putative DNA binding mutant was generated at a later time and was successfully epitope tagged and detected. It is expressed at close to the native levels and preliminary chromatin spin-down experiments indicated that it failed to bind chromatin. All of the mutants have been moved into an integrating vector and been successfully crossed into the chromosome. The mutants are in the process of being epitope tagged to allow visualization of protein levels. Once this is completed, the following experiments will be performed to determine the molecular nature of the defects in the mutant strains. Once I have investigated the effect of the mutations on the known protein-protein interactions of Rev1, I will further characterize their physiological role to determine the mechanism and biological relevance of these interactions.

Analysis of physical interactions in the Rev1 mutants

First, I will address whether the newly characterized Rev1 C-terminal region has an analogous function in binding TLS polymerases in yeast as in mammalian systems. Yeast has only two other TLS polymerases, one of which does not appear to interact physically or genetically with Rev1 [(6, 38); R. Woodruff, unpublished data]. Therefore, to address whether the C-terminal ~100 amino acids of yeast Rev1 does indeed mediate an interaction with other TLS polymerases, coimmunoprecipitation experiments with Rev1 and the Rev7 subunit of polymerase ζ will be employed as described previously (5). I expect that the site-directed mutants described above (*rev1-108*, *rev1-109*, *rev1-110*, *rev-111*, *rev1-112*) will abolish or reduce this interaction. More refined mutations than the alanine-patch substitutions can also be generated to probe the interaction surface in greater detail.

Similar coimmunoprecipitation experiments will be used to confirm that the UBMs of *S. cerevisiae* interact with ubiquitin. Very recently, an interaction between the UBMs of mouse Rev1 and ubiquitin has been demonstrated (16). Once the interaction is established in yeast, the UBM mutants (*rev1-105*, *rev1-106*, *rev1-107*) will be assayed to determine if Rev1 binding to ubiquitin is impaired. In mouse cells, the UBMs have been demonstrated to promote the monoubiquitination of Rev1 (16). Therefore, I will also test the ability of the UBM mutants to mediate monoubiquitination of yeast Rev1. I expect that for these assays, the UBM1 mutant

(*rev1-105*) will show little to no alteration relative to the wild-type while the UBM2 mutants (*rev1-106*, *rev1-107*) will show a dramatic defect. It is possible that the two motifs will show a specialization of function such that one UBM mediates an interaction with ubiquitin while the other promotes or stabilizes monoubiquitination of Rev1. Such an observation would have interesting implications for the multiplicity of the UBM repeats observed in different organisms.

Additionally, the catalytic dead, DNA binding, and BRCT mutants (*rev1*-AA, *rev1-113*, and *rev1-1* respectively) will also be analyzed in the experiments described above to investigate their role(s) in interacting with DNA polymerase ζ, ubiquitin, and in mediating monoubiquitination.

Investigation of the biological relevance of disrupting key protein-protein interactions

Concurrent with determining the molecular nature of the mutant phenotypes, I will also characterize the physiological effects of the Rev1 mutations in more detail. The experiments described below will establish the contribution of the catalytic activity of Rev1 and its several protein-protein and protein-DNA interactions to the function(s) of Rev1 *in vivo*.

Cell-cycle expression of mutant alleles of REV1

First, the effect of the UBM mutants on Rev1 cell-cycle dependent expression will be analyzed. Rev1 protein levels are regulated throughout the cell-cycle and are unexpectedly highest in G2/M rather than during replication (59). The molecular mechanisms controlling the cell-cycle regulation of Rev1 are currently unknown, but it appears that Rev1 levels are controlled in a post-transcriptional manner (59). Ubiquitin-mediated degradation is a common mode of cell-cycle regulation and several lines of evidence tie Rev1 to regulation by ubiquitin. Firstly, the UBM repeats of Rev1 not only bind to ubiquitin but also promote the monoubiquitination of Rev1 (16). Secondly, multiple E2 ubiquitin conjugating and E3 ubiquitin ligase enzymes show genetic interactions with *REV1* (56). Finally, the pattern of Rev1 expression fits the canonical profile for a Cdh1 substrate of the anaphase-promoting complex/cyclosome (APC/C) (61). Therefore, it will be very interesting to determine if the UBM mutants alter the cell-cycle regulation of Rev1.

Although ubiquitination is clearly involved in regulating Rev1 activity (10, 15, 16, 56), I might not expect that monoubiquitination mediated by the UBMs controls the cell-cycle expression of the Rev1 protein. Mutants defective in DNA damage tolerance ubiquitination showed no change in Rev1 expression throughout the cell cycle (Appendix B). As the Rad6-Rad18 heterodimer monoubiquitinates PCNA, it seems a likely candidate for the monoubiquitination of Rev1 as well. Since Rev1 expression showed no change in *rad6Δ* and *rad18Δ* strains (Appendix B), I expect that the UBM mutants may not show an alteration in the cell-cycle dependent expression of Rev1. Nonetheless, it is a critical experiment to carry out, especially given that monoubiquitination may serve as a platform for additional polyubiquitination, which is likely to be involved in the cell-cycle expression of Rev1. (For further discussion of this point, see Appendix B.)

In addition to the UBM mutants, it will be interesting to determine if the polymerase interaction mutants or the catalytic dead, DNA binding, and BRCT mutants affect the cell cycle regulation of Rev1 protein. These mutations may disrupt an interaction with a regulatory factor not involved in ubiquitination.

Cell-cycle sensitivity to DNA damaging agents

Next, the mutants will be examined for a cell-cycle dependence in sensitivity to DNA damaging agents. A *rev1Δ* null mutant displays hypersensitivity to UV irradiation after release from G1 arrest, but is only modestly sensitive after release from G2 arrest (59). We have argued that this pattern of cell-cycle dependent sensitivity is consistent with a gap filling role for *REV1* after replication of UV photoproducts (59). Repriming downstream of the lesion can produce gaps opposite to lesions (35) which may persist throughout replication into G2/M. Considering Rev1 levels are highest during G2/M (59), Rev1-dependent gap filling might only occur after the bulk of replication has been completed. In contrast, an alternate model states that Rev1 facilitates polymerase switching during S-phase to resolve stalled replication forks (6, 31, 32). This role of Rev1 in polymerase switching is thought to be mediated by its various protein-protein interactions: with multiple TLS polymerase through the C-terminus, with PCNA through the BRCT domain, and with ubiquitin through the UBMs (6, 14-16, 55).

To examine whether Rev1's protein-protein interactions contribute to a recruitment role for Rev1 at sites of gaps opposite lesions in G2/M, I will examine the pattern of UV sensitivity in the predicted polymerase interaction mutants, BRCT mutant, and UBM mutants. I expect that these mutants will show a similar phenotype to the *rev1Δ* mutant. Additionally, the DNA binding mutant is hypothesized to specifically affect the proposed gap filling ability of Rev1 in G2/M (see below) and will likely recapitulate the phenotype of the *rev1Δ* mutant. It will be especially interesting to determine the UV sensitivity of the catalytic dead mutant throughout the cell cycle. In unsynchronized cells, the catalytic dead mutant exhibits survival frequencies similar to the wild-type strain (Fig. 5B, F). However, in synchronized cells which display hypersensitivity to UV irradiation after G1 arrest, more modest changes in survival may become more pronounced. Any minor contribution that the catalytic activity of Rev1 makes to lesion bypass might be expected to occur during S-phase. It will be interesting to see whether the catalytic dead mutant is slightly defective for UV survival at all stages of the cell cycle or whether it displays specific defects at particular stages. Investigating Rev1 function during the cell cycle may prove a more robust assay to distinguish subtle phenotypes that have hitherto been unappreciated.

Significance and implications of the mutant phenotypes

Importance of the finding that the C-terminus of REV1 is critical for its function.

In higher eukaryotes, Rev1 is thought to mediate polymerase switching by virtue of its ability to interact with multiple polymerases (6, 14, 31, 55). As its catalytic activity is dispensable for DNA damage resistance or mutagenesis (18, 51), while its protein-protein interaction modules are required (15, 16, 28, 33, 51), Rev1 is proposed to function mainly as an assembly and coordinating factor for lesion bypass. In yeast there are only two TLS polymerases, polymerase ζ and polymerase η. Rev1 interacts with polymerase ζ through multiple contacts with the Rev7 subunit outside of the extreme C-terminal ~100 amino acids (1, 5) and appears to not interact with polymerase η either genetically or biochemically [(6, 38); R. Woodruff, unpublished data]. Consequently, the C-terminal polymerase interaction motif might not be expected to be conserved between yeast and vertebrates. However, the results presented

here argue for sequence, structural, and functional conservation of the C-terminal interaction region of Rev1 from yeast to humans. Besides confirming that *S. cerevisiae* is a useful model organism in which to study DNA damage tolerance and mutagenesis, these findings suggest that the function of Rev1 requires its ability to recruit at least one translesion polymerase to sites of DNA damage. Alternatively, the C-terminus of yeast Rev1 may perform a critical function distinct from the recruitment of polymerase ζ to unreplicated regions remaining in G2/M, either by recruiting unknown factors or by mediating an entirely novel activity. The experiments described above will help to address this point.

Importance of the observation that the putative DNA binding mutant of REV1 lacks activity in vivo.

Two models postulate different roles of *REV1* in DNA damage tolerance and mutagenesis. The polymerase switching model envisions that the protein-protein interactions critical to *REV1* function coordinate the activity of TLS polymerases to resolve stalled replication forks during S-phase. In contrast, the gap filling model proposes that Rev1 serves as a sensor for sites of incomplete replication caused by replication repriming downstream of a lesion. In this model, Rev1 functions during G2/M to recruit or stimulate TLS polymerases, and likely other factors, after replication at sites of DNA damage. The putative DNA binding mutant of Rev1 was designed to probe the contribution of Rev1 to each type of lesion bypass.

In the polymerase switching model, Rev1 is thought to serve as a scaffold for other TLS polymerases. In general, scaffolding proteins function to orchestrate a substrate handoff between two enzymes by positioning the proteins optimally for substrate transfer rather than by interacting directly with the substrate. This would be especially true for a substrate which has only one binding interface or for which the two enzymes have an overlapping recognition site. Interaction of the scaffolding protein with the substrate would thus block interaction of the recipient enzyme and prevent an effective handoff. Since DNA polymerases are optimized to bind DNA at a primer terminus rather than at regions of dsDNA or ssDNA, there is arguably only one way of effectively engaging a TLS polymerase at primer opposite a lesion. Therefore a protein coordinating lesion bypass by promoting handoffs of the primer terminus between polymerases would not be expected to bind the primer terminus itself.

Since the catalytic activity of Rev1 is dispensable for its function *in vivo* while its protein-protein interaction modules are required, Rev1 could function in assembly of a TLS complex without ever engaging the primer terminus. Although a transient interaction cannot be ruled out, it seems logical that in order to promote polymerase switching, Rev1 would not need to gain access to the primer terminus. In fact, DNA binding by the catalytic domain of Rev1 might prevent effective translesion synthesis by another polymerase, since Rev1 has only limited polymerase activity. Therefore, it might be expected that the DNA binding ability of the catalytic domain of Rev1 would be unnecessary if the primary function of Rev1 was to regulate access of polymerases to sites of DNA damage during polymerase switching during S-phase.

A caveat to this hypothesis is that if Rev1 were serving a recruitment role during S-phase to bring the other TLS polymerases to sites of DNA damage, DNA binding by the polymerase domain of Rev1 might be necessary to its function. However, as yet, no unique interactions for Rev1 have been determined that indicate that Rev1 relocalizes to damage prior to other factors. It is also important to note that a role for the non-catalytic domains of Rev1, such as the BRCT domain, in interacting with DNA during polymerase switching would not necessarily negatively affect the polymerase handoff. However, it seems logical that DNA binding by the polymerase domain of Rev1 would not be required, and might even be detrimental, to orchestrate effective translesion synthesis by other TLS polymerases.

In contrast, DNA binding by the polymerase domain of Rev1 is crucial for *REV1* activity in the gap filling model. Without the ability to bind to primer termini, Rev1 would be unable to recruit other TLS polymerases to sites of incomplete replication opposite DNA lesions. Engagement of the lesion by the active site of Rev1 would not prevent lesion bypass by other TLS polymerases in this situation. In fact, it may serve as a mechanism for the cell to delay potentially mutagenic translesion bypass until other high fidelity mechanisms, such as nucleotide excision repair, had a chance to repair the lesion in a more accurate manner.

The fact that the predicted DNA binding mutant in the catalytic domain of Rev1 was completely unable to promote survival or mutagenesis after DNA damage (Fig. 5B-F) supports a role for Rev1 in recruitment of other TLS polymerases rather than a role solely in regulation of access of TLS polymerases. Although the ability of Rev1 to recruit TLS polymerases to stalled replication forks in S-phase is not impossible, currently no evidence points towards this function of Rev1 in polymerase switching. A direct test of the order of recruitment has only been

performed with DNA polymerases η and ι (21) and would be most interesting to extend to the remainder of the TLS polymerases. Therefore, the phenotype of the putative DNA binding mutant supports the recruitment role of Rev1 in gap filling in G2 and throughout mitosis.

Materials and Methods

Strains and Plasmids. Strains used were derivatives of W1588-4C, a W303 strain corrected for *RAD5* (64), and are described in Table 1. The *rev1Δ* strain was generated by moving the *rev1::kanMX4* cassette from the deletion library into a W15488-4C strain containing a *bar1::LEU2* disruption. All plasmids were transformed into the *rev1Δ bar1Δ* strain, either to be stably maintained as low-copy plasmids or to be integrated into the *REV1* promoter to restore the *REV1* gene with appropriate site-directed mutations.

The pRS416-REV1 plasmid was generated using primers pRS-Rev1-fwd and pRS-Rev1-rev to amplify the *REV1* gene by PCR from the genome. The PCR fragment was digested with BclI and KpnI and cloned into the pRS416 vector digested with BamHI and KpnI. This produced a 3.4 kb region containing 210 bp of the *REV1* promoter and 217 bp of the 3′ UTR. It has previously been shown using deletion analysis that *REV1* constructs containing 210 bp of the *REV1* promoter or 27 bp of the 3′ UTR are able to complement a *rev1-1* strain (28). Upon sequencing, two point mutations were found. Quikchange mutagenesis was used to restore the *REV1* construct to the wild-type sequence.

The pRS306-REV1 plasmid was created by digestion of pRS416-REV1 with SpeI and KpnI, gel purification of the 3.4 kb *REV1* fragment, and ligation into pRS306 backbone digested with SpeI and KpnI. Since the subsequent restriction digestion was inhibited by Dcm methylation, the plasmid was miniprepped from XL1Blue and transformed into GM2163 (New England Biolabs) which is a *dam⁻ dcm⁻ E. coli* host strain. The pRS306-REV1 plasmid, and site-directed mutant derivatives, were miniprepped from GM2163, linearized at the SexAI site in the 210 bp *REV1* promoter, and transformed into the *rev1Δ bar1Δ* strain. Integrated constructs were selected on SC-Ura media and verified by PCR using the primers pRS-Rev1-fwd and pRS-Rev1-rev.

Ongoing work is directed towards tagging the integrated *REV1* and mutated *rev1* alleles using pYM10 as described previously (24, 59).

Site-Directed Mutagenesis. Site-directed mutations were generated according to the protocol of Quikchange Mutagenesis kit (Stratagene) with the following changes. Since multiple amino acid changes were made, it was found to be necessary to lower the annealing temperature to 50 °C.

Additionally, the extension time was found to require 2 min/kb and for plasmids larger than 6 kb, a 20 min. extension was necessary. However, 25 min. extension time was too long and resulted in decreased efficiency of recoverable mutants. Primers are listed in Table 2 and a summary of the mutations is listed in Table 3.

UV Survival and Mutagenesis Assays. Three independent cultures of each strain were grown to saturation and appropriately diluted aliquots were plated on SC media to monitor survival. Mutation frequencies were analyzed by plating undiluted aliquots on SC-Trp or SC-Ade media to score for reversion of the *trp1-1* allele or the *ade2-1* allele respectively. Plates were irradiated at 1 J/m^2/sec using a G15T8 UV lamp (General Electric) at 254 nm and grown for 3 or 6 days at 30 °C for survival and mutagenesis assays respectively. The cell-cycle sensitivity assays will be performed as previously described (59).

MMS Sensitivity Assay. Strains were grown to saturation, serially diluted 10-fold, and 2.5 μL of each dilution was spotted onto YPD media with or without 0.02% MMS (Sigma).

Immunoblots. Whole cell extracts were prepared by TCA precipitation (24) and were separated by SDS-PAGE. Proteins were transferred to PVDF membrane (Millipore) using a Mini-PROTEAN II transfer apparatus (Bio-Rad). Antibodies used were rabbit PAP antibody (Sigma) against the protein A tag, anti-HA.11 (Covance), and anti-phosphoglycerate kinase (Molecular Probes).

Computational Analysis. Manual alignments were generated using the Lasergene suite of sequence analysis programs. Helical wheels were adapted from http://cti.itc.virginia.edu/~cmg/Demo/wheel/wheelApp.html. Secondary structure predictions were made using the 3D-PSSM program at http://www.sbg.bio.ic.ac.uk/~3dpssm/.

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Table 1: Strains used in this study

Strain	Genotype	Source
W1588-4C	*MAT a ade2-1, ura3-1, his3-11,15, trp1-1, leu2-3,112, can1-100, RAD5*	Rothstein lab (62)
rev1Δ bar1Δ	*rev1::kanMX4, bar1::LEU2*	L. Waters (57)

Table 2: Primers used in this study

Primer Name	Primer Sequence	Mutation Generated
pRS-Rev1-fwd	GCTTTGAGTTGGGGTAGATTATCGC	none
pRS-Rev1-rev	GTGTTGGTACCAAAGGAGGAGTCGGCCATTCC	none
rev1-1_fwd2	TACACGAGATGATAGTTTTACATGGCaGAAAATTTT TACACTATTTGTCTTC	rev1-1
rev1-1_rev2	GAAGACAAATAGTGTAAAAATTTTCtGCCATGTAAA ACTATCATCTCGTGTA	rev1-1
Rev1_D467A E468A	GATTTTACCTATATCTATTGcTGcAGCTGTTTGTGTG AGGATAATCCC	rev1-AA
Rev1_D467A E468A-r	GGGATTATCCTCACACAAACAGCTgCAgCAATAGAT ATAGGTAAAATC	rev1-AA
SC1	GTGACGAACAGAGCTTTCGAAGCCGCCGCGGCAGC TGTAAAAAATGACATTAACAACG	rev1-105
SC1-r	CGTTGTTAATGTCATTTTTTACAGCTGCCGCGGCGG CTTCGAAAGCTCTGTTCGTCAC	rev1-105
SC2	CTATGGAAGAACAGTTTATGAATGCAGCCGCGGCC GCAATTCGAGCAGAAGTAAGGCACG	rev1-106
SC2-r	CGTGCCTTACTTCTGCTCGAATTGCGGCCGCGGCTG CATTCATAAACTGTTCTTCCATAG	rev1-106
SC3	TATGAATGAACTACCAACCCAAGCTGCCGCGGCAG CAAGGCACGACTTGAGAATTCAG	rev1-107
SC3-r	CTGAATTCTCAAGTCGTGCCTTGCTGCCGCGGCAGC TTGGGTTGGTAGTTCATTCATA	rev1-107

SC4	CGGTTCAAAAAAATTTGTCAAGCCGCGGCACAAGC GGCTGCCGAAACTTTAGGTGATGGA	rev1-108
SC4-r	TCCATCACCTAAAGTTTCGGCAGCCGCTTGTGCCGC GGCTTGACAAATTTTTTTGAACCG	rev1-108
SC5	CGAAACTTTAGGTGATGGAGGGGCGCATGCAGCAG CTGCTAAATTATTCGTGAAATATTT	rev1-109
SC5-r	AAATATTTCACGAATAATTTAGCAGCTGCTGCATGC GCCCCTCCATCACCTAAAGTTTCG	rev1-109
SC6	GCCGCATGAAAAAGATGTTAAATTATTCGCGGCCG CGGCGGCTAAACTTTGCGATTCTAATAGAGTCCAT	rev1-110
SC6-r	ATGGACTCTATTAGAATCGCAAAGTTTAGCCGCCGC GGCCGCGAATAATTTAACATCTTTTTCATGCGGC	rev1-110
SC7	TATTTGATTAAACTTTGCGATTCTAATGCAGCGCAT GCAGCTGCTCATTTATCAAACCTAATATCAAGGG	rev1-111
SC7-r	CCCTTGATATTAGGTTTGATAAATGAGCAGCTGCAT GCGCTGCATTAGAATCGCAAAGTTTAATCAAATA	rev1-111
SC8	CCACTTTTAAACAGAAATAAACATGCTGCCCAGGC CGCGGCTAAACTTGACATGGACTTTGAAG	rev1-112
SC8-r	CTTCAAAGTCCATGTCAAGTTTAGCCGCGGCCTGGG CAGCATGTTTATTTCTGTTTAAAAGTGG	rev1-112
QC-R620L K621L	CTGTACGATCCCAAAGAAGTCTTACAACTCTTAAGC TTATCAATTGATATCAATTGGGGAATC	rev1-113
QC-R620L K621L-r	GATTCCCCAATTGATATCAATTGATAAGCTTAAGAG TTGTAAGACTTCTTTGGGATCGTACAG	rev1-113
QCRev1A	CCAGATTAGAAGTAAATGATTTGCCCAGTACTATGG	nt 2763 *REV1*
QCRev1B	CCATAGTACTGGGCAAATCATTTACTTCTAATCTGG	nt 2763 *REV1*
QCRev1C	GGAGAGAAGAGAGCCTACAGAACGAG	nt 2616 *REV1*
QCRev1D	CTCGTTCTGTAGGCTCTCTTCTCTCC	nt 2616 *REV1*

Table 3: Site-directed mutants used in this study

Allele	Amino acid changes	Location	Reference
rev1-1	G193R	BRCT domain	(28, 33)
rev1-AA	D466A E467A	pol domain	(18)
rev1-105	L763A P764A E765A D766A	UBM1	this study
rev1-106	E820A L821A P822A T823A Q824A	UBM2	this study
rev1-107	I825A R826A E828A V829A	UBM2	this study
rev1-108	L889A V890A K891A W893A V894A	C-terminus	this study
rev1-109	P903A E905A K906A D907A V908A	C-terminus	this study
rev1-110	V912A K913A Y914A L915A I916A	C-terminus	this study
rev1-111	R923A V924A L926A V927A L928A	C-terminus	this study
rev1-112	T972A Y973A T975A V976A R977A	C-terminus	this study
rev1-113	R620L K621L	pol domain	this study

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Chapter Four

Discussion

Summary of Results

DNA damage elicits a network of responses, most specialized to promote rapid and accurate repair of DNA lesions. However, certain genes actively promote the generation of mutations, both spontaneously and after DNA damage. In eukaryotes, the vast majority of mutagenesis requires Rev1 and DNA polymerase ζ. My work has focused on the regulation and molecular mechanisms of the *S. cerevisiae* translesion polymerase Rev1.

In Chapter Two, I demonstrated the novel and unexpected cell-cycle regulation of Rev1 in *S. cerevisiae*. Characterization of Rev1 expression levels revealed that Rev1 was highly expressed not during the bulk of replication, but rather afterwards in G2 and throughout all of mitosis. This pattern of Rev1 accumulation was observed not only in normally growing cells, but also after DNA damage, indicating that Rev1 operates in a similar manner even when replication forks encounter DNA lesions during S-phase. Moreover, *rev1Δ* cells displayed a cell-cycle dependent sensitivity to UV irradiation, showing that the observed regulation of protein levels was relevant to its function. These results challenged our understanding of translesion synthesis as a process operating predominantly during S-phase to enable replication to continue in the face of DNA lesions. Rather, the finding that Rev1 was not present at high levels during S-phase led to a model in which Rev1 promotes gap filling after replication at sites of DNA damage where repriming events produced a region of ssDNA opposite to a lesion.

In Chapter Three, I described experiments designed to address the contribution of various domains to *REV1* function *in vivo*. Novel motifs were identified by extensive sequence alignments of Rev1 proteins from across diverse lineages throughout eukaryotes. Site-directed mutations were generated to disrupt the motifs. Initial results suggest that protein-protein and protein-DNA interactions are critical for *REV1* activity *in vivo*, while the catalytic activity of Rev1 is dispensable for survival and mutagenesis. In particular, mutations in the C-terminus of Rev1 indicate that this region is conserved between yeast and vertebrates, contrary to some indications in the literature. Additionally, the phenotype of a mutation of the catalytic domain predicted to disrupt DNA binding lends support to the gap filling model of Rev1 activity proposed in Chapter Two. Further experiments were discussed which will elucidate the molecular mechanisms and physiological functions disrupted in the mutant strains.

Molecular mechanism(s) controlling Rev1 cell-cycle regulation

As yet, we have only hints towards understanding what may be regulating the cell-cycle dependent expression of Rev1. Since Rev1 protein levels fluctuate ~50-fold while the mRNA levels display only a ~3-fold change during the cell-cycle, we believe that the majority of the Rev1 cell-cycle expression is regulated at the post-transcriptional level (65). Data presented in Appendix B and recent work from our lab have revealed roles for *CDC7* and *UMP1* in the accumulation of Rev1 protein (L.Waters, M.E. Wiltrout, unpublished data). Both have been implicated through epistasis analysis to function in the *REV1/REV3/REV7* pathway of the *RAD6* post-replicative repair group (46, 51, 53). *CDC7* encodes a serine/threonine kinase required for the initiation of S-phase (38). Both hypomutable and hypermutable alleles of *CDC7* exist (22), suggesting that Cdc7, together with its cell-cycle regulated accessory factor Dbf4 (38), regulate mutagenic translesion synthesis. Since Dbf4 contains a BRCT domain (14), it may heterodimerize with the BRCT domain of Rev1 to promote Cdc7-dependent phosphorylation of Rev1. *UMP1* encodes a nonessential subunit of the proteasome (54). An *ump1Δ* strain displays an increase in spontaneous and UV-induced mutagenesis which is *REV3*-dependent (46, 53). As *ump1Δ* mutants also exhibit defects in proteolysis (54), it seems likely that Ump1 may degrade Rev1. In the absence of Ump1, aberrantly high levels of Rev1 may accumulate and promote mutagenesis. Though we have evidence that *CDC7* and *UMP1* control Rev1 levels (see Appendix B), we do not yet know whether these genes regulate Rev1 levels in a cell-cycle-dependent or independent manner. The roles of *CDC7* and *UMP1* in the regulation of Rev1 are currently being investigated in our lab.

Several lines of evidence link Rev1 to ubiquitin. The *RAD6* epistasis group, of which *REV1* is a member, contains genes encoding three E2 ubiquitin-conjugating enzymes (Rad6, Mms2, and Ubc13) and two E3 ubiquitin ligases (Rad18 and Rad5) (61). Additionally, in vertebrate systems Rev7 has been shown to interact with and inhibit the anaphase-promoting complex/cyclosome (APC/C)—a multisubunit E3 ubiquitin ligase (7, 52). Thus, it seemed likely that one or more of these proteins might regulate the accumulation of Rev1 levels by targeting it for proteasomal degradation. Accordingly, the pattern of Rev1 cell-cycle accumulation was analyzed in the appropriate deletion mutant backgrounds (see Appendix B). However, no change in Rev1 protein levels was observed in any of the mutant backgrounds, indicating that

ubiquitination mediated by post-replication repair factors is not responsible for regulating Rev1 accumulation.

Though ubiquitin-mediated degradation is a common mechanism controlling cell-cycle dependent expression (70), preliminary data suggests that the accumulation of Rev1 during the cell cycle may not be completely controlled by degradation. Initial experiments using *REV1* under the control of a galactose-inducible promoter indicate that the half-life of Rev1 protein expressed in G1-arrested cells is as long, if not longer, than in G2-arrested cells (Appendix B). It should also be noted that *REV1* transcription has been reported to initiate from two start sites, differing by 24 nucleotides (30). Since the RT-PCR analysis used primers which amplified the 3′ end of the gene, the results reported in Chapter Two for the fold change of the *REV1* transcript represent the total levels of all *REV1* mRNA's. It is possible that one *REV1* transcript displays pronounced cell-cycle regulation while the other is produced at constant levels. If so, differential regulation of translation by the two 5′ UTR's may explain part of the observed cell-cycle dependent accumulation of Rev1 protein levels. Therefore, regulation at the transcription and translation levels may be partially responsible for the fluctuations in Rev1 protein levels, in addition to degradation or post-translational modifications. RT-PCR experiments using primers located in the 5′ UTR of *REV1* are in progress to address the cell cycle levels of each transcript.

In summary, no definitive pathway has been identified to date that controls the cell-cycle dependent regulation of Rev1. So far, we have focused on investigating the role of post-translational modifications and degradation to the differential accumulation of the Rev1 protein through the cell cycle. However, we are now also considering alternate models of regulation including translational control and multiple *REV1* transcripts. Thus far, we have employed a candidate gene approach, but unbiased screens for regulators of Rev1 protein levels are also planned. Identifying the genes involved in controlling the cell-cycle dependence of *REV1* function will greatly expand our knowledge of how this enigmatic translesion polymerase mediates its function in survival and mutagenesis *in vivo*.

Why is Rev1 cell-cycle regulated?

As shown in Chapter Two, Rev1 protein levels are almost undetectable in G1 and slowly accumulate throughout S-phase. As cells complete replication, Rev1 levels rise rapidly and are maintained at high levels throughout mitosis until telophase (shown in more detail in Appendix D). As discussed in Chapter Two, the presence of high levels of Rev1 in G2/M indicates that its true substrate may be a gap opposite to a lesion which would only be produced after replication. However, this does not explain why Rev1 exhibits cell-cycle regulation. Instead of asking "Why is Rev1 upregulated in G2/M?" one could as easily pose the question "Why is Rev1 not present in G1?" It is fortuitous for us in elucidating Rev1 function that Rev1 does show a cell-cycle dependent accumulation, but why might Rev1 be detrimental to the cell if present significant amounts in G1 and/or throughout S-phase? Additionally, the cell-cycle regulation of Rev1 appears to control polymerase ζ activity as well, suggesting that polymerase ζ activity may also be deleterious during G1 and/or S-phase. Several possible negative effects of Rev1 expression during G1 and S-phase are discussed below.

Firstly, Rev1 may interfere with replication in S-phase. The physiological effects of overexpression of TLS polymerases on DNA replication have not yet been addressed. However, as the polymerization rate of TLS polymerases *in vitro* is very low relative to replicative DNA polymerases (2, 13, 24, 34), it is probable that over- or misexpression of TLS polymerases *in vivo* may act as a "molecular brake" for DNA replication, analogous to what has been recently described for polymerase α (31). Especially considering the limited polymerase activity of Rev1 (48), inappropriate expression of Rev1 allowing it excess access to the primer terminus may inhibit processive DNA synthesis. Additionally, the primary function of Rev1 is mediated by its protein-protein interactions with PCNA and other TLS polymerases (18, 19, 30, 33, 57). These interactions are thought to promote polymerase switching during S-phase (9, 17, 32, 60). We have proposed that, instead, Rev1 serves primarily to recruit TLS polymerases in G2/M to mediate gap filling (65). Inappropriate expression during S-phase might therefore promote aberrant polymerase handoffs, allowing TLS polymerases unwarranted access to the DNA at sites of normal replication pausing. Inappropriate TLS polymerase activity may not only perturb normal DNA replication but also may increase the frequency of mutations, since TLS

polymerases display substantially reduced replication fidelity on undamaged DNA relative to replicative DNA polymerases (15, 27).

The presence of Rev1 may also be detrimental in G1. If expressed in G1, Rev1 may inhibit a G1-specific process. Given the high affinity of Rev1 for regions of ssDNA (K_d < 1 nM, (40)) and primer template termini (K_d ~ 2.5 nM, (42)) , it is conceivable that Rev1 could interfere in the initiation of replication by binding to unwound DNA at origins or nascent replication forks and inhibiting further polymerization.

Finally, the cell-cycle regulation of Rev1 may be a mechanism to reduce the cellular potential for mutagenesis. Outside of lab culture conditions designed to optimize cell growth, many cells spend significant amounts of time in G1, either due to nutrient limitation or to specialized functions in multicellular organisms which require terminal differentiation. Low Rev1 levels in these cells may reflect a strategy to minimize mutagenesis in cells which have a low proliferation potential and are less likely to produce daughter cells for which adaptive mutations may be beneficial. Additionally, as discussed in Chapter Two, delaying potentially mutagenic Rev1-dependent lesion bypass until late in the cell cycle may promote the action of more highly accurate repair or damage tolerance systems, such as NER or DNA polymerase η.

Other roles of *REV1*, *REV3*, and *REV7*

Links between REV1 and cell proliferation

Rev1 may be regulated by various cell growth pathways. In global microarray experiments, *REV1* transcript levels have been shown to be modulated by glucose signalling (63), the Ume6 transcription factor involved in induction of meiosis (67), rapamycin (23), filamentous growth (37), and perturbations of various MAPK pathways (55). These networks all monitor the carbon and nitrogen sources available and transmit downstream signals, using overlapping kinase cascades. *REV1* is upregulated by the addition of glucose (63) and is strongly repressed after treatment with rapamycin (23), which mimics nutrient starvation. Uniquely among the *UME6*-regulated genes, *REV1* seems to require Ume6 for activation of transcription during vegetative growth (67). Taken together, these data suggest that *REV1* functions mainly during times of active cell proliferation. Integrating the data from alterations in the filamentous

growth and MAP kinase pathways into an interpretable model is more challenging. However, it is clear that multiple metabolic and cell proliferation pathways impinge upon the *REV1* gene in a way that, so far, has not been observed for *REV3* or *REV7*. As Rev1 is the only TLS polymerase known to be cell-cycle regulated thus far, these microarray studies may highlight a unique role for *REV1* in response to the regulation of cell growth.

The Ume6-dependent transcription of *REV1* is particularly intriguing in light of the fact that *REV1*, as well as *REV3* and *REV7*, has been shown to be upregulated during sporulation in yeast (4, 8, 58). All of the TLS polymerases also show increased levels in meiotic tissues in mice (29). However, only *REV1* was found to require Ume6 for its transcription during vegetative growth (67). Interestingly, the *REV1* gene contains an URS1 consensus binding site for the Ume6 transcription factor (4). Consistent with its unusual pattern of Ume6-dependent regulation, the URS1 site is located in the Rev1 open reading frame rather than in the promoter region. A site-directed mutant which silently removes the URS1 consensus without altering the protein sequence has been generated and will be tested for its effect on survival and mutagenesis after DNA damage, as well as cell-cycle regulation.

Translesion polymerases and RNA metabolism

Multiple seemingly tangential links between the TLS polymerases and RNA metabolism exist. In a directed yeast two-hybrid with splicing components, yeast Rev1 and Rev7 were each reported to interact with a component of the spliceosome: Snp1 and Smd3 respectively (12). Human Rev7 also interacts with trichosanthin, a protein related to ricin which binds to the 23S and 28S rRNA subunits of the ribosome (6). Additionally, in a recent proteomics study polymerase η was identified to interact with Lsm12, which likely binds RNA, and Snu13, which participates in splicing and rRNA processing (26). Thus, TLS polymerases may bind to RNA interacting proteins. The biological significance of any of these interactions is currently unknown. However, a functional correlation between *REV1* and RNA metabolism was shown in the two microarray analyses which analyzed glucose signalling and rapamycin effects, where *REV1* clustered tightly with genes involved in ribosome biogenesis and RNA metabolism (23, 63). Given that translesion polymerases mediate their function by interacting with a structurally

similar nucleotide polymer, it is worth keeping in mind that several studies have found connections between TLS polymerases and RNA.

In contrast to the poorly characterized interactions described above, the interaction of vertebrate Rev7 with the human papillary renal cell carcinoma-associated protein PRCC has been well documented to be functionally important (62, 66). Interaction with PRCC is required for the nuclear import of Rev7; in the absence of PRCC, human and Xenopus Rev7 exhibit exclusively cytoplasmic localization (62, 66). Interestingly, the normal function of the PRCC protein appears to be in pre-mRNA splicing, since it colocalizes and interacts with several components of the spliceosome (59). Chromosomal translocations fusing the *PRCC* and *TFE3* genes are strongly correlated with renal cancers (59, 62, 66). The PRCC-TFE3 or TFE3-PRCC fusions fail to interact with Rev7 which prevents Rev7 from translocating into the nucleus (62, 66). Intriguingly, cells transfected with either the *PRCCTFE3* or *TFE3PRCC* fusion exhibit mitotic checkpoint defects (62, 66). These results suggest that Rev7 may play a role in RNA splicing and/or that Rev7 function is important for proper mitotic progression in vertebrate cells.

Interactions with membrane proteins

Although the physiological relevance is uncertain, it is worth noting that Rev1 and Rev7 have been shown to associate with multiple membrane proteins. Human Rev1 was identified by a two-hybrid screen to interact with the cytoplasmic domain of several integrin proteins (68). Integrins are a class of transmembrane receptors which mediate signalling from the extracellular matrix to the cytoskeleton (68). In similar two-hybrid screens, human Rev7 was found to interact with the cytoplasmic domains of two metalloprotease-disintegrin proteins (49) and with the adenovirus death protein ADP (69), all of which are membrane proteins. Human Mad2, which is homologous to human Rev7 (1), also showed a two-hybrid interaction with a disintegrin (49). The biological relevance of the Rev1-integrin and Rev7-disintegrin interactions is unknown. Indeed, these interactions may well represent nonphysiological artifacts, since *in vivo* Rev1 and Rev7 are unlikely to be found in the cytoplasm. However, given that in yeast *REV1* appears to be regulated by cellular proliferation signals (see above) and that in vertebrates, *REV7* has been shown to inhibit cell-cycle progression (7, 52), it is possible that interaction with these cell surface proteins, which relay environmental signals, may provide a mechanism to integrate

cell growth cues with their molecular effectors. In a more complete study, human Rev7 was shown to coimmunoprecipitate with the adenovirus death protein ADP and overexpression of Rev7 was demonstrated to antagonize cell lysis caused by ADP expression (69). As ADP localizes to nuclear membranes and the ER, this interaction may represent a valid physiological function of the Rev7 protein upon viral infection, possibly to coordinate cell cycle arrest with resistance to viral infections (69).

Why is the novel catalytic activity of Rev1 dispensable yet conserved?

Rev1 from both yeast and mammals displays limited polymerase activity, restricted primarily to the insertion of dCMP (20, 35, 48, 71). Additionally, both yeast and mammalian Rev1 prefers to bind DNA containing a template G (20, 39, 41, 71). Crystallization of yeast Rev1 with DNA has revealed an elegant explanation for the predominant insertion of C's, as well as for the preferential binding to a template G (47). A template G is selected for by specific contacts with the backbone and sidechains of amino acids in the "G loop" of the little fingers domain (also called the polymerase associated domain or PAD) (47). The preferential insertion of C relies on hydrogen bonds formed between the incoming nucleotide and an arginine residue in Rev1, rather than between the incoming nucleotide and the template base (47). This mechanism of substrate choice using the protein itself for a template is utterly unprecedented in DNA polymerases and argues for a high level of specialization of the Rev1 active site. Yet, bizarrely, this novel mechanism of polymerization using the enzyme itself as a template does not seem to be required for the function of Rev1 *in vivo*.

Intriguingly, strains bearing a catalytically inactive version of Rev1 (42) display no significant decrease in survival or mutagenesis after exposure to the alkylating agent MMS (21). I confirmed this finding in Chapter Three and extended the observation to another DNA damaging agent, UV irradiation. In a more direct assay for translesion synthesis past various lesions, the catalytic dead allele of Rev1 showed only a slight decrease in the frequency of lesion bypass *in vivo*, however the spectrum of nucleotides inserted opposite the lesion was significantly altered (50). Additionally, the catalytic activity of Rev1 was shown to be employed in somatic hypermutation initiated by AID-mediated deaminations, however the *in vivo*

phenotype of the catalytic dead Rev1 was not investigated in this system (56). Therefore, although it contributes to lesion bypass, the highly specialized and conserved dCMP transferase activity of Rev1 is dispensable for its function in survival and mutagenesis after DNA damage. These findings indicate that another activity of Rev1 mediates its function *in vivo*, which is believed to be protein-protein interactions as discussed in Chapters Two and Three.

The observation that the catalytic activity of Rev1 does not contribute to its function *in vivo* suggests that we do not understand a fundamental property of Rev1's mechanism of action inside cells. Why would such a unique and highly optimized enzymatic activity that is conserved from yeast to humans exist if it did not perform an important role in the cell? Perhaps we have not examined *REV1* function using as assay that can detect the contribution of its catalytic activity. A related question is why would a cell evolve and maintain a DNA polymerase whose only activity is to insert one of the four nucleotides? The answers are still a mystery, but I discuss some possibilities below.

The cognate lesion for Rev1 may be an adducted G

Since the discovery of low fidelity TLS polymerases, a recent paradigm shift has moved from considering TLS polymerases as "sloppier copiers" (15) to enzymes optimized for accurate bypass of particular types of DNA damage termed a "cognate lesion" (10). Evidence for the specialization of TLS polymerases for a particular cognate lesion has now been provided for two TLS polymerases (25, 28, 43, 45). The characteristic features of cognate lesion bypass by TLS polymerases are a high level of accuracy and an increased efficiency of polymerization opposite the lesion relative to other substrates (44). These parameters are thought to ensure that a particular translesion polymerase preferentially synthesizes DNA opposite its cognate lesion, rather than undamaged DNA or non-cognate modified bases (25, 28, 43, 45).

Studies of the kinetic parameters of Rev1 have shown that Rev1 is optimized not only to insert a C but also to do so opposite a template G, an abasic site, or certain adducted G's (20, 39, 41, 71). These findings may give us a clue as to the function of Rev1's catalytic activity *in vivo*. Preliminary work from our lab reveals that the catalytic dead mutant of Rev1 displays a reduction in survival after exposure to 4-nitroquinoline oxide (M.E. Wiltrout, unpublished data) which, among other products, generates N^2- and *C8*-adducted G's (11). This finding, together

with the finding that Rev1 can bypass N^2-adducted G's *in vitro* and *in vivo* (64, 71, 72), suggests that a cognate lesion for the dCMP transferase activity of Rev1 is adducted G's. Future work using purified Rev1 in primer extension assays with templates containing various adducted G lesions will determine the particular lesion structures that Rev1 can bypass most efficiently. As Rev1 preferentially inserts C's, bypass of adducted G's would be accurate. The fidelity of Rev1 opposite its proposed cognate lesion would partially explain why its catalytic activity does not seem to contribute towards mutagenesis *in vivo*. However, the hypothesis that adducted G's constitute a cognate lesion for Rev1 does not address the contribution of the catalytic activity of Rev1 towards survival. If adducted G's were a major source of spontaneous damage, such that cells needed a specialized enzyme for their bypass, *rev1Δ* strains might be expected to show a growth defect, which they do not (L. Waters, unpublished observation). Additionally, Rev1 has been shown to efficiently bypass other lesions as well, including adducted A's and abasic sites. Insertion of C opposite these lesions would not result in accurate bypass.

Other lesions bypassed by Rev1 in vivo?

Kinetic and structural parameters indicate that other lesions besides adducted G's may also preferentially bound by Rev1. The crystal structure of Rev1 suggests that the steric discrimination used to select for binding to a template G over an A, T, or C would provide no selection against an abasic site (47). The K_M for Rev1 template binding opposite to abasic sites indicates that they may be a relevant substrate *in vivo*. In both yeast and mammalian systems, purified Rev1 displays only a 2.5 – 25-fold discrimination for template G's relative to template abasic sites (20, 39, 41, 71). Thus, abasic sites may also serve as a significant source of Rev1 lesion bypass events *in vivo* for all organisms. Indeed, insertion of C's opposite to abasic sites is specifically lost in cells bearing a catalytically inactive Rev1 (50, 56).

Additionally, *in vitro* mammalian Rev1 shows only a ~10-fold discrimination for template G's over template A's or template U's (39, 41, 71). In contrast, yeast Rev1 exhibits a ~600-fold discrimination against A's *in vitro* (20) which is borne out by the crystal structure showing loss of stabilizing bonds and potential steric clashes with a template A (47). Activity opposite a template U has not been measured for yeast Rev1. Therefore, adducted A's or C's which have deaminated to U's may also be lesions which employ the catalytic activity of Rev1 in

mammals. Since bypass by Rev1 of abasic sites, adducted A's, and inappropriate U's would be predominantly mutagenic, it is not clear whether these substrates would be considered as cognate lesions. It is also not clear why cells might use Rev1 to bypass these lesions.

Since abasic sites are non-informational lesions, they cannot be bypassed in an accurate manner. However, if the base lost most often endogenously to produce an abasic site were a G, then insertion of a C by Rev1 would only rarely result in a mutation. While it has been shown that purines are hydrolyzed from DNA ~20-fold more frequently than pyrimidines, it does not seem that G's are lost more frequently than A's *in vivo* (3, 11). One possible scenario for predominant loss of G's would be that upon deamination of C's, the loss of a hydrogen bond would destabilize the G-C base pair and allow hydrolysis of a G more frequently than an A. Still, it seems reasonable that cells would not have selected for a polymerase optimized only to insert C's opposite to abasic sites unless the vast majority of abasic sites were produced by hydrolysis of G. Moreover, repair of abasic sites by base excision repair would be occur with much higher fidelity since the undamaged strand is used as a template to restore the damaged sequence. Thus, it seems more likely that cells co-opted an already existing dCMP transferase to perform translesion synthesis over an abasic site when no other option was available. This may be especially relevant in mammals, which generate immunoglobulin diversity by targeted deamination events which are processed to generate abasic sites (5). Abasic sites are likely to be a particularly severe problem for cells, as base hydrolysis leading to abasic sites is the most common form of spontaneous DNA damage, occurring in each mammalian cell ~10,000 times a day (36). Possibly the misincorporation of C's across from spontaneously generated abasic sites contributes to an advantageous basal level of mutagenesis which allows the generation of variation necessary for evolution.

Whether Rev1 is employed in mammals to bypass adducted A's and U's found inappropriately in DNA is unknown. Ethenoadenine adducts are commonly produced after exposure to oxidative stress, which is commonly encountered during inflammation in mammalian tissues and is associated with cancer (16). *In vivo*, ethenoadenine adducts are highly mutagenic (16) and human Rev1 has been shown to bypass a 1, N^6-ethenoadenine adduct by insertion of a C (71). Why mammalian cells would preferentially use Rev1, which would bypass adducted A's and C's in a highly inaccurate manner, over another TLS polymerase, which might do so more accurately, is an open question.

Importance of Rev1's unique activity

Given that all eukaryotic cells possess a highly optimized DNA polymerase specialized for DNA synthesis of only one base pair—insertion of a C opposite a template G, it seems reasonable that this activity is used for an important cellular function which is relevant to a wide variety of organisms living under many diverse conditions. This function may include such processes as replication of G quartets or maintenance of G-rich telomeres. Alternatively, Rev1 activity may be needed to bypass a particular DNA lesion. Since the sequence of the catalytic domain of Rev1 is very highly conserved across all eukaryotes (L. Waters, unpublished observation), it is likely that the dCMP transferase activity of Rev1 is likewise very similar throughout many organisms. Therefore, the lesions which Rev1 bypasses are likely to be similar across all eukaryotes and fundamental to the process of living itself. These lesions may occur either frequently and pose a constant problem for cells or rarely but have serious consequences. Future studies elucidating the as-yet mysterious function of Rev1's specificity for C insertion opposite a template G will provide critical insight into one of the most enigmatic and fundamental players in mutagenesis.

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Appendix A

Preliminary Characterization of the Lethal Event in *rev1Δ* cells after DNA damage

Introduction and Results

As shown in Chapter Two, *rev1Δ* strains are hypersensitive to UV irradiation after release from G1 arrest while showing only mild survival defects after release from a G2 arrest. At first, this seemed counterintuitive since Rev1 levels are low in G1 and high in G2. I might have predicted that the requirement for Rev1-mediated lesion bypass would occur during the period in which its levels are highest and therefore that cells lacking Rev1 would show an increased sensitivity to DNA damaging agents during G2/M. However, we have proposed that the true substrate for Rev1 is a gap opposite to a lesion generated by repriming of the replicative polymerase downstream of the damaged base (1). This substrate would only be generated after replication on a damaged DNA template. Thus, cells which were UV irradiated prior to DNA replication would immediately encounter DNA lesions and produce gaps opposite to the TT dimers. In contrast, cells irradiated after replication would require a substantial period of time corresponding to G2, M, and G1 until replication generated the proposed substrate for Rev1-mediated lesion bypass. During this time, nucleotide excision repair or other high fidelity processes will repair the TT dimers and reduce the number of lesions encountered by replication forks. This hypothesis predicts that *rev1Δ* cells exposed to UV irradiation after release from G1 would arrest during late replication or G2/M when the proposed gaps opposite to lesions are being processed in a Rev1-dependent manner.

To test this prediction, I observed the cell cycle stage of *rev1Δ* cells after release from G1 arrest following a dose of UV irradiation which killed ~99 % of *rev1Δ* cells. To monitor cell cycle progression, I quantified the percentage of budded cells at various times after UV irradiation by microscopic inspection. While wild-type cells showed significant accumulation of budded cells 2 hrs after release, *rev1Δ* cells lagged behind, perhaps indicating a defect in initiating or progressing through S-phase (Fig. 1A). After 24 hrs, the wild-type cells had all formed microcolonies and by 48 hrs had grown into a lawn covering the entire plate. In contrast, even after 48 hrs, ~90 % of *rev1Δ* cells were arrested as budded cells (Fig 1A). The observation that *rev1Δ* cells arrest as budded cells supports the hypothesis that Rev1 activity is required specifically after replication on damaged DNA templates produces gaps opposite to DNA lesions. Interestingly, *rad30Δ* cells, which lack DNA polymerase η, do not display a bias in

arrest stage (Fig 1A), consistent with the fact that Rad30 is not subject to cell-cycle regulation at the protein level and does not show differential sensitivity to UV irradiation throughout the cell cycle (1). These results might be confounded by alterations of the DNA damage checkpoints in the *rad30Δ* and *rev1Δ* cells, however it is unclear at this time how loss of TLS polymerases affect checkpoint function.

Further examination of the *rev1Δ* cells after 48 hrs revealed that approximately half of the budded cells were arrested with a small bud indicative of S-phase and half with a large bud indicative of G2/M (Fig. 1B). Additional experiments using DAPI staining and anti-tubulin immunofluorescence will be performed to address more precisely where *rev1Δ* cells arrest after DNA damage. In the future, methods which more robustly reveal replication status, such as DNA combing or incorporation of radiolabelled nucleotides, could be employed to determine at what point during replication Rev1 is required for viability.

Materials and Methods

Strains. Strains used were derivatives of the wild-type strain W1588-4A: *MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 RAD5* (2). All strains were *bar1::LEU2* and additionally contained *rev1::kanMX4* or *rad30::kanMX4* deletions.

Cell cycle arrest assay. Logarithmically growing *bar1Δ* yeast were arrested with 50 ng/mL α-factor for 4 hours at 25 °C or until cultures displayed at least 95 % unbudded cells. Cells were washed twice to remove α-factor and plated on SC media. Plates were irradiated at 1 J/m^2/sec using a G15T8 UV lamp (General Electric) at 254 nm. A dose of 20 J/m^2 was used since this has been previously shown to result in ~99 % lethality (1). Plates were examined by light microscopy at the indicated timepoints.

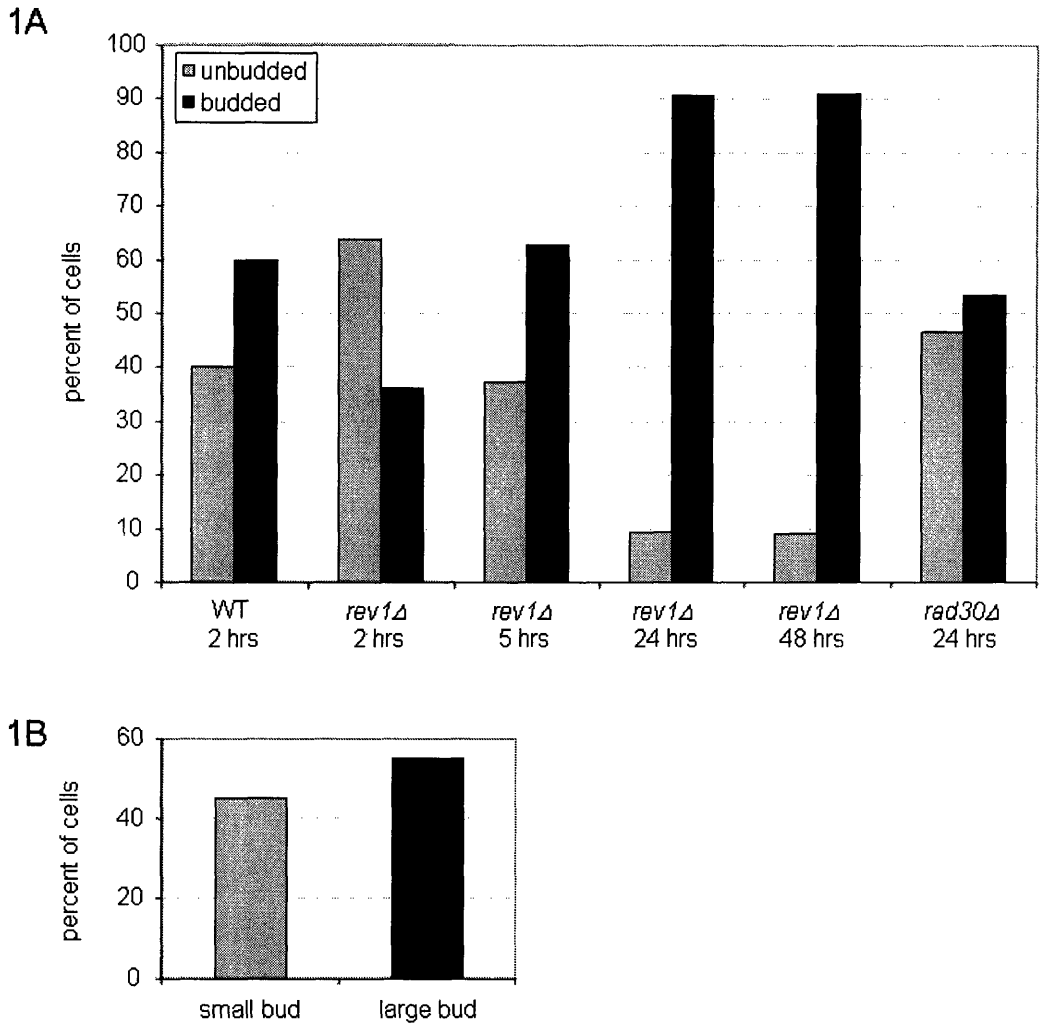

Fig. 1 *rev1Δ* cells accumulate as budded cells after UV irradiation following release from G1 arrest. (*A*) Quantitation of unbudded vs. budded cells reveals that *rev1Δ* cells predominantly arrest as budded cells following UV irradiation, while *rad30Δ* cells show equal numbers of arrested unbudded and budded cells. (*B*) *rev1Δ* cells accumulate both as small budded and as large budded cells.

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Appendix B

Initial Characterization of the Molecular Mechanism of the Regulation of Rev1

Introduction

As discussed in Chapter Two, Rev1 is subject to pronounced cell-cycle regulation, fluctuating ~50-fold between G1 and G2/M (26). The molecular mechanisms of this regulation are currently unknown, however the majority of the cell-cycle dependent oscillation in the concentration of the Rev1 protein appears to occur at the post-transcriptional level (26). *REV1* is a member of the poorly characterized *RAD6* epistasis group of post-replication repair genes, which includes many genes involved in various types of post-translational regulation: multiple E2 ubiquitin conjugating enzymes and E3 ubiquitin ligases; a modifier of a major E3 ubiquitin ligase controlling cell cycle progression; a cell-cycle regulated kinase required for the initiation of replication; and a proteasomal subunit.

When I undertook this work, it was known that five members of the *RAD6* pathway were E2 ubiquitin conjugating enzymes (*RAD6*, *MMS2*, and *UBC13*) or E3 ubiquitin ligases (*RAD18* and *RAD5*), however their targets were unknown (11, 25). Since that time, it has been shown that the Rad6/Rad18 heterodimer is responsible for monoubiquitinating PCNA after DNA damage and that Rad5/Mms2/Ubc13 polyubiquitinate PCNA through non-canonical K63 linkages (9, 11). Since ubiquitination often leads to degradation, either directly or indirectly, I hypothesized that *RAD6*, *RAD18*, *RAD5*, *MMS2*, or *UBC13* may affect the cell-cycle dependent regulation of Rev1.

The pattern of Rev1 expression I observed in wild-type cells was consistent with degradation by the anaphase-promoting complex/cyclosome (APC/C), an E3 ubiquitin ligase which is responsible for regulation of the cell cycle through timed ubiquitination of cyclins and other cell cycle factors (27). Rev1 interacts with the Rev7 subunit of DNA polymerase ζ in yeast, flies, and mammals (1, 4, 6, 8, 17, 19, 23, 24). In higher eukaryotes, Rev7 also interacts with the spindle checkpoint protein Mad2 (18). Moreover, similar to Mad2, Xenopus Rev7 interacts with components of the APC/C and inhibits cell-cycle progression (3, 21). Although yeast Rev7 has not been shown to have such a function, I wanted to investigate whether *REV7* is responsible for any part of the cell-cycle-dependent expression of Rev1.

One of the most intriguing members of the *RAD6* epistasis group is *CDC7*, a kinase required for the initiation of S-phase. Different mutant alleles of *CDC7* can display either a *hypermutator* or a *hypomutator* phenotype after DNA damage (12). The fact that *CDC7* mutants

show both an increase and a decrease in mutagenesis implies that *CDC7* may be involved in regulating mutagenic TLS. Indeed, *cdc7Δ mcm5-bob1* mutants were recently found to be epistatic to *rev3Δ* mutants for survival after UV irradiation, although they show an additive relationship for survival after exposure to MMS (20). Although Cdc7 levels do not change during the cell-cycle, its interacting partner Dbf4, which is required for Cdc7 kinase activity, displays cell-cycle regulation, peaking during late G1/early S-phase (14). Interestingly, Dbf4 contains a BRCT domain (5) and it is attractive to speculate that the Cdc7-Dbf4 complex binds to the BRCT domain of Rev1 in order to phosphorylate Rev1 and alter its levels and/or activity. In order to analyze the effect of *CDC7* on Rev1 expression, two temperature-sensitive alleles can be employed: *cdc7-1* which displays a reduced frequency of mutagenesis and *cdc7-4* which displays an increased mutation frequency (12).

I also initiated an investigation into the role of a proteasomal subunit in Rev1 cell-cycle regulation. *UMP1* has been implicated in *REV3*-dependent mutagenesis (16, 22). Since an *ump1Δ* strain and other proteasomal mutants show a slight *hypermutator* phenotype (16, 22), I wondered if the proteasome was responsible for degrading Rev1 in a cell-cycle dependent fashion and further, if aberrantly high levels of Rev1 caused by proteasome dysfunction might result in an increase in mutation frequency.

The above genes were mostly implicated in ubiquitin-mediated degradation as a mechanism controlling cell-cycle expression. To directly address whether Rev1 cell-cycle expression was mediated by degradation, I employed a modified pulse-chase analysis to compare the half-life of Rev1 protein in G1 relative to G2.

The preliminary results from these experiments have begun to provide us with an explanation for the cell-cycle regulation of Rev1. Understanding of the regulation of Rev1 will provide us with valuable insight into its function *in vivo*, which is still unclear despite decades of research.

Results

RAD6, *RAD18*, *MMS2*, *UBC13*, and *REV7* have little effect on Rev1 expression levels

To investigate the effect of several genes known to mediate ubiquitination or cell cycle arrest, I moved kanMX4 disruption cassettes from the deletion library into the Rev1-TEV-ProA-His_7 strain and assayed for the expression of Rev1 throughout the cell cycle. An abbreviated set of timepoints were taken to gain a quick look into whether Rev1 levels were aberrantly high in G1 or whether they failed to accumulate in G2/M. The results showed no striking difference in the pattern of Rev1 expression (Fig. 1A), indicating that the majority of the cell-cycle regulation of Rev1 is independent of these factors or depends on a redundant function not disrupted in the single mutant strains. However, some subtle alterations were observed. The maximal levels of Rev1 were somewhat lower in the *mms2Δ* and *ubc13Δ* strains. Moreover, the last 120 min. timepoint representing M phase may show a reduction in Rev1 levels. As this experiment was only performed once, replicates are needed to determine whether these observations are reproducible. Both the *rad6Δ* and *rad18Δ* strains exhibited slow growth; in particular the *rad6Δ* strain grew so slowly that it did not achieve > 95 % unbudded cells until after 8 hrs in α-factor arrest at 30 °C, relative to only 4.5 hrs at 25 °C for the other strains. Due to the extremely slow growth of the *rad6Δ* strain, only the first timepoint immediately after release from α-factor was taken for cell cycle analysis. The *rad6Δ* mutant showed no detectable Rev1 in G1. The slow growth and pleiotropic phenotypes of *rad6Δ* mutants have led many groups to use *rad18Δ* strains instead to analyze *rad6Δ* effects specific to DNA damage tolerance, as Rad6 requires Rad18 to monoubiquitinate PCNA (9). In this experiment, the *rad18Δ* strain showed an essentially wild-type Rev1 pattern, however the overall levels of Rev1 may be reduced.

Subsequently, the effect of a *rev7Δ* mutant on Rev1 expression was investigated. Overall, Rev1 levels were low after release from G1 and accumulated as cells entered G2, indicating that *REV7* does not play a major role in the cell-cycle regulation of Rev1 (Fig. 1B). However, relative to the previously observed pattern, the *rev7Δ* strain showed higher levels of Rev1 in G1 and a more gradual accumulation through the cell cycle. These results require repeating, especially since the *rev7Δ* strain grew uncharacteristically slowly in this experiment.

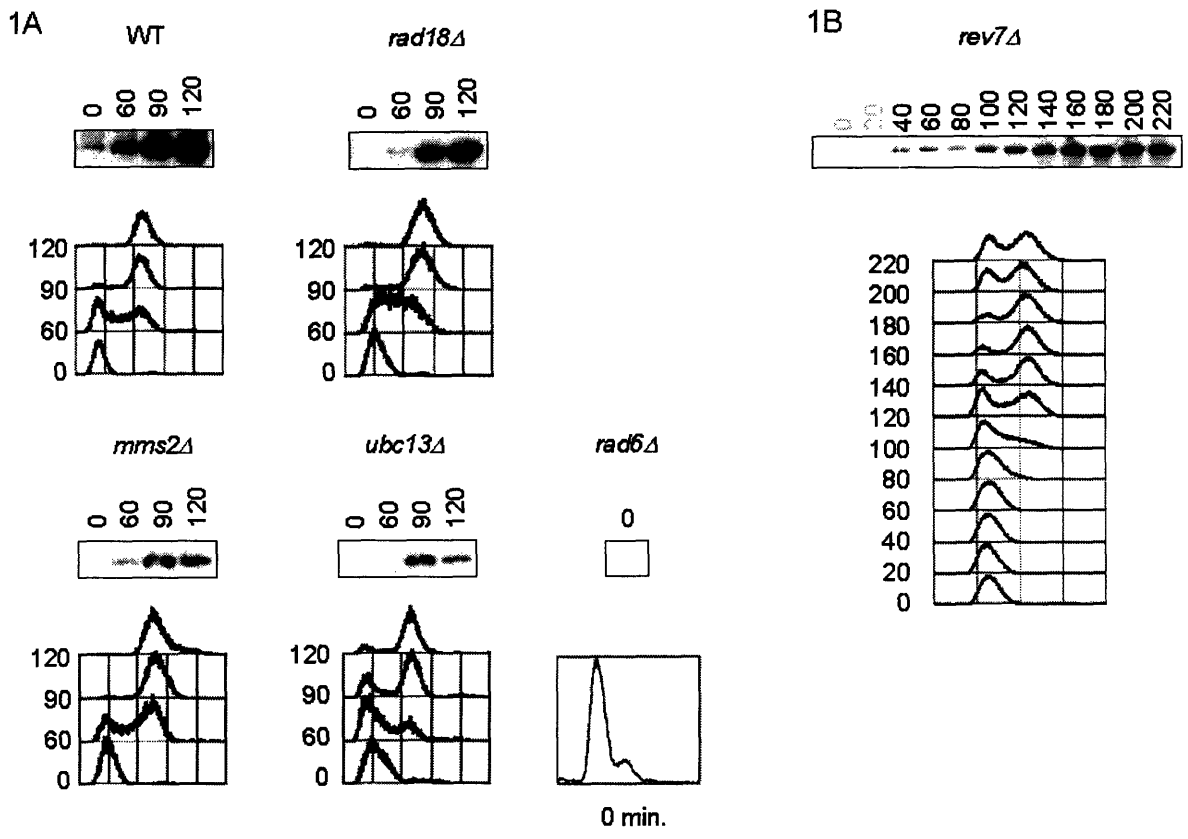

Fig. 1 Rev1 expression is not significantly altered in *rad6Δ*, *rad18Δ*, *mms2Δ*, *ubc13Δ*, or *rev7Δ* strains. (*A*) Immunoblot shows that Rev1 levels are low during G1 and rise as cells enter G2 in the indicated mutant backgrounds. FACS analysis monitors cell cycle progression. Due to its extremely slow growth, only the 0 min. timepoint was taken for the *rad6Δ* strain. (*B*) Immunoblot showing Rev1 cell cycle expression in a *rev7Δ* strain. A bubble prevented transfer of protein in the region of the 0 and 20 min. timepoints, as indicated by the grey type. FACS analysis shows DNA content of cells as they progress through the cell cycle.

CDC7 and *UMP1* alter Rev1 levels, perhaps in a cell-cycle dependent manner

The role of *CDC7* in the cell-cycle regulation of Rev1 was analyzed by moving the Rev1-TEV-ProA-His_7 epitope tagged construct into a strain bearing the temperature-sensitive *cdc7-4* allele. Rev1 expression throughout the cell cycle was monitored at the permissive temperature to allow cell cycle progression. It should be noted that the original *cdc7-4* mutant in the Hartwell strain background was only back-crossed 3 times with W303 to generate the strain used here. Therefore, the *cdc7-4* strain used in the experiment may be slightly different from our W303-based lab strain used as a control, so the effects observed may result from a mutation present in one genetic background relative to the other. As an additional complicating factor, the strains released poorly from α-factor, as seen in the FACS analysis. However, with these caveats in mind, the *cdc7-4* strain did show ~5-fold or more increase in Rev1 levels in G1 relative to the wild-type (Fig. 2). Interestingly, the *cdc7-4* allele is reported to have an increased mutation frequency (12). As higher levels of Rev1 were observed, the *cdc7-4* background may promote Rev1 accumulation and hence mutagenesis. It is difficult to compare Rev1 levels at any other timepoint since the *cdc7-4* did not display the same kinetics of cell cycle progression as the wild-type, therefore correlating cell-cycle stages between the two strains is too subjective. In order to perform this experiment in a more robust manner, the *cdc7-4* allele will be moved to our lab strain background. The *cdc7-1* allele will also be moved and tested for its effect on Rev1 expression. As the *cdc7-1* strain displays a reduction in mutagenesis (12), it will be fascinating to determine if Rev1 levels are lower in this mutant background.

Finally, the role of *UMP1* on Rev1 expression was investigated in collaboration with Mary Ellen Wiltrout. Preliminary evidence clearly indicates that Rev1 levels are significantly higher in an *ump1Δ* strain, as well as after treatment with the proteasome inhibitor MG132 (M.E. Wiltrout, unpublished data). However, at this time it is unclear whether this represents a constant degradation throughout the cell cycle or whether the proteasome is employed at specific times to promote cell-cycle dependent turnover of Rev1.

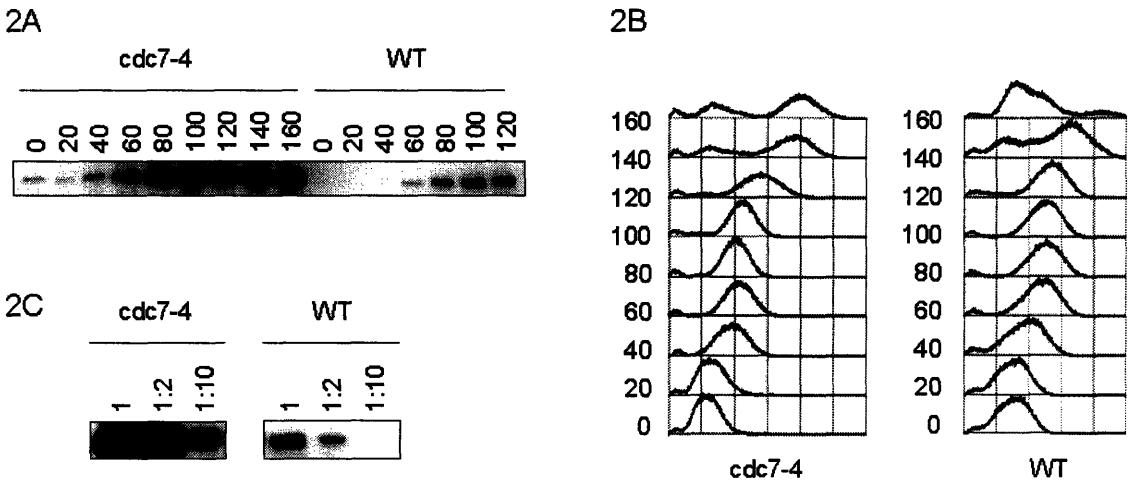

Fig. 2 Rev1 levels are ~5-fold higher in G1 in a *cdc7-4* mutant strain. (*A*) Immunoblot shows that Rev1 displays cell-cycle dependent expression in the *cdc7-4* mutant strain. (*B*) FACS analysis monitoring cell cycle progression in the *cdc7-4* and WT strains. (*C*) Quantitative dilutions of the 0 min. timepoint from part A shows that Rev1 is expressed at a higher level in the *cdc7-4* mutant background. Ponceau-S staining of the membrane revealed that the total protein concentration in the *cdc7-4* whole cell lysates was twice that of the protein concentration in the WT samples. Thus, Rev1 is present at ~5-fold higher levels in the *cdc7-4* strain just after release from G1 arrest.

Rev1 degradation may not contribute to its cell-cycle expression

To monitor the half-life of the Rev1 protein in G1 arrested cells relative to G2 arrested cells, I employed a modified pulse-chase protocol using a C-terminally HA-tagged Rev1 construct under the control of the galactose-inducible promoter. Rev1 expression was induced with galactose and cells were arrested in G1, S, or G2. At time zero, glucose was added to the cultures to prevent further Rev1 expression and timepoints were taken as indicated. The galactose induction corresponds to a pulse of Rev1 expression followed by a chase phase when Rev1 is repressed by the addition of glucose. However, unlike a classical pulse-chase experiment where radiolabelled amino acids specifically monitor protein stability, in this assay, mRNA stability influences the outcome of the protein levels as well. To remove the complicating factor of the mRNA stability, in the second experiment cycloheximide was added to prevent further translation from any remaining *REV1* transcripts. Despite the difference in experimental design, both replicates of the assay consistently showed that, under these conditions, the Rev1 protein is reasonably stable throughout the cell cycle (Fig. 3). In fact, the half-life of Rev1 in G1-arrested cells appeared to be slightly longer than in G2-arrested cells (Fig. 3). The Rev1-1 protein, which contains a mutation in the BRCT domain which inactivates its function *in vivo*, shows equivalent stability to the wild-type protein (Fig. 3B). Interestingly, under these conditions, Rev1 is able to be overexpressed stably in G1 and is only present at ~3-fold lower levels than in G2, despite being present at ~50-fold lower levels in G1 than in G2 under normal chromosomal regulation (26). Thus, misregulation of Rev1 by overexpression in G1 is possible. This finding can inform future experiments probing the effect of ectopic expression of Rev1 in G1. Though these preliminary experiments do not have a control to confirm that the assay worked properly, the results indicate that Rev1 may not be subject to differential degradation throughout the cell cycle. Future work will confirm and extend these findings.

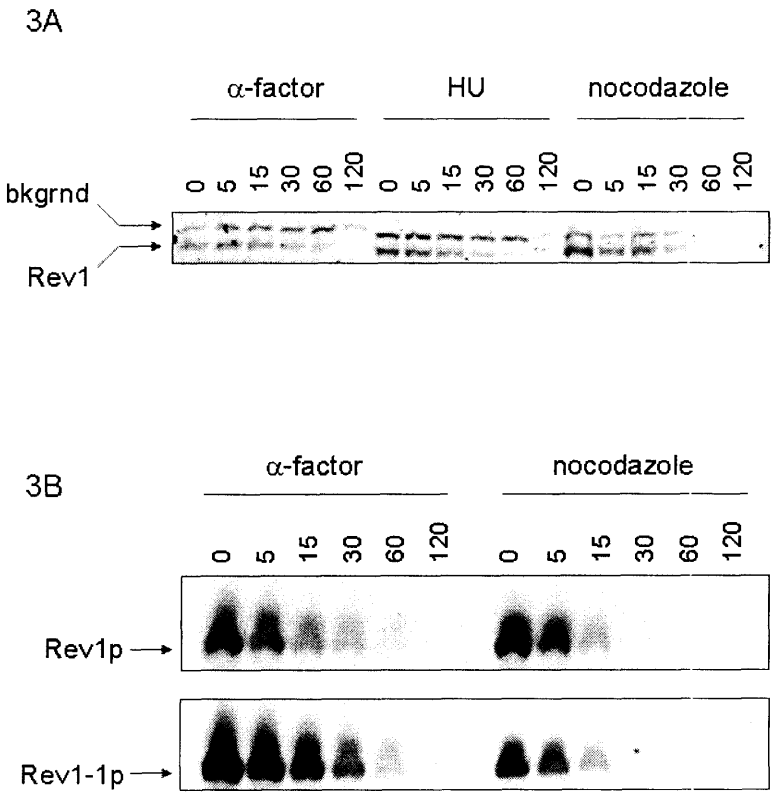

Fig. 3 Rev1 cell-cycle expression does not seem to be controlled significantly by degradation. (*A*) Immunoblot shows Rev1 stability is roughly the same cells arrested in G1, S, or G2. (*B*) Immunoblot showing Rev1 and Rev1-1 protein stability after addition of cycloheximide in G1 or G2 arrested cells.

Discussion

Summary of results

A candidate gene approach was taken in order to determine the molecular mechanisms controlling the cell-cycle regulation of Rev1. For several of the genes investigated, no significant alteration in Rev1 levels was observed. In retrospect, the lack of an effect may not be surprising in the case of the *mms2Δ* and *ubc13Δ* mutants or the *rad6Δ* or *rad18Δ* mutants since these genes mediate K63-linked polyubiquitination and monoubiquitination respectively, neither of which are not thought to be involved in protein degradation (10, 11). An indirect role of these modifications in promoting protein degradation is not excluded, however since no major change in Rev1 expression was observed, it is not likely that such an indirect effect occurs. More subtle alterations of Rev1 protein levels may be important and need to be investigated further.

In contrast, two less characterized members of the *RAD6* epistasis group appear to be involved in regulating Rev1: *CDC7* and *UMP1*. Since Rev1 appears to increase slightly after cells initiate replication and slowly accumulates throughout S-phase (26), I speculated that Cdc7-Dbf4 may phosphorylate Rev1 upon entry into S-phase to allow accumulation of very low levels during replication. A second Rev1 modification as replication is completed may promote significant stabilization of Rev1 as its levels rapidly increase during late S-phase as cells enter G2. Indeed, preliminary data supports a role for *CDC7* in controlling Rev1 levels. Although the Cdc7 kinase is only activated after G1 when its regulatory subunit Dbf4 is expressed, I observed increased Rev1 levels in the *cdc7-4* background immediately after release from G1. This may indicate a non-catalytic role for Cdc7 in Rev1 accumulation during G1 or that Cdc7 can be activated by another subunit in G1. This result is very preliminary, but the difference observed is worthy of further experiments to verify the importance of *CDC7* to Rev1 expression.

Additionally, degradation by the proteasome, mediated by the Ump1 subunit, reduces Rev1 levels by ~10-fold (M.E. Wiltrout, unpublished data). This may be a continuous process throughout the cell cycle to keep Rev1 levels low even during G2/M, or Rev1 may be subject to proteasomal degradation only during G1 and S-phase. The preliminary results from the modified pulse-chase assay indicate that Rev1 protein is as stable in G1 and S-phase as in G2, supporting the former possibility. However, this experiment may suffer from artifacts imposed by the long

galactose induction necessary to observe Rev1 or by the overexpression of Rev1 from a high copy plasmid. Thus, further experiments are underway to investigate this question (M.E. Wiltrout, unpublished data).

Model for regulation of REV1 activity by ubiquitin

Rev1 binds to monoubiquitin through recently identified ubiquitin-binding motifs (UBMs) (7). Additionally, the UBMs mediate self-monoubiquitination of Rev1 (7). The biological relevance of this modification is currently unknown. I speculate that, similar to the auto-inactivation exhibited by Src kinase (2), the UBMs of Rev1 may mediate its monoubiquitination and then, via in an intramolecular interaction, bind to its own monoubiquitin moiety. This would prevent the recruitment of Rev1 to monoubiquitinated PCNA and inactivate its localization to sites of DNA damage. Upon DNA damage, a deubiquitinating enzyme (DUB) may relieve the inhibition of Rev1 localization by removing the monoubiquitin moiety from Rev1. Thus freed from self-interaction, the UBMs of Rev1 would facilitate recruitment of Rev1 to monoubiquitinated PCNA at sites of DNA damage. Once present at a blocked primer terminus, Rev1 would promote translesion synthesis and subsequently might serve as its own E3 ubiquitin ligase to inactivate itself once more, preventing further unnecessary and potentially mutagenic TLS. A convenient source of monoubiquitin would be provided by PCNA itself. Transfer of the monoubiquitin moiety from PCNA to Rev1 would thus accomplish two levels of inhibition of TLS—inactivation of Rev1 activity and removal of the recruitment signal on PCNA for translesion polymerases. Although highly speculative, this model is consistent with the current evidence that, while Rad6 and Rad18 are required to activate mutagenic TLS after DNA damage, they do not control the cell-cycle expression of Rev1.

Materials and Methods

Strains and Primers. Strains used are shown in Table 1. All strains are derivatives of W1588, a W303 strain corrected to be *RAD5* (28). Briefly, kanMX4 disruption cassettes from the yeast deletion collection were amplified by PCR and transformed into the *REV1-TEV-ProA-*

7His::HIS3MX6, bar1::LEU2 strain. For the *cdc7-4* strain, the *REV1-TEV-ProA-7His::HIS3MX6* cassette was amplified by PCR and transformed into strain OAy711. As the *cdc7-1* strain is already His^+ (15), the epitope tagged Rev1 construct could not be moved into the *cdc7-1* strain background by this method. Primers are shown in Table 2.

Cell Synchronization. Logarithmically growing *bar1Δ* yeast were arrested with 50 ng/mL α-factor for 4 hours at 25 °C or until cultures displayed at least 95 % unbudded cells. Cells were washed twice to remove α-factor and released into YPD media. Timepoints were removed as indicated for TCA precipitation and FACS analysis.

FACS Analysis. Following fixation in 67% ethanol at 4 °C for up to one week, cells were washed in 50 mM sodium citrate and incubated overnight at 50 °C with 250 μg/mL RNaseA (Qiagen). Proteinase K was added to 500 μg/mL and incubated at 50 °C for 1 hour. Cells were sonicated, stained with 16 μg/mL propidium iodide (Sigma), and analyzed on a Becton Dickson FACSCalibur flow cytometer.

Immunoblots. Whole cell extracts were prepared by TCA precipitation (13) and were separated by SDS-PAGE. Proteins were transferred to PVDF membrane (Millipore) using a Mini-PROTEAN II transfer apparatus (Bio-Rad). Ponceau-S staining confirmed that the total protein loaded in each lane was equivalent. Antibodies used were rabbit PAP antibody (Sigma) against the protein A tag, anti-HA.11 (Covance), and anti-phosphoglycerate kinase (Molecular Probes).

P_{GAL} Pulse-Chase Assay. A *rev1Δ* strain bearing pAC311-REV1-HAC or pAC-rev1-1-HAC (4) was grown in SC-Trp media containing 2 % sucrose as a carbon source. Approximately eighteen hours prior to arrest, cells were resuspended in SC-Trp media containing 2 % galactose to induce Rev1 expression. Logarithmically growing *bar1Δ* yeast were arrested with 50 ng/mL α-factor (SynPep), 200 mM hydroxyurea (Sigma), or 15 μg/mL nocodazole (Sigma) for 4 hours at 25 °C or until cultures displayed at least 95 % unbudded cells for G1 arrest or at least 95 % budded cells for S-phase and G2 arrest. At time zero, glucose was added to a final concentration of 2 % and timepoints were removed as indicated for TCA precipitation. In Fig. 3B,

cycloheximide (Calbiochem) was added to a final concentration of 50 μg/mL at the same time as glucose addition.

Acknowledgements

I thank Sanjay D'Souza for the gift of pAC311-REV1-HAC, pAC-rev1-1-HAC, and primers REV7DELAFWD and REV7DELDBKWD. I thank the R. Woodruff for the gift of strain JAy19 and the Bell lab for the gift of strains RM14-3a and OAy711.

Table 1: Strains used in Appendix B

Strain	Genotype	Source
W1588-4C	*MATa ade2-1, ura3-1, his3-11,15, trp1-1, leu2-3,112, can1-100, RAD5*	Rothstein lab (27)
JAy19	*bar1::LEU2, REV1-TEV-ProA-7His:HIS3MX6, RAD30-TEV-ProA-7His:HIS3MX6*	R. Woodruff
rad6Δ Rev1-tag	*rad6::kanMX4, bar1::LEU2, REV1:TEV-ProA-7His:HIS3MX6*	this study
rad18Δ Rev1-tag	*rad18::kanMX4, bar1::LEU2, REV1:TEV-ProA-7His:HIS3MX6*	this study
mms2Δ Rev1-tag	*mms2::kanMX4, bar1::LEU2, REV1:TEV-ProA-7His:HIS3MX6*	this study
ubc13Δ Rev1-tag	*ubc13::kanMX4, bar1::LEU2, REV1:TEV-ProA-7His:HIS3MX6*	this study
rev7Δ Rev1-tag	*rev7::kanMX4, bar1::LEU2, REV1:TEV-ProA-7His:HIS3MX6*	this study
ump1Δ Rev1-tag	*ump1::kanMX4, bar1::LEU2, REV1:TEV-ProA-7His:HIS3MX6*	M.E. Wiltrout
rev1Δ bar1Δ	*rev1::kanMX4, bar1::LEU2*	L. Waters (25)
RM14-3a	*MAT a cdc7-1, bar1, ura3-52, trp1-289, leu2-3,112, his6*	Bell lab, (14)
OAy711	*MAT a ade2, ura1, ura3, his3, his7, leu2, cdc7-4*	Bell lab
cdc7-4 Rev1-tag	OAy711 *REV1-TEV-ProA-7His:HIS3MX6*	this study

Table 2: Primers used in Appendix B

Primer Name	Sequence
Rad6-f	GGTGACTACATTTCCCGGATTAG
Rad6-r	CGGGTATCGGCAGTTATAACC
Rad18-f	CTTGCCCCGTTGCCTTGC
Rad18-r	CAGCACTTAACGTGGAGATCAC
Mms2-f	CATTGCAATGCCGCTCTCACATC
Mms2-r	CTTGGGTGCAACAGTCTTTCTG
Ubc13-f	CCGCATCCGTATTGTTACCCG
Ubc13-r	CTTACATTAGTGTAGGACGGTCG
REV7DELAFWD	AGTATGTATTTCTTTTCCCCTTGCT
REV7DELDBKWD	CGCCACTTACAAAATATTCAAGACT
Rev1D	GTGAAACAATGGGTTGCCGAAACTTTAGGTGATGG
Rev1E	GGCGAGGTCTTTCGGAATGGTGG
Ump1-fwd	CAGGATTTAAGAAGTCCATACCGCAGG
Ump1-rev	CCTCCAACTGGATTCAACTGAAACTGG

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Appendix C

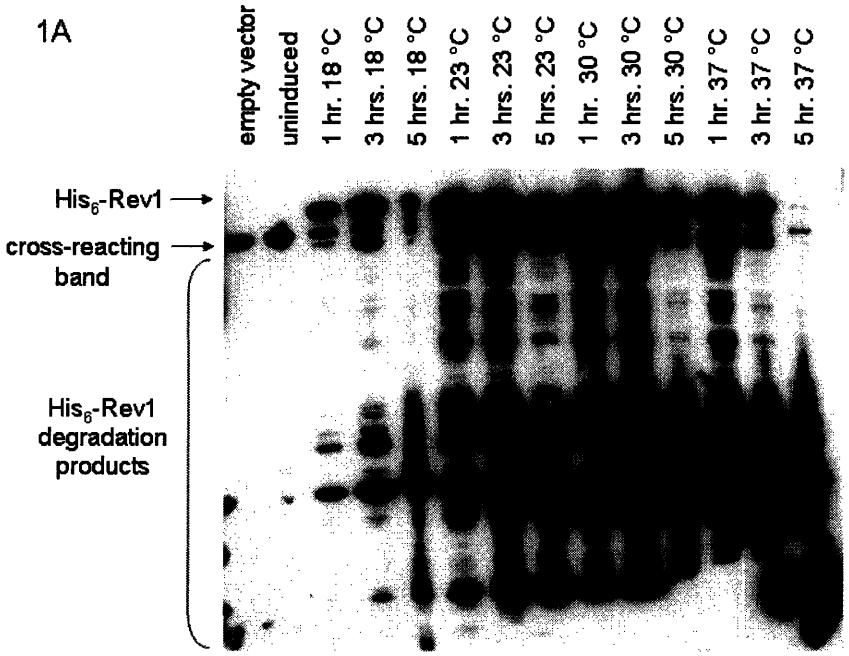
Purification of Rev1

Introduction and Results

A major focus of my thesis research was to identify the interacting partners of Rev1 in *S. cerevisiae*. Additionally, I wanted to produce antibodies to Rev1 that could detect its endogenous levels. To that end, I generated epitope tagged constructs for Rev1 expression both in *E. coli* and yeast and attempted several purification schemes outlined below.

Overexpression of Rev1 from plasmids in *S. cerevisiae* and *E. coli*

I was able to purify a small amount of Rev1 from overexpressing plasmids in yeast and *E. coli*, but the protocols were never optimized and were not pursued in order to focus on purifying native levels of Rev1 and its interacting partners. First, I set out to purify Rev1 from yeast using pJN60, a high-copy plasmid containing an N-terminal GST fusion to Rev1 under a galactose-inducible promoter (4). The yield of Rev1 was poor, so I turned to bacterial expression systems. To purify Rev1 from *E. coli*, three plasmids were created: pGEX-Rev1, pET16b-Rev1, and pET11T-Rev1. pGEX-Rev1 encoded an N-terminal GST fusion to Rev1 under the P_{Tac} promoter and included a thrombin protease site in the linker between GST and Rev1. The thrombin digestion was very ineffective and overnight incubation resulted in the disappearance of Rev1. Additionally, much of Rev1 expressed in *E. coli* seemed to be insoluble and precipitated in the pellet after centrifugation of the crude lysate. Thus, I turned to using an N-terminal His_6 tag to allow purification of Rev1 under denaturing conditions in order to obtain large amounts for antibody production. To this end, I constructed pET16b-Rev1 and pET11T-Rev1 which both expressed His_6-Rev1 from the T7 promoter. The pET11T vector contains a terminator upstream of the T7 promoter which substantially reduces background expression levels (5) and has been used successfully to express moderately toxic proteins at high levels (2). Both of these plasmids seemed to express equivalent amounts of Rev1. I transformed the His_6-Rev1 plasmids into multiple *E. coli* strains optimized for expression of eukaryotic proteins and found that optimal induction of Rev1 from pET16b required short inductions at low temperatures to prevent rampant degradation (Fig. 1). Initial purification attempts were thwarted by low yield. It was decided to pursue antibodies directed against Rev1 synthetic peptides and to purify Rev1 and its interacting partners from natively expression levels in yeast.



Optimal induction conditions: 1-3 hours at 18 – 23 °C.

Fig. 1 Optimum expression of full-length Rev1 in *E. coli* requires short induction times at low temperatures. (*A*) Immunoblot directed against the N-terminal His_6 tag of Rev1. Cells were grown to OD_{600} ~0.5 and IPTG was added to a final concentration of 1 mM. Lane 1 contains whole cell lysate from a strain bearing the empty vector pET16b, lane 2 shows lysate from an uninduced culture. Both lanes 1 and 2 exhibit a cross-reacting band just below full-length His_6-Rev1. Lanes 3 – 15 show different induction conditions as indicated. Cells were harvested and boiled in loading buffer to prepare samples for SDS-PAGE.

Purification of natively expressed Rev1 in *S. cerevisiae*

For purification of Rev1 expressed from its native promoter in the chromosome, I used a C-terminal protein A epitope tag which has a high affinity for IgG. I grew large volumes of cells to mid-late log phase to obtain a dense culture in which cells were still actively growing, since Rev1 is predominantly expressed during G2/M. Cells were collected, lysed, and the lysate applied to IgG beads. The beads were washed and a pre-elution sample was taken to analyze the amount of Rev1 bound to the beads before elution with TEV protease. The beads were also boiled to assess the efficiency of TEV cleavage by observing the amount of Rev1 remaining on the beads. Samples were analyzed by SDS-PAGE followed by either Sypro Ruby staining or by Western blot detection against the protein A epitope tag. Sypro Ruby can detect ~1 ng or less of protein per band. Even with this level of sensitivity, it was very difficult to detect Rev1 on stained gels, although Rev1 could be easily seen by Western blotting, indicating that the yield of the purification was very poor (<1 ng/L).

I repeated this purification, with some variations, nine times in an attempt to optimize the protocol, however I was only able to observe Rev1 by Sypro Ruby staining in three experiments (Fig. 2). In two experiment could I detect potentially copurifying bands specifically found in the eluates from the Rev1-tag strain and not found in the WT eluates (Fig 3). The identity of these bands is unfortunately unknown. In order to visualize the bands, I loaded the entire sample onto the gels and thus had no eluate fraction remaining to send for mass spectrometry analysis. Sypro Ruby staining is only detectable by fluorescence imaging so I could not cut out the bands, and silver staining was not sufficiently sensitive to detect these proteins. It is possible that the ~68 kD bands correspond to Rad30, which encodes DNA polymerase η in yeast, although genetic and biochemical evidence indicates that Rev1 and Rad30 do not interact [(1, 3); R. Woodruff, unpublished data]. Alternatively, the ~68 kD band may represent the Ddc1 subunit of the alternative 9-1-1 sliding clamp. The ~175 kD band may correspond to the Rev3 catalytic subunit of DNA polymerase ζ. Unfortunately, leaching of IgG molecules from the beads prevented identification of any bands smaller than 50 kD. To test the identity of these bands or to detect proteins present at less than 1 ng, a candidate Western blot approach could be used to assay for enrichment of particular proteins, such as PCNA or Rev7, bound to the Rev1-tag beads relative

to the WT control beads. Due to progress in other areas of my research, I did not employ this method, although we did obtain antibodies to several candidate proteins.

By Western blot, I could determine that at least 50 % of Rev1 present in the crude extract was precipitating in the pellet after centrifugation (Fig. 4A). However, Rev1 was substantially enriched on the beads prior to elution, indicating that the purification was effective but needed to be optimized (Fig. 4A). During the optimization of the purification (Fig. 4B), I discovered that Rev1 was expressed differentially throughout the cell cycle and began to pursue the characterization of Rev1 regulation. Due to advances in other areas of my project, the purification was never performed after DNA damage or under G2 arrest when Rev1 levels are high. It would be very interesting to repeat the purification of Rev1 using the new knowledge we have gained regarding its cell-cycle regulation and proteasomal degradation. Indeed, a combination of approaches using G2-arrested yeast as well as proteasomal inhibitors like MG132 may increase the starting concentration of Rev1 in cell lysates to the point where copurification of Rev1 and its interactors may be feasible.

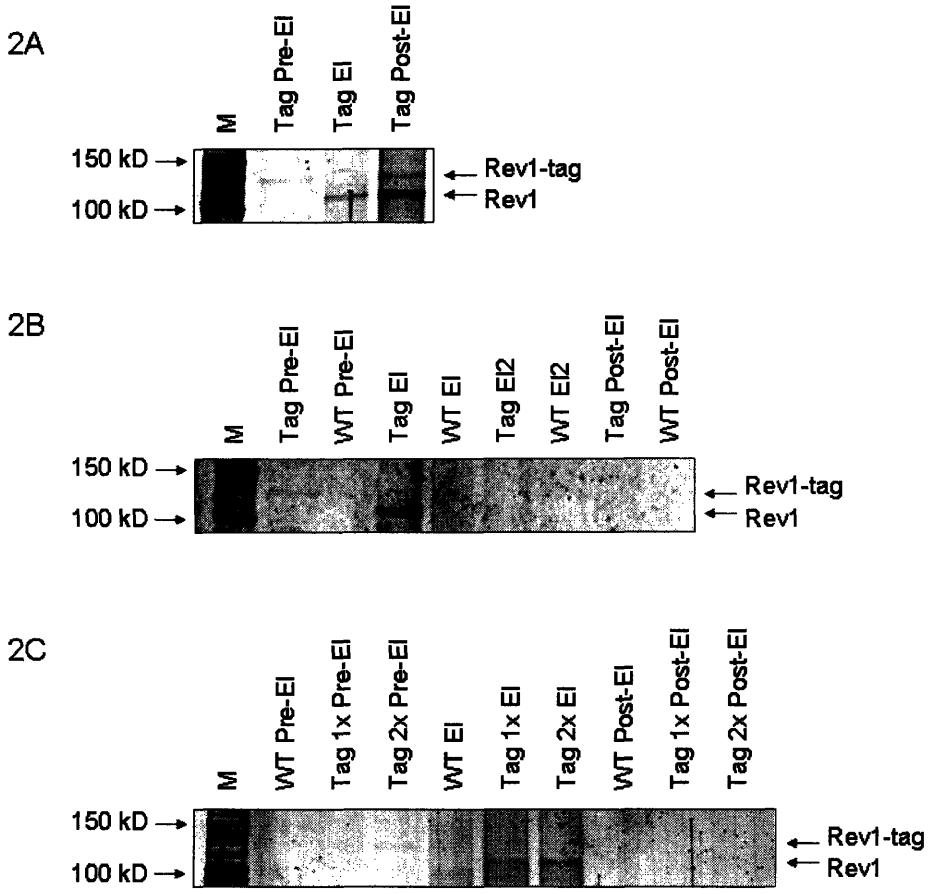


Fig. 2 Visualization of Rev1 and Rev1-TEV-ProA-His_7 in purified fractions. (*A*) Sypro Ruby stained gel of the pre-elution, elution, and post-elution fractions from the first trial of the protein A purification. (*B, C*) Sypro Ruby stained gel from the third (*B*) and sixth (*C*) protein A purifications showing bands specifically present in the tagged strain corresponding to the uncleaved Rev1-TEV-ProA-His_7 in the pre-elution fractions and the shorter Rev1 in the elution fractions. No bands are visible in the post-elution fraction, indicating that TEV protease effectively digested all the Rev1 bound to the beads. In (*C*), cell lysates from 2 L of cells (2x) compared to lysates from 1 L of cells (1x) did not reveal a corresponding increase in the amount of Rev1 purified in this experiment.

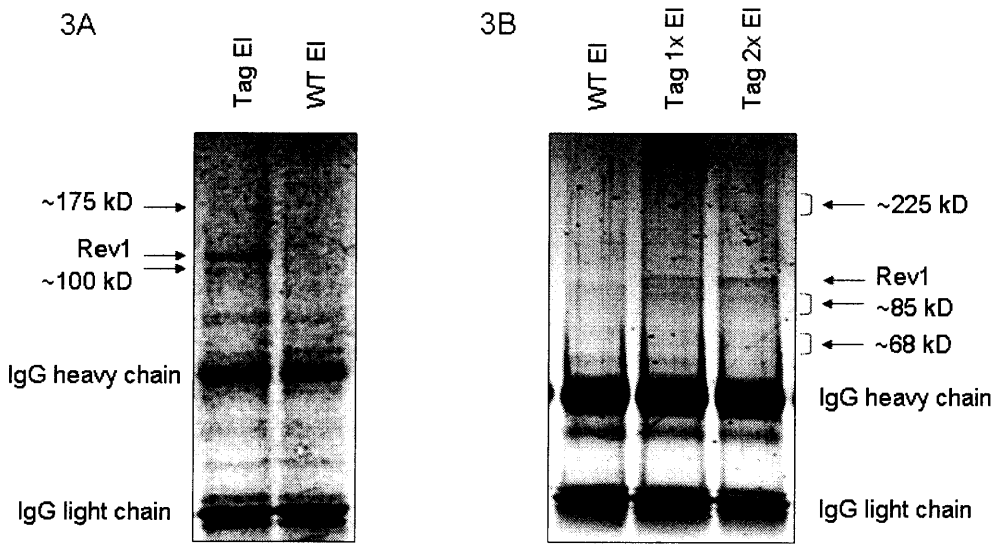


Fig. 3 Visualization of proteins copurifying with Rev1. (*A, B*) Sypro Ruby stained gels from the third (*A*) and sixth (*B*) protein A purifications reveal bands specifically present in lysates from Rev1-TEV-ProA-His_7 cells relative to WT cells.

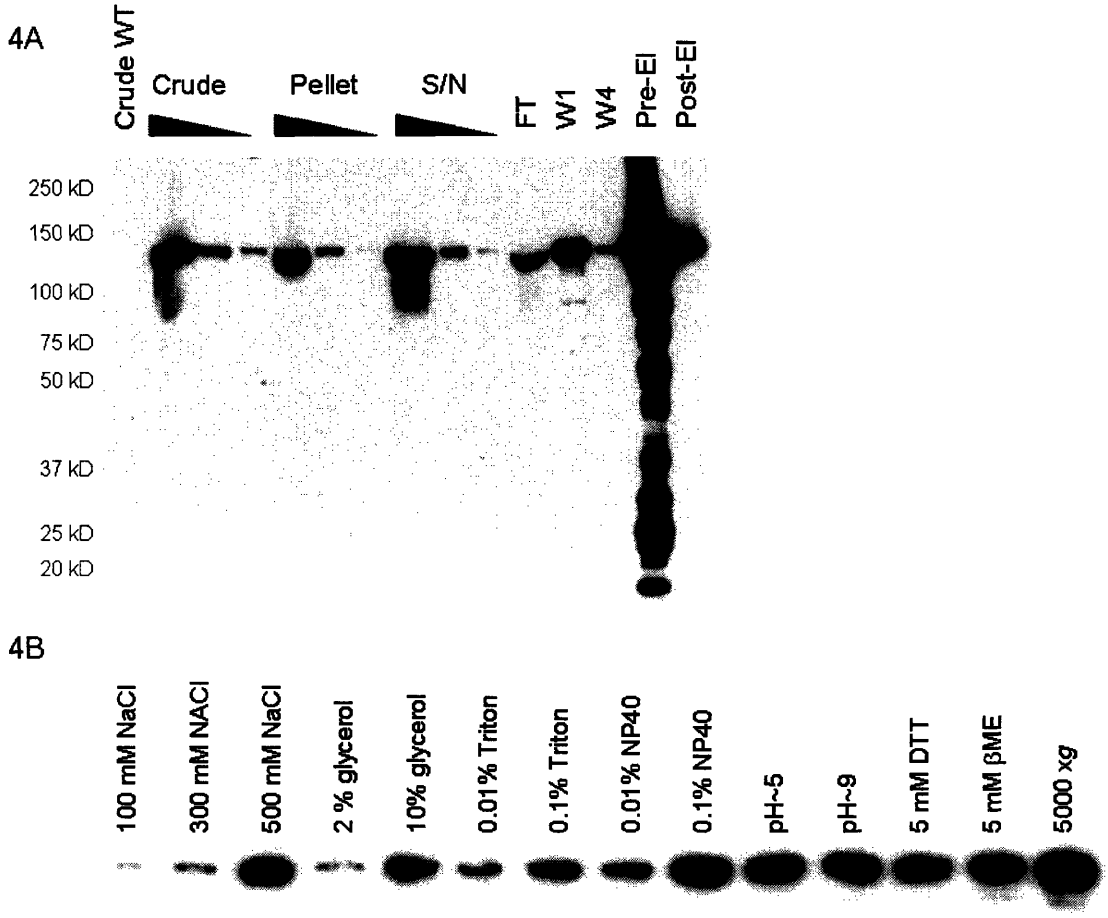


Fig. 4 Optimization of lysis conditions to reduce insoluble Rev1. (*A*) Immunoblot directed against the protein A epitope of Rev1 shows the efficiency of purification in the fourth protein A purification. Dilutions (1, 1:10, and 1:50) of the crude lysate, insoluble pellet, and clarified supernatant fractions reveal that approximately half of the Rev1 protein in the crude lysate precipitates after centrifugation. (*B*) Immunoblot against protein A reveals the amount of soluble Rev1 after centrifugation in various lysis buffers. Cells were lysed in a French pressure cell in a buffer consisting of 50 mM NaH_2PO_4, 100 mM NaCl at pH 7.0. Aliquots were taken and adjusted to the indicated concentration of salt, glycerol, detergents, reducing agents, or pH. Samples were spun at 15,000 x *g*, or for the sample in the last lane, at 5000 x *g*, for 20 minutes. The supernatant was collected and subjected to immunoblot analysis.

Materials and Methods

Strains and Primers. Strains used were derivatives of W1588-4C, a W303 strain corrected for *RAD5* (6), and are described in Table 1. Primers used to create the strains are shown in Table 2.

Protein A Purification. Yeast cells bearing the Rev1-TEV-ProA-His_7 epitope tagged construct under the native promoter in the chromosome were grown to mid-late log phase (OD_{600} ~0.7 - 0.9) to obtain a dense culture in which cells were still actively growing to enrich for higher levels of Rev1 in cycling cells. At least 1 L of cells were harvested and resuspended in lysis buffer containing 50 mM NaH_2PO_4, 300 mM NaCl at pH 7.0 and protease inhibitors consisting of 5 μg/mL pepstatin, 5 mM benzamidine, 10 mM EDTA, 1 mM EGTA, 1 tablet Mini-Complete Protease Inhibitors – EDTA (Roche) per 30 mL lysis buffer, and 10 μg/mL E-64. Usually, 100 μg/mL bacitracin and 0.1 % NP40 were also added. Cells were lysed either in French pressure cell by three passes at 15,000 psi or by grinding with dry ice. In the latter case, cells were frozen dropwise in LN_2 and stored at -80 °C until the LN_2 had evaporated. Cells were then added to a coffee grinder (Krups) filled with dry ice and ground for 5 min. at 4 °C. The lysate was thawed for 1-2 hrs at 4 °C to remove dry ice. No difference was observed in the amount of Rev1 in the crude lysate was observed between these two lysis procedures. The lysate was clarified by centrifugation either at 15,000-20,000 x *g* or at 1500-2000 x *g* and the supernatant applied to magnetic beads (Dynal) coated with rabbit IgG (Sigma). The beads were washed and a pre-elution sample was taken to analyze the amount of Rev1 bound to the beads before elution. Between 25 and 100 units of TEV protease (~1 U/μL) (Invitrogen) was added to cleave Rev1 from the protein A tag and incubated between 1 hr and overnight at 4 °C. The beads were also boiled to assess the efficiency of TEV cleavage by observing the amount of Rev1 remaining on the beads. Variations on the procedure included different volumes of cells harvested, an ultracentrifugation step, a DNaseI digestion to clarify the lysate, varying numbers and types of washes, and in later purifications, different purification resins. For two experiments, rabbit IgG coupled to Affi-gel (Bio-Rad) was used and in two other trials, Ni-NTA beads (Qiagen) were used.

Immunoblots. Fractions from the purification were separated by SDS-PAGE. Proteins were transferred to PVDF membrane (Millipore) using a Mini-PROTEAN II transfer apparatus (Bio-Rad). Antibodies used were rabbit PAP antibody (Sigma) against the protein A tag and anti-His (Santa Cruz) to detect the His_6 tag.

Protein Visualization. Fractions from the purification were separated by SDS-PAGE and stained with Sypro Ruby (Molecular Probes). Gels were analyzed on a FluorImager 595 (Molecular Probes).

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Table 1: Strains used in Appendix C

Strain	Genotype	Source
W303	*MAT α, ade2-,1 ura3-1, his3-11,15, trp1-1, leu2-3,112, can1-100*	lab stock
W1588-4A	*MATα, ade2-1, ura3-1, his3-11,15, trp1-1, leu2-3,112, can1-100, RAD5*	Rothstein lab (6)
tag10	W1588-4A *REV1-TEV-ProA-7His:HIS3MX6*	this study
ySC7	W303 *lys2::hisG, bar1::hisG, pep4::kanMX4*	Bell lab (S. Chen)
#9	tag10 *pep4::kanMX4*	this study
BL21-RIL	*E. coli* B F^- *ompT hsdS*(r_B^- m_B^-) *dcm*$^+$ Tet^R *gal endA* Hte [*argU ile& leuW* Cam^R]	Stratagene

Table 2: Primers used in Appendix C

Primer Name	Sequence
PEP4-5'	GCGGTTATTGAATCTATGGAGAGGCTG
PEP4-3'ap	GGGCAGCAGCATAGAACAATGG
KanMX4-5'ap	GTATTCTGGGCCTCCATGTCGC
KanMX-D	CGATACTAACGCCGCCATCCAG

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Appendix D

Fine Timecourse of Rev1 Cell-Cycle Dependent Expression

Results

To investigate the precise timing of Rev1 accumulation during the cell-cycle, I monitored Rev1 expression as described previously (1) at five or ten minute intervals during the cell cycle (Fig. 1). Cell cycle progression was assessed by FACS analysis as described previously (1), as well as by microscopic analysis to determine the percent of budded cells. Quantitative dilutions were used to estimate Rev1 protein levels relative to the peak value at 120 min. The results show that Rev1 levels are nearly undetectable during G1, but rise slightly just prior to or simultaneously with the initiation of replication at 40 min. after release from α-factor. During S-phase, Rev1 levels rise slowly but are still quite low even when the bulk of replication has been completed at 80 min. Following the completion of replication, Rev1 protein begins to accumulate more rapidly in G2. Rev1 levels appear to peak during mitosis and begin to decrease as cells exit from mitosis and re-enter G1.

References

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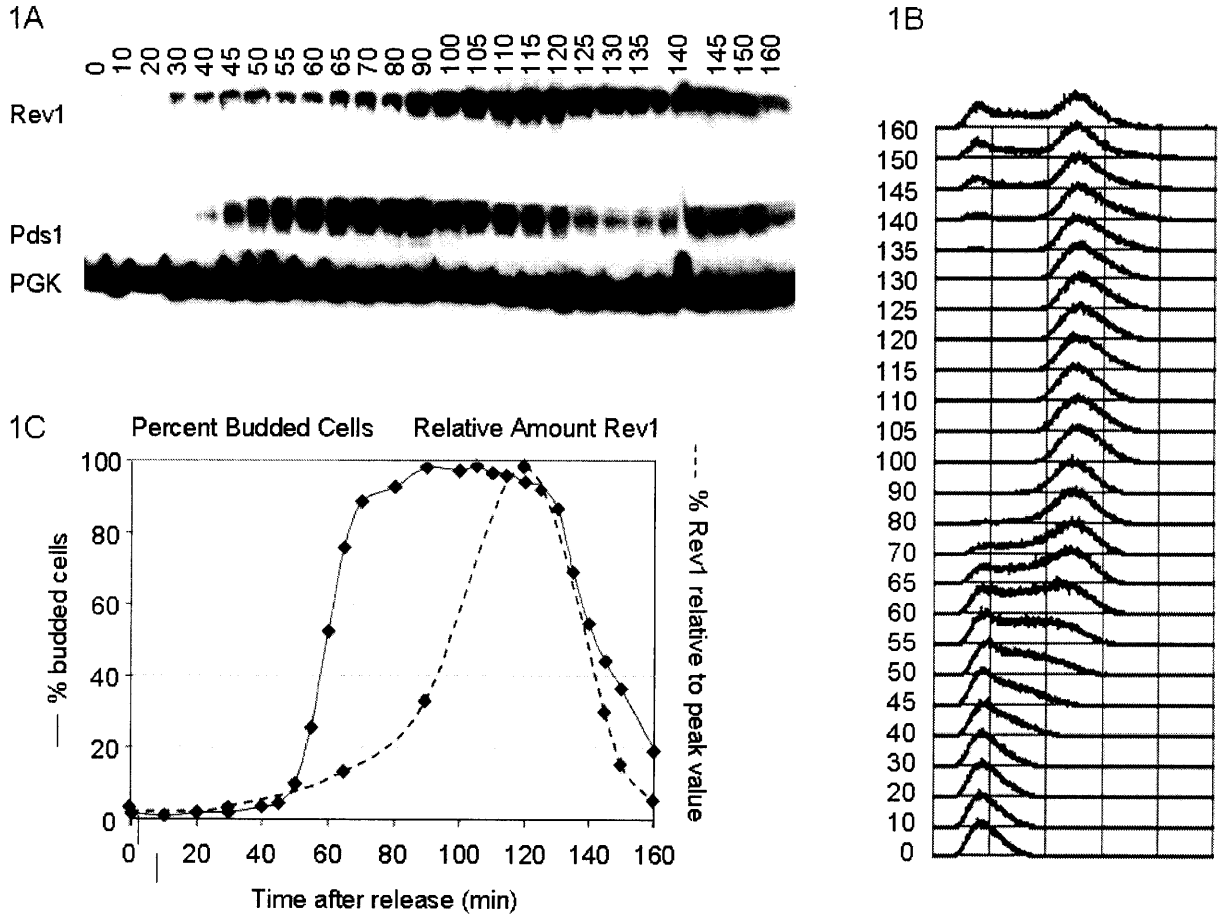

Fig. 1 Fine Timecourse of Rev1 Cell-Cycle Dependent Expression (*A*) Immunoblot showing Rev1 protein levels throughout the cell cycle. Note that a long exposure was chosen to emphasize the difference between G1 and S-phase Rev1 expression. In order to detect Rev1 in G1 and S, the bands in G2/M are highly overexposed. Pds1 was used as a marker for cell-cycle progression and PGK (phosphoglycerate kinase) as a loading control. (*B*) FACS analysis monitoring DNA content throughout the timecourse. (*C*) Plot showing the percent budded cells (solid line) and the percent of Rev1 protein relative to peak value (dashed line) as a function of time.