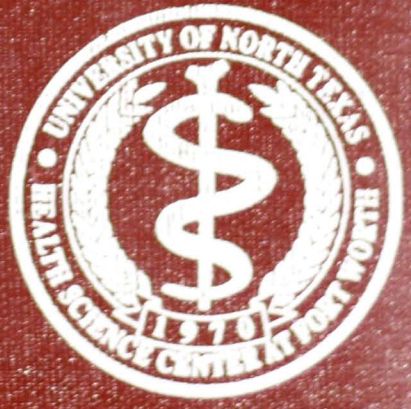
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ABSTRACT

Pabla, Ritu., Tolerating DNA damage: Translesion polymerase eta (η) and its regulation in *Saccharomyces cerevisiae*. Doctor of Philosophy (Cell Biology and Genetics), May 2008, 137 pp., 21 illustrations, bibliography, 151 titles

RAD30 gene encoded DNA polymerase eta (Polη) is the only eukaryotic polymerase that can bypass UV-induced thymine-thymine (T-T) dimers in a predominantly error-free manner. The unique ability of reading bulky and geometrically distorted bases in the template makes the polymerase low-fidelity and error-prone for an undamaged template. The purpose of this study is to delineate the mechanism(s) by which activity of Polη is regulated.The increase in RAD30 transcript after UV damage is not reflected at the protein levels. Instead, Polη is monoubiquitinated constitutively. This posttranslational modification is upregulated in G1 phase and downregulated on entry into S phase of the cell-cycle. This downregulation is further accelerated in response to UV induced DNA damage. A missense mutation (L577Q) of the ubiquitin binding domain (UBZ) results in reduced degree of ubiquitination of the mutant protein outside of G1 and a complete failure to stably interact with ubiquitinated substrates. This mutation renders the strain more UV sensitive and mutagenic, a phenotype resembling a complete RAD30 deletion. In other words, UBZ motif and its interaction with ubiquitinated PCNA is critical for Polη function *in vivo*. In addition to nucleus, the polymerase localizes in mitochondria suggesting its role in damage tolerance in mitochondria. No drastic changes in the localization of polymerase are observed during cell-cycle progression and after UV damage.

TOLERATING DNA DAMAGE: TRANSLESION POLYMERASE ETA (η) AND ITS REGULATION IN *SACCHAROMYCES CEREVISIAE*

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TOLERATING DNA DAMAGE: TRANSLESION POLYMERASE ETA (η) AND ITS REGULATION IN *SACCHAROMYCES CEREVISIAE*

DISSERTATION

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas

Health Science Center at Fort Worth

in Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

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May 2008

ACKNOWLEDGEMENTS

Words fail me to express my profound gratitude and reverence to Dr. Wolfram Siede, my major professor, whose unstinted co-operation, expert guidance, constructive criticism and moral support enabled me to successfully complete my study. He has always inspired me to become more committed, sincere and genuine not only in research but also in daily life.

I gratefully acknowledge the support and encouragement extended to me by my committee members, Dr. Rafael Alvarez, Dr. Rustin Reeves, Dr. Raghu Krishnamoorthy and Dr. Dan Dimitrijevich.

I express my deep sense of appreciation for my friends, lab members, faculty, staff and students of Department of Cell Biology and Genetics for providing me best and adequate facilities.

I could not have finished this arduous task without the encouragement, positive attitude and moral support of my dear husband Srinivas Gottipati. He stood by me in the toughest of the moments and his belief in me helped me accomplish this dissertation.

I must owe my deep sense of gratitude to Almighty God whose blessings and benedictions enabled me to complete my dissertation in time.

This dissertation is dedicated with deepest love and affection to my family. Their love, vulnerability, wisdom and moral strength have inspired me to be the best I can be and to share what I have learned from them.

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LIST OF ABBREVIATIONS

BER	Base excision repair
CC	Cytosine-cytosine
CPD	Cyclobutane pyrimidine dimmer
CPEO	Chronic progressive external ophthalmopelia
CPT	Camptothecin
CYH	Cycloheximide
DAPI	4', 6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	deoxy nucleoside triphosphate
DIC	Differential interference contrast
DUBs	Deubiquitinating enzymes
FA	Fanconi anemia
GAL	Galactose
GFP	Green fluorescent protein
HTLV-I	Human T-cell leukemia virus type I
HU	Hydroxyurea
KSS	Kearns-sayre syndrome
LHON	Leber's hereditary optic neuropathy
MMC	Mitomycin C
MMR	Mismatch repair

MMS	Methylmethane sulphonate
mRNA	messenger ribonucleic acid
mtDNA	Mitochondrial deoxyribonucleic acid
MTS	Mitochondrial targeting signal
Mut	Mutant
NER	Nucleotide excision repair
NLS	Nuclear localization signal
PAD	Polymerase associated domain
PAGE	Poly acrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PIP	PCNA interacting peptide
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RR	Recombinational repair
SDS	Sodium dodecyl sulphate
ssDNA	Single-strand deoxyribonucleic acid
SUMO	small ubiquitin related modifier
TAP	Tandem affinity purification
TC	Thymine-cytosine
TLS	Trans-lesion synthesis

TSS	Tax speckled structure
T-T	Thymine-thymine
Ub	Ubiquitin
UBD	Ubiquitin-binding domain
UBM	Ubiquitin-binding motif
UBZ	Ubiquitin-binding zinc finger domain
UIM	Ubiquitin-interacting motif
UV	Ultraviolet
WT	Wild-type
XP	Xeroderma pigmentosum
XPV	Xeroderma pigmentosum variant

CHAPTER I

INTRODUCTION

DNA damage and repair pathways:

Cellular DNA is damaged by a variety of external sources like ultraviolet (UV) radiation, ionizing radiation, heat and chemical agents. Also the damage can be "spontaneous" due to inherent instability of DNA and attack by intermediates of cellular metabolism resulting in alkylation, oxidation, deamination and loss of DNA bases, intra- and inter strand crosslinks, and strand breakage [1]. DNA damage induced by UV irradiation are photolesions which are primarily of two types: cis-syn **cyclobutane pyrimidine dimers** (CPDs) and pyrimidine (6-4) pyrimidone photoproducts. These lesions are the result of covalent linkage of adjacent pyrimidine residues. In general, cells have evolved four complex systems to respond to DNA damage: 1) DNA repair, 2) cell cycle checkpoints, 3) apoptosis, and 4) damage tolerance.

DNA repair forms the most effective defense system and removes the lesion or damage. It comprises at least five mechanisms: a) base excision repair (BER); b) nucleotide excision repair (NER); c) mismatch repair (MMR); d) recombinational repair (RR) and e) direct reversal of damage. In response to DNA damage, the progression of the cell cycle into S phase is delayed by G1 cell cycle checkpoint control, whereas progression into M phase is delayed by the G2 checkpoint. Prolongation of G1 and G2

phases allows more effective DNA repair and thus avoids DNA synthesis and mitosis in the presence of DNA damage. If the DNA damage is excessive then cells undergo apoptosis to protect the genomic integrity [2].

Despite of fully functional DNA repair and cell cycle checkpoint control, some DNA lesions often persist through replication of the genome. To counter the blockage of the replication apparatus, cells have evolved a damage tolerance system which allows cells to complete replication in the presence of DNA damage (Figure 1).

Damage Tolerance

The multiple pathways by which the arrested replication is relieved are collectively referred to as DNA damage tolerance mechanisms [3].These mechanisms tolerate the damage and hence differ from repair mechanisms. Replication fork regression (template switching) is one such damage tolerance mechanism which is rarely associated with alterations in the nucleotide sequence of the newly synthesized DNA strand.

In contrast, lesion bypass or Trans-Lesion Synthesis (TLS) involves two distinct classes of events: mutagenic or error-prone and error-free. TLS is achieved via specialized TLS [4] polymerases. Bacteria such as *E.coli* have two such TLS polymerases, Pol IV (DinB) and Pol V (comprising the UmuC-UmuD'complex) which mediate TLS in conjunction with RecA protein and form a part of SOS response [5]. Eukaryotes are endowed with a variety of TLS polymerases like Pol η, Pol ζ and Rev1 in yeast and Pol η, Pol ζ, Pol ι, Polκ and Rev1 in humans. All these polymerases share properties that allow them to bypass DNA lesions on the template that are non-instructional for high-fidelity replicative polymerase. Due to their low-fidelity TLS polymerases are able to read a variety of bulky

adducts formed at the template bases. This functional divergence can be attributed to their special structural features [6]. Replication through a DNA lesion can be handled by a single TLS polymerase or it may require the sequential action of two DNA polymerases, in which one inserts the nucleotide opposite the lesion site, and the other extends from the inserted nucleotide. Hence, there are polymerases that function specifically as inserters or as extenders in the lesion bypass process [7].

For example, Pol η performs error-free TLS through a CPD by inserting the correct base opposite the 3' damaged nucleotide and then extends from the inserted nucleotide. However, replication through a [6-4] TT photoproduct requires two DNA polymerases, in which Pol η misinserts a G opposite the 3'-T and Pol ζ extends from this mispaired primer terminus by incorporating an A opposite the 5'-T, resulting in an error-prone bypass of the photoproduct [4].

DNA Polymerase eta (Pol η)

The *RAD30* gene of the yeast *Saccharomyces cerevisiae* encodes for Pol η. The gene was identified as a homologue of *DINB* of *E.coli* and designated as *RAD30* in 1997 [8, 9]. Rad30 is the seventh eukaryotic DNA polymerase to be described and hence is named DNA polymerase η. NER is the major pathway that removes UV induced photolesions during G1 and G2 phases of the cell-cycle. The basic repair mechanism involves damage recognition, endonuclease incisions at invariable distance 5' and 3' of the lesion and release of single stranded DNA fragment containing the lesion. The single strand gap is filled by repair synthesis using the opposite strand as a template. However, when such lesions are encountered during replication, in yeast and humans, Pol η can replicate past

UV induced cis-syn thymine-thymine (TT) dimers in largely an error-free manner [10, 11]. Also, genetic studies with yeast have indicated a role of Pol η in the error-free bypass of cyclobutane pyrimidine dimers of TC and CC nature [12]. The yeast Polη can also efficiently incorporate 'C' opposite 8-oxoguanine formed by oxidative damage [13]. Pol η may play a role in bypassing additional DNA lesions, some of which can be error-prone [12, 14].

Structure of *S.cerevisiae* DNA Polymerase η:

Pol η is ~ 71 kilodaltons protein consisting of 632 amino acids. The catalytic core of Pol η encoded by N-terminal 1-513 amino acids containing five motifs is highly conserved in all Y-family polymerases. The core has a shape of polydactyl right hand consisting of palm, finger and thumb domains which are similar to all DNA polymerases. In contrast to high-fidelity replicative polymerases, Pol η harbours a unique Polymerase Associated Domain (PAD) which mimics an extra set of fingers. The DNA binding groove is thus defined by four domains: palm, fingers, thumb and the PAD. The palm domain carries the active site residues that coordinate two divalent metal ions for the nucleotidyl transfer reaction. Yeast Rad30 and its counterparts from other species contain nine invariant or highly conserved acidic residues. In *S. cerevisiae* Pol η, residues D30 and E39 are in motif I, E79 is in motif II, and D155, E156, and D160 are in motif III while D228 and D235 are located between motifs III and IV and D293 is located between motifs IV and V. The fingers and the thumb domain are small and stubby. The most astonishing aspect of fingers domain is its lacking of equivalent of helices "O" and "O1" which play a central role in closing off the active site and in the fidelity of replicative

polymerases. The PAD is joined to the thumb by a flexible tether and inclusion of this unique domain increases the potential DNA binding surface of Pol η [15].

The C terminus of Pol η is divergent in all Y-family polymerases. The first 513 amino acids are enough for the polymerase activity of the protein. Although the first 320 amino acids of Rad30 contain all of the conserved motifs I to V and there is no apparent amino acid conservation between residues 452 and 513, the requirement of this C-terminal region for Pol η activity may reflect a role for this portion in adopting the proper three dimensional structure of the protein. The presence of a bipartite nuclear targeting motif encompassing amino acids 601 to 617 supports an essential role for this C-terminal region in the targeting of the protein to the nucleus. Deletion of the last 54 amino acids of Pol η renders the protein non-functional despite of having an intact DNA polymerase activity and TT dimer bypass ability [16]. In addition C terminus also contains Proliferating Cell Nuclear Antigen (PCNA) and other TLS polymerases interacting sequences. This emphasizes the role of C terminus in regulating the interaction of Pol η with other proteins.

Recently in 2005, ubiquitin binding domains (UBM and UBZ) were identified at C terminus which are evolutionarily conserved in all Y-family TLS polymerases [17]. Human and yeast Pol η harbour a ubiquitin-binding zinc finger domain or UBZ domain which represents a novel member of the C_2H_2 zinc finger family completely distinct from the presumed DNA binding family. The UBZ domain enables human Pol η to interact with ubiquitinated substrates to regulate TLS and it is responsible for its own

monoubiquitination [17]. In humans, UBZ domain is critical for the localization of Pol η at the site of DNA damage (Figure 2).

Lesion bypass by DNA polymerase η

Polη is unique among DNA polymerases in its ability to replicate through a *cis-syn* TT, TC and CC dimers in an error-free manner. Steady-state kinetic studies have shown that both yeast and human Pol η insert an A opposite the 3'-T and the 5'-T of the TT dimer with the same efficiency and accuracy with which they insert an A opposite a T in the undamaged sequence [18, 19].

The structural and biochemical features of TLS polymerases suggest metal-assisted mechanism of catalysis. The three consecutive acidic residues in the palm domain coordinate the binding of two divalent metal ions, and this domain also binds to the incoming dNTP. The fingers make intimate contacts with the incoming dNTP. The thumb domain contacts the duplex portion of the DNA on the minor- groove side while the PAD contacts the duplex DNA on the major-groove side. The long loop that connects the thumb to the PAD lies on the underside of the DNA. The PAD increases the DNA binding surface of the enzyme by almost two times which explains the indispensability of this region for DNA synthesis. In contrast to replicative polymerases which can hold only one unpaired template base in their active site, the active site of Polη is very open and unrestricted that it can accomadate two template nucleotides. Since the linkage between TT dimer is covalent, the 5'-T of the dimer can't be flipped out. Hence the ability of Polη to accommodate two template bases makes this polymerase uniquely suited for the bypass of this lesion [4, 15].

RAD30 is a member of the *RAD6* pathway:

DNA repair pathways are highly conserved from yeast to humans. Three principal pathways have been classified according to genetic relations of DNA repair mutants in *Saccharomyces cerevisiae* .

The *RAD3* group mediates nucleotide excision repair, the *RAD52* group directs double-strand break repair through homologous recombination. The *RAD6* group functions in damage tolerance. Members of the *RAD6* group act on the stalled replication fork that has encountered a lesion on the template and allow replication to resume [3, 20]. The damage tolerance mediated by *RAD6* pathway can be error-prone if accomplished via specialized translesion polymerases that may insert incorrect or correct nucleotides (depending on the type of lesion) across a damaged site. In contrast *RAD6*-dependent mode is error-free if the information of the undamaged sister duplex is used at the replication fork to resume replication (template switching).

Currently 24 genes have been assigned to the *RAD6* group. For example *RAD5, RAD6, RAD18, MMS2, UBC13, UBC9, REV1, REV3* and *REV7* are members of the group.

The UV sensitivity of the strains carrying *rad30* deletion has classified *RAD30* as a member of *RAD6* epistasis group. The UV survival curves of both the *rad30rad6* and *rad30rad18* double deletion strains are identical to that of the single *rad6* and *rad18* single deletion strains, respectively, signifying that both *RAD6* and *RAD18* are epistatic to *RAD30* [8]. This means that *RAD30* participates in DNA damage tolerance mechanism that is dependent on both *RAD6* and *RAD18.* Similarly, UV survival and UV-induced mutagenesis studies have shown that *RAD30* plays a role in damage tolerance in a

REV/RAD5 independent manner. Also PCNA (*POL30*) may be necessary for most if not all error-free damage tolerance [21].

PCNA: a dancer with many partners:

The cellular response to DNA damage is well characterized in the yeast *Saccharomyces cerevisiae.* Like all higher eukaryotes, yeast also coordinates different facets of the DNA damage response by posttranslational modification of proteins. Besides phosphorylation, ubiquitination has emerged to be an important modification involved in various critical roles. One of the most striking examples of how ubiquitination can influence protein function is that of PCNA in yeast as well in humans [22]. Proliferating Cell Nuclear Antigen (PCNA), the sliding clamp forms a homotrimer that encircles DNA as a ring and functions as a processivity factor for replicative polymerases δ and ε [23]. In addition, the clamp acts as a central signal integrator for the coordination of replication, repair, and postreplicational chromatin assembly at the replication fork. Most TLS polymerases interact directly with PCNA through PCNA interacting peptide (PIP) sequence motifs [24]. It is generally thought that the PCNA interaction motif might serve as a tether that allows a loose association of TLS polymerases with the replication fork even in the absence of DNA damage. This would facilitate the rapid exchange of replicative polymerases with TLS polymerases following DNA damage [25].

PCNA is a target for ubiquitination by the members of *RAD6* pathway:

A connection between DNA damage tolerance and the ubiquitin system was first recognized when the *RAD6* gene from *Saccharomyces cerevisiae* was discovered to

encode a ubiquitin-conjugating enzyme (E2). RAD6 is the principal mediator of both Trans-lesion synthesis as well as error-free damage avoidance pathway [26]. Yeast PCNA, encoded by *POL30* gene, is modified at a single, highly conserved lysine residue, K164 [22]. The modification requires the members of RAD6 pathway, Rad6p, Rad18p, Rad5p, Ubc13p, and Mms2p.

Following DNA damage, the homotrimeric PCNA encircling the replication fork is monoubiquitinated by E2-E3 pair comprising of Rad6p-Rad18p. Rad18p has single-strand DNA (ssDNA) binding activity in addition to RING finger (E3 activity) and always exists in a stable complex with Rad6p [27, 28]. Monoubiquitination of PCNA may serve as a signal for the switch between replicative polymerase and a TLS polymerase (Pol η) due to enhanced affinity of Polη for the modified clamp [29, 30]. Polζ, another TLS polymerase, is also activated by modified PCNA by a more indirect mechanism [31, 32]. Also *UBC13* and *MMS2*, members of *RAD6* pathway, possess ubiquitin-conjugating activities. The conjugation reaction is a two-step process in which Rad6p and Rad18p attach the first ubiquitin moiety or monoubiquitinate PCNA at K164 which is then extended to a multimeric chain by another E2-E3 pair (Ubc13/Mms2-Rad5). The E2 activity of Ubc13p and Mms2p heterodimer and the ligase activity of Rad5p results in the formation of a multiubiquitin chain in which ubiquitin moieties are linked via K63 of ubiquitin [22].

Attachment of a single ubiquitin moiety conveys distinct, proteasome- independent signals. For example, monoubiquitination of plasma membrane proteins triggers their selective uptake by endocytosis and subsequent degradation in the lysosome or vacuole

[33]. Recently, a repair-associated Fanconi anaemia protein FANCD2 was identified as a target for monoubiquitination signifying the role of this modification in the DNA damage response in higher organisms [34]. Not only monoubiquitination, but also multiubiquitin chains can convey signals unrelated to proteasomal degradation. Ubiquitin itself comprises of seven lysine residues and each of them can serve as an attachment site for further ubiquitin moieties. It is apparent that ubiquitin chains linked uniformly through one particular lysine will adopt distinct conformations depending on their topology [35, 36]. While the canonical linkage through K48 usually triggers proteasomal degradation of the modified target, multiubiquitin chains linked through K63 have been implicated in the inflammatory response, in endocytosis, in ribosome biogenesis and in DNA damage tolerance [36].

Independent of DNA damage, yeast PCNA is also subject to sumoylation at the same residue K164 and to a lesser extent, at K127 [22]. This reaction involves the SUMO-specific E2 Ubc9p and the SUMO ligase Siz1p. Low levels of sumoylated PCNA are detected in S phase, but not in G1, G2 and mitosis. Interestingly, sumoylation of PCNA has so far only been observed in *S. cerevisiae* [36].

Consequences of PCNA modifications

All three modifications affect the same lysine residue of PCNA, suggesting that they label PCNA for alternative functions. Damage induced PCNA ubiquitination is elementary for DNA repair and occurs at the same conserved residue in yeast and humans [22]. PCNA ubiquitination is elementary for *RAD6*-dependent DNA repair. Stalled

replication machinery at the lesion following DNA damage may be switched to different modes of repair through distinct PCNA modifications.

Monoubiquitination of PCNA results in an increased affinity of TLS polymerases (Pol η and Pol ζ) for the replication clamp, which leads to polymerase switch and hence mediates translesion synthesis [22]. It was recently proved that PCNA gets monoubiquitinated at K164 in response to malfunction of normal replicative machinery due to mutations in replication genes even in the absence of DNA damage. In this instance, Pol ζ is recruited to the primer terminus and contributes to spontaneous mutagenesis [37].

Multi-ubiquitination of PCNA at K164 is pivotal for the error-free branch of *RAD6*-dependent DNA repair. Triggered by DNA damage, Rad18p recruites Rad6p to DNA bound PCNA and their E2-E3 activity initially monoubiquitinates PCNA at K164. DNA damage also stimulates nuclear translocation of Ubc13p and Mms2p which form a heterodimer and associate with chromatin-bound Rad5p. Also through Rad5 and Rad18 interaction, Ubc13- Mms2 is brought in contact with Rad6. In a second enzymatic reaction, this assembly of two ubiquitin-conjugating enzymes and two ubiquitin ligases (together with ubiquitin-activating enzyme) catalyses the conjugation of additional ubiquitin molecules onto the previously added ubiquitin moiety of monoubiquitinated PCNA [22]. It is speculated that K-63 linked ubiquitin chain may stimulate interaction with proteins associated with the undamaged sister duplex resulting in a transient template switch to the undamaged sister chromatid. Whereas PCNA ubiquitination mediates damage bypass, modification with SUMO, which occurs even in the absence of

exogenous DNA damage, appears to be a guarding mechanism. SUMO-modified PCNA recruits Srs2 which is a helicase and inhibitor of recombination and thus prevents unwanted recombination during replication in S phase [38] (Figure 3).

Significance for human health

The human and yeast Rad30 proteins share 23% identical and 53% conserved residues, and the two proteins have several highly conserved motifs throughout their length [39]. Mutations that inactivate the RAD30 gene in yeast, result in moderate increase in UV sensitivity and an increase in the frequency of UV–induced mutations [8]. Mutational inactivation of human homologue hRAD30A or POLH gene, located on chromosome 6p21.1-6p12 causes the **variant form of Xeroderma Pigmentosum (XP-V)** [40]. Xeroderma Pigmentosum (XP), a rare genetic disease, is classified into seven complementation groups (XP-A to XP-G) that correspond to defects in one of the seven genes involved in NER [3]. The disease is characterized by sun sensitivity, early onset of freckling, and subsequent onset of skin cancers especially basal and squamous cell carcinomas [41]. The eighth genetic complementation group for XP is called XP-V. However XP-V patients, who account for approximately 25% of XP, carry out normal NER but are defective in their replication of UV-damaged DNA [42].

XP-V is an autosomal recessive disorder associated with increased incidence of sunlight-induced skin cancers. XP-V cells are characterized by delayed completion of DNA replication following UV irradiation. A considerable number of mutations like single-base pair substitutions, small insertions and deletions resulting in frameshifts

have been found to be associated with XP-V phenotype [43]. In the majority of cell lines derived from XP-V patients, Pol η is severely truncated causing premature termination of translation. This results in a non-functional protein lacking polymerase activity. In the absence of active Pol η in XP-V patients, UV induced lesions in the DNA may be replicated by error-prone bypass polymerase such as Pol ζ, which frequently misinserts a base at the lesion site, resulting in hypermutability following UV exposure [44]. Pol η is the first DNA polymerase shown to possess a tumor suppressor activity in humans.

Why is regulation of Pol η important for a cell?

Replicative DNA polymerases must duplicate DNA templates with extraordinary fidelity in order to preserve genomic integrity. However, to achieve such exquisite DNA replication fidelity in turn prevents these polymerases from incorporating nucleotides opposite lesions on a DNA template, and from elongating from primers that are not properly paired with the template [45]. Therefore, cells have also evolved low-fidelity TLS polymerases which rescue cells by bypassing DNA lesions during replication, although the bypass may or may not involve misinsertions.

In humans and *Saccharomyces cerevisiae*, Pol η inserts correct nucleotides opposite CPDs (TT, TC and CC) and hence function in error-free replication of UV-damaged DNA. Surprisingly, the accuracy of DNA synthesis opposite the damaged DNA by Pol η is nearly indistinguishable from that opposite undamaged DNA, with frequencies of misinsertion of about 1/100 to 1/1000 [19]. This low fidelity of Pol η results from a

relaxed requirement for correct base-pairing geometry and a more open active site, which enables the polymerase to accommodate distorted and bulky lesions [15]. Additionally, processivity (a measure of the number of deoxynucleotides that a polymerase incorporates before dissociating from the DNA template) of Pol η on both non-damaged and damaged templates is quite low, suggesting that Pol η incorporates just a few deoxynucleotides across from dimer after which the enzyme falls off and action of replicative polymerase is resumed [19].The low processivity of the enzyme restricts DNA synthesis to short patches to prevent mutations. Unlike replicative polymerases, Pol η lacks an intrinsic 3' to 5 proofreading exonuclease activity [11]. The amazing fact is that Pol η has low fidelity for an undamaged template but it is involved in a process that reduces UV-induced mutagenesis. Although Pol η bypasses CPDs without any error, for most of the other lesions and undamaged template, Pol η is an error-prone polymerase. Pol η could elongate DNA chains from mispaired template-primers *in vitro* when excess amounts of Pol η were added to the reactions [46]. This may mean that excess amounts may cause repeated attacks of the Pol η on the mispaired 3' terminus of the primer resulting in the elongation of DNA chains containing a mispaired template-primer. The rate of spontaneous mutagenesis increases *in vivo* upon overproduction of Rad30p in wild-type yeast strain. Interestingly, mutator effects are even observed upon overproduction of Rad30p that is inactive as a DNA polymerase [47]. This suggests that much of the mutator effect results from indirect perturbation of replication rather than from direct misincorporation by Pol η. While excess wild-type Pol η predominantly causes base substitutions, excess inactive Pol η induces both base

substitutions and frameshifts. In other words, more than one mutagenic mechanism is operating when Rad30 is overexpressed [47]. There have been several hypotheses to explain the elevated mutation rates due to Pol η excess. For example, when present at a higher than normal concentration, low fidelity Pol η may compete with replicative polymerase and directly misincorporate nucleotides during DNA replication or Pol η present in excess could bind to DNA at sites where replication pauses (after an error is made by replicative polymerase) and inhibit appropriate proofreading. Pol η may compete with replicative polymerase to fill gaps generated during base and nucleotide excision repair. But these hypotheses can not account for the stronger mutator effect exerted by the catalytically inactive Pol η excess. This fact emphasizes that polymerase activity of Rad30p is not essential for its mutagenic activity when overproduced. In that case, a third possibility is that excess Rad30p may indirectly elevate mutation rates by perturbing interactions that are normally important for replication fidelity. The wild-type and the mutant form of Pol η may sequestrate or inhibit proteins that are essential for chromosomal DNA replication such as replicative polymerase or its accessory factors like PCNA. The difference in specificity of mutator phenotypes could be explained by different kinetics of such inhibition by the two polymerase variants [47].

As TLS is inherently mutagenic for an undamaged template, how can we account for the important role of TLS in lesion bypass in mammalian cells? TLS may not be the best option for a cell , nevertheless, TLS is a very efficient process and ensures that replication through a lesion site continues unabated and the coordinated synthesis of leading and the lagging strands by Pol δ is not significantly impaired. In the absence of

TLS, interruptions in the newly synthesized DNA strand would persist for long periods leading to the formation of double-strand breaks in DNA, repair of which by non-homologous recombination would generate gross genomic rearrangements. Furthermore, the mutagenic consequences of TLS opposite an undamaged template may not be as significant as it is error-free in many instances, as for example, the bypass of CPDs by Pol η. Moreover, a large proportion of the mammalian genome is noncoding, which ensures that a vast majority of mutations will have no adverse effects on cellular physiology [4].

Specialized DNA polymerase, Pol η likely evolved to promote mutation avoidance in the presence of unrepaired DNA damage especially by UV radiation. However, in light of its mutagenic potential for an undamaged template and non-CPD lesions, control and regulation of its action is critical for maintaining genomic stability.

Pol η function may be controlled at the level of mRNA synthesis. Our preliminary data confirms the previously reported upregulation of Pol η transcript after UV damage in a diploid strain [8, 9]. In our studies, we found a substantial increase in the message at 60 minutes post-UV.

Message stability, protein turnover, protein stability or posttranslational modification may be other possible mechanisms involved in regulating Pol η. For example in *E.coli*, TLS polymerases Pol IV and Pol V are maintained at low levels under normal growth conditions. In response to DNA damage, their transcript and protein levels are upregulated [48]. The translesion synthesis function of Pol V may be further controlled

by a posttranslational mechanism. Cleavage of UmuD protein is regarded as an important mechanism controlling the activity of Pol V in lesion bypass [48].

The controlled synthesis of Pol η may be achieved by maintaining a balance between its synthesis and turnover. It is possible that rate of degradation of Pol η may be an important regulatory factor. Infact protein levels of several DNA repair proteins including NER are known to be regulated by ubiquitin-mediated proteasomal degradation [26, 49, 50]. In addition to targeting for proteasome-mediated degradation, ubiquitination influences the interaction(s) of a protein with other proteins, three dimensional structure of a protein, protein activity or location [51, 52]. As discussed before, Rad6, a ubiquitin conjugase (E2), ubiquitinates PCNA after UV damage and influences its interaction with Polδ. Posttranslational modifications like phosphorylation and ubiquitination are known to play an important regulatory roles in DNA repair pathways and checkpoint control mechanisms. For example, from the group of proteins involved in Fanconi Anemia (FA), FANCD2, is phosphorylated upon DNA damage by ionizing radiation (IR) resulting in the activation of the S-phase checkpoint. Mitomycin c (MMC), which acts a cross-linking agent and also produces double strand breaks like IR, monoubiquitinates FANCD2 and targets the protein to chromatin-associated nuclear foci [52].

Project Hypothesis

Recent studies in the DNA repair field have highlighted the expanding roles of posttranslational mechanisms in the regulation of diverse DNA- repair processes and pathways. Control over the activity of eukaryotic DNA-damage tolerance pathways is exerted by two systems of protein modification: the ubiquitin and the SUMO conjugation systems [36]. Recently novel Ubiquitin binding domains UBM and UBZ were discovered in TLS polymerases which mediate interaction with ubiquitinated substrates as well as with free Ub moiety itself [17]. It is crucial for cells to limit the function of mutagenic lesion bypass polymerase Pol η to maintain genomic stability. As a caretaker of the genome, Pol η must react in a rapid and efficient manner to execute its function. It is hypothesized that **ubiquitination regulates function of Pol η and its interaction with ubiquitinated substrates and hence necessary for efficient bypass by the enzyme.** Yeast is a simple yet sophisticated and more feasible eukaryotic model for molecular analysis. Since most of the regulatory pathways are conserved among eukaryotes, this study would provide further insight into the role of this important cancer susceptibility gene in humans.

Figure1. Multiple mechanisms play roles in response to DNA damage.

In response to extrinsic or intrinsic DNA damage, cells have evolved multiple systems to preserve genomic stability. DNA repair is the most prefered choice that includes Base excision repair, Nucleotide excision repair, Mismatch repair, Recombinational repair and Direct reversal of damage. Cell-cycle checkpoints in G1 and G2 phases ensure that cells enter S and M phase of the cell-cycle free of damage. Apoptosis is the last resort that protects cells from incurring major genomic rearrangements, deletions or insertions. Damage tolerance is a unique system that prevents blockage of replication fork by bypassing DNA lesions that are incurred during replication. This damage bypass can be error-free or error-prone and are carried out via specialized translesion polymerases like Pol η, Pol ζ and Rev1 in *Saccharomyces cerevisiae*.

In addition, there is yet another system of damage avoidance in which the opposite undamaged daughter strand is used as a template. Such an error-free mechanism is dependent on combined actions of Rad5, Ubc13 and Mms2 proteins.

Figure1.

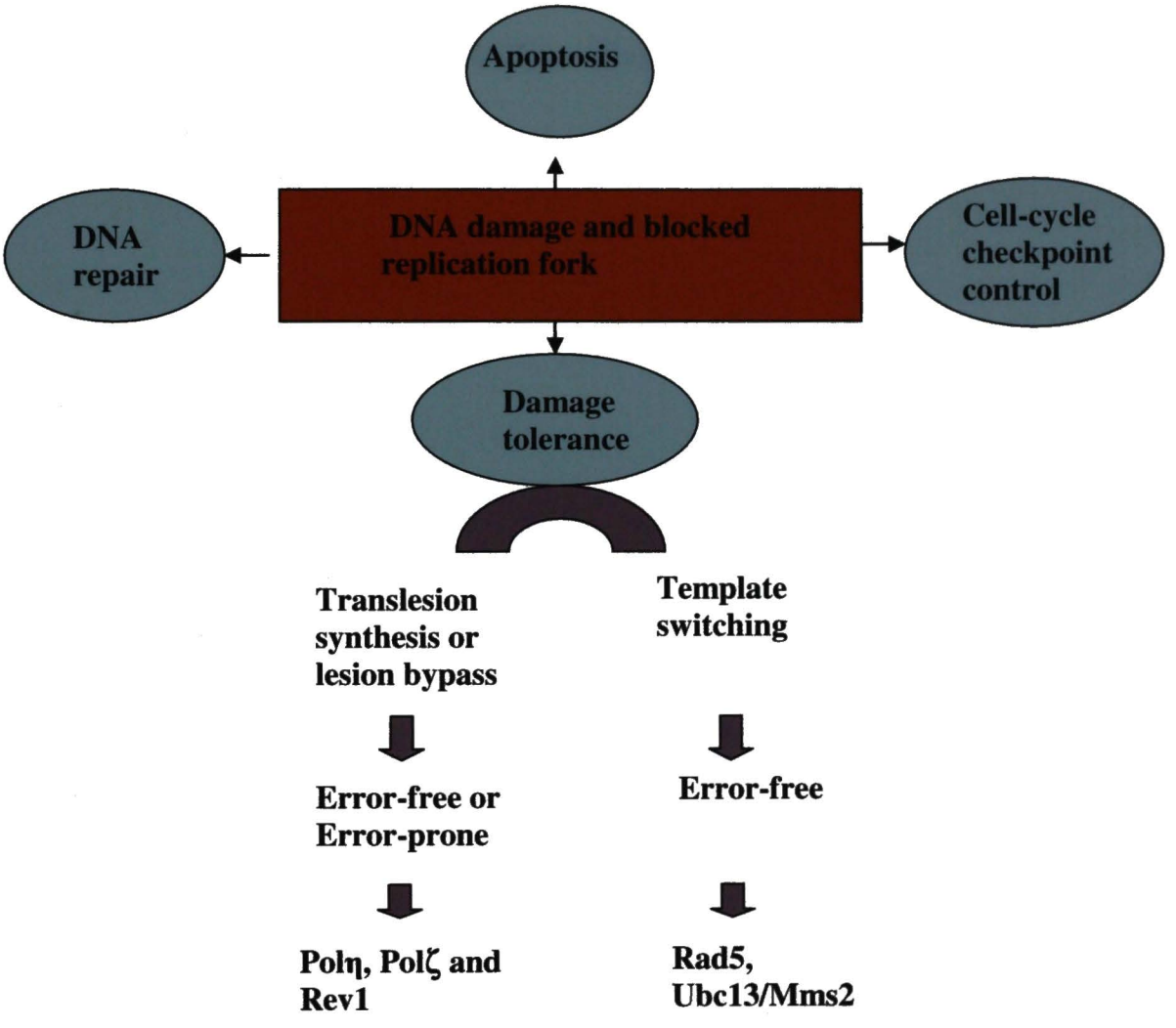

Apoptosis
DNA repair
DNA damage and blocked replication fork
Cell-cycle checkpoint control
Damage tolerance
Translesion synthesis or lesion bypass
Template switching
Error-free or Error-prone
Error-free
Polη, Polζ and Rev1
Rad5, Ubc13/Mms2

Figure 2. Mapping of Pol η regions

The N-terminus conserved polymerase domains of yeast Rad30/ Polη are indicated as I, II , III, IV and V. The Polymerase Associated Domain (PAD) is also shared among Y-family polymerases. Also shown in the figure are C-terminus C_2H_2 UBZ motif, Nuclear Localization Signal (NLS) and PCNA Interacting Peptide (PIP) sequences. Yeast Pol η is 632 amino acid long.

Figure 2.

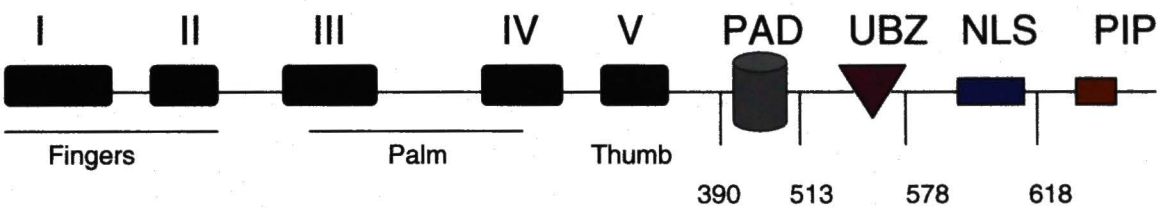
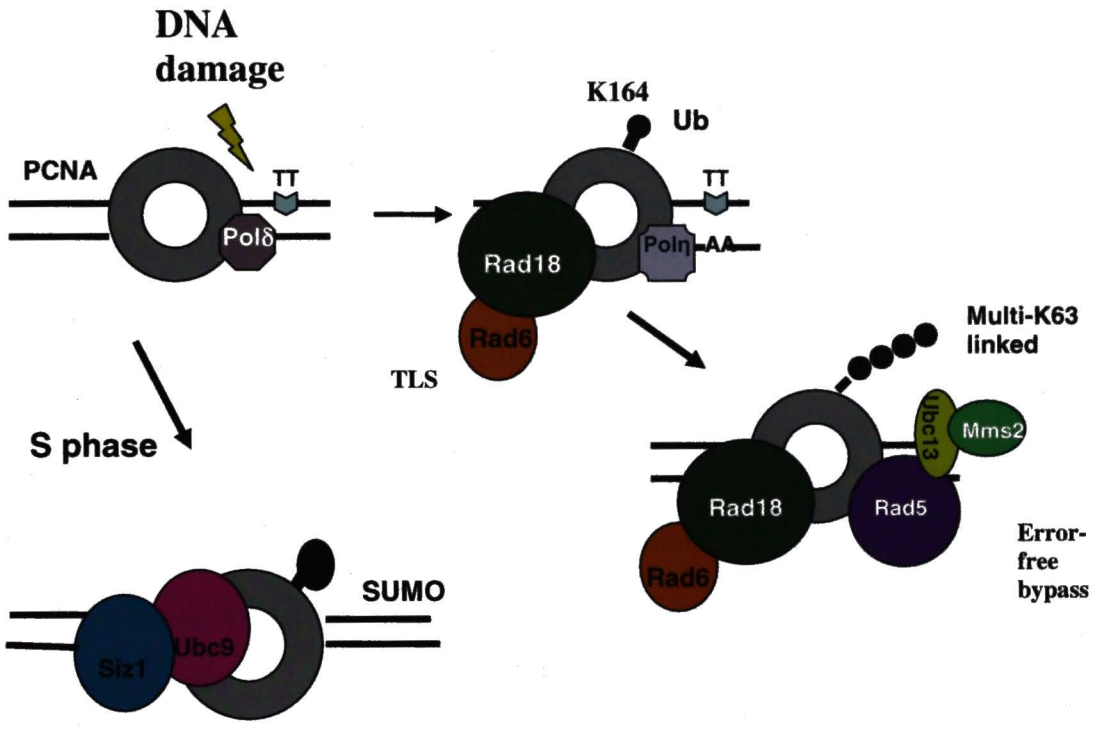


Figure 3. Model for ubiquitination and SUMO modification of PCNA

DNA damage caused by UV includes T-T dimers in template strand during replication. Due to high fidelity, Pol δ is unable to read the distorted bases gets stalled at the site of lesion. DNA damage induces monoubiquitination of PCNA at K164, which is catalysed by combined actions of ubiquitin activating enzyme Uba1(not shown), Rad6 (E2, conjugating enzyme) and Rad18 (E3, ligase). Monoubiquitinated PCNA signals recruitment of Pol η at the site of lesion by trading places with replicative polymerase Pol δ. DNA damage also induces nuclear import of Ubc13 and Mms2 (E2 complex), which are recruited to the chromatin by Rad5 (E3, ligase). The enzyme assembly catalyzes K63-linked polyubiquitination which signals transient template switch to the undamaged sister chromatid. In the absence of DNA damage, PCNA is modified by SUMO during S phase, involving Ubc9 and Siz1 (SUMO ligase).

Figure 3.


DNA
damage
PCNA
TT
Polδ
K164
Ub
TT
Rad18
Polη
AA
Rad6
TLS
S phase
Multi-K63
linked
Ubc13
Mms2
Rad18
Rad5
Rad6
Error-
free
bypass
SUMO
Siz1
Ubc9

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CHAPTER II

REGULATION OF *SACCHAROMYCES CEREVISIAE* DNA POLYMERASE η TRANSCRIPT AND PROTEIN

PREFACE

The UV component of sunlight is a major factor in the development of skin cancer. The two major types of DNA damage induced by UV radiation are the bipyrimidine photolesions: *cis-syn* cyclobutane pyrimidine dimers and pyrimidine (6-4) pyrimidone photoproducts. Human and yeast DNA polymerase η have unique ability to bypass *cis-syn* photodimers in an error-free manner. For all other types of DNA lesions, Pol η is essentially an error-prone polymerase. Polη has a more open active site and may not require strict shape complementarity to bypass bulky lesions. Human Polη also lacks intrinsic 3'→ 5' exonuclease activity. Polη is characterized by low processivity and carries out DNA synthesis in short patches. The low-fidelity character of Polη is believed to be an inevitable consequence of their biological function in translesion synthesis. Due to its low-fidelity, cells may have evolved controls to limit the levels of Pol η to prevent inaccurate synthesis of undamaged template. In *E.coli*, TLS polymerases DinB (Pol IV) and UmuD'$_2$C (Pol V) are subject to tight transcriptional regulation as part of the SOS response after UV damage. Moreover, UmuD protein is posttranslationally processed to its mutagenically active form UmuD'. A dimer of

UmuD' and a monomer of UmuC interact to form UmuD'$_2$C, a functional Pol V, to replicate past a blocking lesion in DNA. However, very little is known about the regulation of eukaryotic TLS polymerases. Thus, the objective of the work in the following section was to study the regulation of DNA polymerase η in rapidly dividing cells as well as after UV damage.

REGULATION OF *SACCHAROMYCES CEREVISIAE* DNA POLYMERASE η TRANSCRIPT AND PROTEIN

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Running Title: Regulation of DNA polymerase η

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Acknowledgements: This study was supported by grant ES011163 from the National Institutes of Health. We thank Fyalon Kerr for technical help. We thank Dr. Graham Walker's group for communicating unpublished results. We appreciate Dr. Pei Zhou for contributing an illustration.

Key words: Pol η/ Rad30, UV, ubiquitination, UBZ

Pabla, R., Rozario, D., and Siede, W. Regulation of Saccharomyces cerevisiae DNA polymerase η transcript and protein. Radiat Environ Biophys., 47(1): 157-168, 2008

ABSTRACT

RAD30-encoded DNA polymerase η functions as a translesion polymerase that can bypass the most frequent types of UV-induced pyrimidine photoproducts in an error-free manner. Although its transcript is UV-inducible in *Saccharomyces cerevisiae*, Rad30 (studied as a Rad30-Myc fusion) is a stable protein whose levels do not fluctuate following UV treatment or during cell cycle progression. Rad30 protein is subject to monoubiquitination whose level is upregulated in G1 and downregulated during S-phase reentry. This downregulation is accelerated in UV-treated cells. A missense mutation (L577Q) of the ubiquitin binding domain (UBZ) confers a reduced degree of ubiquitination outside of G1 and a complete failure to stably interact with ubiquitinated substrates. This mutation confers a phenotype resembling a complete *RAD30* deletion, thus attesting to the significance of the UBZ motif for polymerase η function in vivo.

INTRODUCTION

Prokaryotic and eukaryotic cells have developed various mechanisms to repair spontaneously occurring or externally introduced DNA damage. Additional pathways exist to tolerate but not repair DNA damage, primarily in order to permit completion of DNA replication in the presence of a damaged template [1, 2]. During recent years, the discovery of translesion polymerases has provided essential insights into the mechanisms of one class of such tolerance pathways. It is now clear that such polymerases have special features to allow bypass of damaged bases that are not accepted by high-fidelity replicative polymerases [3]. The necessity to bypass a non-coding or miscoding template base or to extend from an imperfectly matched primer/template junction may result in a sequence change and indeed, an error-prone bypass polymerase such as polymerase ζ accounts for most of the DNA-damage induced mutations in *Saccharomyces cerevisiae*. However, such bypass does not have to be error-prone. Polymerase η represents an example of the evolutionary conserved Y-type family of polymerases [4] that is capable of bypassing the most frequent UV photoproducts, pyrimidine dimers of the cyclobutane-type, in a largely error-free manner [5, 6]. In humans, this polymerase contributes significantly to UV resistance and genetic stability since inactivation of Pol η was identified as the underlying cause of XP-V (Xeroderma pigmentosum variant type), a heritable syndrome resulting in UV sensitivity and enhanced skin cancer incidence [7–9].

In this study, we have analyzed aspects of regulation of *S. cerevisiae RAD30*, encoding polymerase η. The absence of proofreading in translesion polymerases

predicted a high spontaneous error rate that was confirmed for human and murine polymerase η [10, 11]. Thus, the activity of such a polymerase should be carefully regulated and restricted to cells or DNA regions containing DNA damage. In *Escherichia coli*, the activity of similar PolY-type translesion polymerases IV and V is strictly limited to cells exposed to DNA damaging agents since their transcript is part of the SOS regulon; thus, their proteins are hardly expressed in untreated cells [1]

At the outset, we wished to determine the significance of the transiently increased *RAD30* transcript abundance following UV radiation [12, 13] in haploid or diploid *S. cerevisiae*. This transcript regulation, however, is not evolutionary conserved. For the orthologous human XP-V transcript, such increase has been found in response to topoisomerase inhibition by camptothecin [14] but not for UV [15]. Transcriptional upregulation of other eukaryotic bypass polymerases following DNA damage has been described, e.g. for polymerase κ in *Schizosaccharomyces pombe* and mice [16, 17].

Another important mode of regulation concerns the control of the assumed polymerase switch [18]. It is widely accepted that a high-fidelity, replicative polymerase will cease activity at a UV-induced pyrimidine dimer and will be replaced temporarily by one of several translesion polymerases which are in general highly non-processive. So far, the best candidate for a signaling and interacting partner that may initiate a polymerase switch is proliferating cell nuclear antigen (PCNA). Monoubiquitination of PCNA (at lysine 164 in yeast) is found after methylmethane sulfonate (MMS) or UV treatment [19, 20] and the requirement of ubiquitinated PCNA for UV mutagenesis has been demonstrated [21]. Monoubiquitination of PCNA is dependent on the Rad6/Rad18

complex and further extension of the ubiquitin chain through lysine 63 linkage is provided by Ubc13-Mms2-Rad5, creating a signal for error-free bypass by a largely unknown mechanism [1]. Domains in Pol η, Pol ι and Rev1 have been identified that bind PCNA or ubiquitinated PCNA and a preferred interaction with monoubiquitinated PCNA has been shown [20, 22–27]. Available in vitro data, however, differ on a possible stimulation of Pol η lesion bypass activity by monoubiquitinated PCNA [28, 29].

In this study, we show that *S. cerevisiae* Rad30 activity is not primarily regulated through its protein level but through its interaction with ubiquitinated substrates. Additionally, we demonstrate ubiquitination of Rad30 itself and a cell cycle stage-dependent regulation of ubiquitination that can be modulated by UV exposure.

MATERIALS AND METHODS

Yeast strains

All protein studies were performed in strains derived from BY4741 (*MATa his3Δ leu2Δ met15Δ ura3Δ*). Rad30 protein was epitope tagged with 13xMyc at its C-terminus following transformation and microhomology-mediated recombination at its chromosomal location with a PCR product, using a plasmid-borne, *KanMX4*-marked module[30]. The same strain containing a TAP-tagged Rad30version was purchased from Open Biosystems. Replacement of *RAD30* ORF with *KanMX4* and of *RAD5*, *RAD6*, *RAD18* and *DOA1* with *HIS3* were constructed by the same technique. Rad30 was also Myc-epitope tagged in strain BY4742 (*MATα his3Δ leu2Δ lys2Δ ura3Δ*) and mated with Myc-tagged BY4741 to create diploid BY4743 containing homozygously tagged Rad30. RNA analysis was performed in (untagged) BY4743 *RAD* and in homozygously deleted *rad9Δ::KanMX4*, *rad17Δ::KanMX4* derivatives which had been obtained from the Euroscarf strain repository. The haploid strain used for some RNA studies was SX46A (*MATa ade2 his3-532 trp1-289 ura3-52*, originally from J. Rine). Mutation analysis of the *trp1-1* nonsense allele was performed in the background of strain Y300 (*MATa ade2-1ura3-1 trp1-1 his3-11,15 leu2-3,112 can1-100*, originally from S. Elledge). In this strain, an additional deletion of *RAD5* was introduced using a plasmid-borne *HIS3*-marked deletion, provided by F.Ahne. Yeast transformation techniques, general growth conditions and media recipes can be found elsewhere [31]. Primer sequences and additional details of strain construction can be provided upon request.

RNA analysis

Extraction of total RNA and Northern blotting were performed as described [12]. Equal loading was confirmed after staining RNA gels with ethidium bromide or by detection of a non-fluctuating control transcript of Pyruvate dehydrogenase alpha (PDA1)[32].

Cell synchronization and inhibitor treatments

Stationary-phase cells were grown at 30°C for 40 h in YPD (1% yeast extract, 2% peptone, 2% dextrose), logarithmic phase cells for 16 h and typically used at a titer of 1-2x10^7cells/ml. Asynchronous logarithmic-phase haploid cells (BY4741) were synchronized in G1 by adding yeast mating factor α (US Biological, dissolved in water) in two aliquots were added, separated by 1.25 h of incubation, up to a final concentration of 10μg/ml. Synchronization was normally achieved after 2.25 h and confirmed microscopically by the absence of small-budded cells. Treatment with the S-phase inhibitor hydroxyurea (US Biological) was performed at 30 mg/ml. For arrest in M-phase, cells were incubated for 2.5 h with 10μg/ml nocodazole (US Biological, dissolved in DMSO). Cycloheximide (Sigma, dissolved in water) was applied at 100-500 μg/ml to block protein synthesis [33]. Camptothecin (Sigma, stock solution in DMSO) was used at 170 μg/ml in YPD.

UV treatment and determination of mutability

For protein and RNA analysis, cell suspensions in phosphate-buffered saline (PBS) were treated with a calibrated germicidal UV lamp (254 nm) as described elsewhere [12].

Following irradiation, logarithmic-phase cells were resuspended in YPD, stationary-phase cells were kept in PBS and samples were collected at given time points. For determining UV-induced reversion frequencies of the *trp1-1* nonsense mutation marker, 1×10^7 cells of Y300 derived strains were spread per plate on synthetic tryptophane-free omission medium [31]. Appropriate dilutions were plated on the identical medium supplemented with tryptophane to determine survival of colony-forming cells. Cells were irradiated directly on solid media plates.

Protein extraction and Western blotting

For Western blot analysis, samples of approximately 2×10^8 cells were lysed by agitation with zirconium beads in TCA or in 50 mM Tris–HCl (pH 7.5), 50 mM NaCl, 0.1% NP-40, 1.5% fungal protease inhibitor cocktail (Sigma) according to published protocols [34]. Mouse monoclonal anti-Myc antibody 9E10 (Covance) was used at a dilution of 1:2,000 or 1:1,000 (for detection following immunoprecipitations, see below). Anti-PAP (peroxidase-anti-peroxidase) antibody (Sigma) was used at 1:1,000 dilution for TAP detection [35]. The secondary antibody was anti-rabbit-IgG-HRP, used at 1:5,000 dilution. Antibodies against the loading controls actin (Act1) (Abcam) or 3-phosphoglycerate kinase (Pgk1) (Molecular Probes) were diluted 1:500 or 1:5,000, respectively. All primary antibody incubations were carried out for 2 h at room temperature.

Ubiquitin-agarose pull-down assay

Lysates of haploid cells (BY4741) expressing Rad30-Myc were subjected to pull-down assay with ubiquitin(Ub)-agarose. Twenty microliters of washed Ub coupled-agarose beads (Boston Biochem) or Protein G-agarose beads (Sigma) as a negative control were incubated for 2 h at room temperature with 600μg of total protein in 50 mM Tris–HCl (pH 7.5), 50 mM NaCl, 0.1% NP-40 (binding buffer) supplemented with 0.5% BSA and 1.5% fungal protease inhibitor cocktail. Beads were washed three times in binding buffer at 4°C, resuspended in 25 μl of 1x SDS loading buffer (100 mM DTT), boiled for 5 min and centrifuged. Eluted proteins were resolved by SDS-PAGE, followed by immunoblotting with anti-Myc antibody.

Immunoprecipitation

Exponential-phase cells were lysed by agitation with zirconium beads in NP-40 buffer (150 mM NaCl, 50 mM Tris–HCl (pH 7.2), 2 mM EDTA, 1 mM DTT, 0.05% SDS, 1% NP-40) supplemented with fungal protease inhibitor cocktail (1.5%), BSA (0.1%) and 5 mM *N*-ethylmaleimide. Cell extracts were clarified by centrifugation at 20,000*g* for 10 min at 4°C. The protein concentration of the supernatant was determined (Bio-Rad assay) and 600 μg total protein were incubated with 3μg of ubiquitin antibody (rabbit polyclonal, Abcam) or purified rabbit IgG (Sigma) as a negative control. After rotating at 4°C overnight, 30μl protein G-agarose (Sigma) were added and incubation was continued for 2 h at room temperature. Bound proteins were eluted as described above. To determine the relative ratios of ubiquitinated Rad30 over total Rad30,

immunoprecipitated Rad30 and total Rad30 of the same sample were analyzed in parallel in the same gel and on the identically treated membrane. Signals were quantified using an Alpha Innotech Fluorochem 8900™ imager.

RESULTS

In contrast to its transcript, Rad30 is a stable protein whose levels do not fluctuate following UV irradiation.

As shown previously by others and us [12, 13], steady state-levels of RAD30 mRNA are increased following UVC irradiation of haploid yeast. Here, we have reproduced this result for diploid cells where we found a generally less variable and more pronounced response as in haploid cells (Fig. 1A). Although many DNA-damage inducible genes of *S. cerevisiae* are regulated by cell cycle checkpoint proteins, post-irradiation *RAD30* transcript levels were not decreased in selected checkpoint mutants (*rad9*, *rad17*). Nevertheless, the kinetics of transcript abundance appears to be altered and a delayed but more persistent response was found (Fig. 1A), independent of genetic background (data not shown). No notable changes in *RAD30* transcript levels were found in the untreated control portion of the cultures (not shown).

Experiments with UV-treated diploid cells kept arrested in M-phase with nocodazole indicate that cell cycle progression is not required to observe *RAD30* transcript fluctuations following UV treatment (Fig. 1B). If compared to untreated cells, we consistently found in diploid and haploid cells a rapid drop of *RAD30* mRNA levels immediately following UV radiation (Fig. 1B, C). The same was not found for a control RNA (*PDA1*, Fig. 1C).

Next, we determined if the protein levels of Rad30 reflect the changes in transcript levels. We tagged chromosomal RAD30 C-terminally with the 13xMyc-epitope in diploid and haploid strains since none of six tested commercial or custom-made Rad30

peptide antibodies gave satisfactory results. As also shown by others [36], such strains retain wild-type properties and the tagged protein can be considered fully functional (Fig. 2 and data not shown). However, indications for a possibly compromised function were obtained for a commercially available TAP-Rad30 fusion in the same genetic background. (TAP stands for "tandem affinity purification", representing a combination of protein A and calmodulin-binding peptide) (Fig. 2). When Rad30 protein levels were detected by Western blotting in asynchronously dividing cells, no notable differences were found in response to UV treatment if compared to the unirradiated control (Fig. 3A). Thus, Rad30-Myc protein levels appear to be unaffected by the significant fluctuations of *RAD30* mRNA levels. We reasoned that protein turnover rate may have accelerated following UV irradiation and an elevated transcript level may be required to maintain a constant protein level. However, by inhibiting protein synthesis with cycloheximide this hypothesis was not confirmed. If cells were incubated in the presence of 100µg/ml cycloheximide, a concentration known to inhibit translational initiation and elongation in logarithmic-phase yeast [33], Rad30 protein levels in UV-treated cells stayed as constant as in untreated cells even during 5 h of incubation (Fig. 3A, lower panels).

We conclude that *RAD30* encodes a protein of long halflife whose level is not affected by short-term mRNA fluctuations. The same situation pertained to stationary-phase cells where a constant level of Rad30 was demonstrated (the level appears to be quite significant although the protein is thought to act primarily in S-phase) (Fig. 3B and data not shown). A possible dependency of Rad30 protein levels on cell cycle position was addressed in haploid cells (BY4741) that were synchronized in G1 with the yeast

pheromone α-factor. Following release from arrest, Rad30 levels remained constant in both the UV-treated culture and the untreated control portion of the culture (Fig. 4A). Since these cells traverse the cell cycle synchronously during the first 80 min (as verified by budding analysis, not shown), this observation also implies that Rad30 protein abundance is independent of cell cycle stage.

Two additional stress treatments besides UV irradiation were investigated. Logarithmic-phase haploid cells were treated with the ribonucleotide reductase inhibitor hydroxyurea (Fig. 4B) and with the topoisomerase I inhibitor camptothecin (Fig. 4C). Neither treatment did result in any reproducible changes of Rad30 protein levels. In haploid cells, we also investigated the level of the Rad30-TAP fusion following UV treatment and in the presence of cycloheximide (Fig. 5). As shown for the Myc fusion, our results indicate no elevated protein level after UV irradiation (Fig. 5A) and stable protein levels that remained unaffected for several hours even if an extremely high concentration of cycloheximide (500 μg/ml) was used to block protein synthesis (Fig. 5B).

Rad30 is ubiquitinated

Since Rad30 protein abundance does not appear to be altered during cell cycle progression or following DNA-damaging treatments, we explored if covalent modifications of Rad30 as a means of regulation of activity can be identified.

The Rad30-Myc protein was probed for ubiquitination by immunoprecipitation of ubiquitinated proteins with a ubiquitin- specific antibody followed by immunobloting using anti-Myc antibody. A ubiquitin-modified fraction of Rad30-Myc was indeed

detectable in whole-cell extracts (Fig.6) and protein mobility suggested a monoubiquitinated species. No polyubiquitinated forms were detected. Ubiquitination of Rad30 was still detectable in strains deleted for Rad5, Rad6, Rad18 or Doa1 which are all involved in ubiquitin transactions, specifically in PCNA mono- and polyubiquitination (Fig. 6). However, we have consistently found a lower Rad30 level in *rad6* mutants; the significance of this observation is under investigation.

Next, we addressed the possible dependence of Rad30 ubiquitination on cell cycle stage. We monitored the degree of ubiquitination during the process of arresting an asynchronously growing culture in G1 (Fig. 7A). When the signal of ubiquitinated Rad30 was normalized for total Rad30 amount in the crude extract, an approximately 3-fold increase in ubiquitination was noted in G1 as compared to asynchronously growing cells.

Next, we analyzed G1-synchronized cultures that were released from α-factor arrest. Typically, the fraction of small budded cells (an estimate for early S-phase cells) amounted to approximately 30% at 1 h after release. This number was reduced to 8% if the culture had been UV irradiated in G1 prior to release into fresh medium. The degree of ubiquitination decreased during cell cycle reentry as expected (Fig. 7B). Interestingly, although the budding pattern indicated an extended G1 arrest, this decrease was accelerated in the UV-treated aliquot of the culture.

UBZ motif regulates Rad30 ubiquitination, binding of ubiquitinated substrates, UV resistance and mutability.

Due to a PCR-introduced sequence alteration during Myc epitope tagging, we generated a point mutation of the ubiquitin- binding motif of Rad30 (UBZ) defined by Bienko et al.

[23]. This mutation (Rad30-Ubz*) results in a change of leucine 577 to glutamine, a novel point mutation of a conserved residue (Fig. 8). Although this mutation conferred a lower degree of ubiquitination in asynchronous logarithmic-phase cells, an increase in Rad30 ubiquitination in G1 during synchronization with α -factor was nevertheless found (Fig. 9A, B). Albeit with slower kinetics than the wild type, mutant Rad30 can ultimately be ubiquitinated to the same (or even higher) extent than the normal protein. In spite of a generally lower level of ubiquitination outside of G1, an accelerated loss of ubiquitination after UV treatment and release from G1 arrest was still evident (Fig. 9 C, D).

Several studies have demonstrated the appearance of ubiquitinated PCNA following UV irradiation and enhanced affinity of Pol η to monoubiquitinated PCNA [20, 26]. Consequently, we studied and confirmed the specific binding of wild-type Rad30 to ubiquitin agarose (Fig. 10). However, the Rad30-Ubz* mutant protein had completely lost this ability. Therefore, we concluded that this mutant version does not stably interact with ubiquitinated substrates although it can still be slowly ubiquitinated, especially during G1 arrest.

Given the potential significance of the Rad30 interaction with monoubiquitinated PCNA for the polymerase switch during translesion synthesis [18], we asked if this mutant version confers reduced UV survival and enhanced mutability. This was indeed the case. If studied in a wild-type background, UV sensitivity of the point mutant (*rad30-ubz**) was comparable to that associated with a *RAD30* deletion mutant (Fig. 11A). For the chosen mutational system (*trp1-1* reversion) the influence of Rad30 on UV

mutagenesis is not readily detectable unless amplified by inactivation of the (mostly) error-free tolerance pathway that depends on *RAD5* [13]. If studied in a *RAD5*-deleted genetic background, reduced UV survival and enhanced *trp1-1* reversion frequencies of the point mutant (*rad30-ubz**) were not notably different from a *RAD30* deletion mutant and essentially identical results were found in logarithmic or stationary-phase cells (Fig. 11 B, C). Only in the low-dose region, a somewhat reduced mutability was noted in logarithmic phase point mutant cells as compared to the deletion mutant (Fig. 11 B).

DISCUSSION

This study addresses the regulation of translesion polymerase η in budding yeast. First, we analyzed the published UV inducibility of the transcript [12, 13] in more detail. We show that *RAD30* transcript increase in yeast is not dependent on a functional checkpoint protein network as found for other UV-inducible yeast transcripts [37, 38]. There is, however, a more subtle influence indicating a delayed but finally more persistent RNA response in *rad9* or *rad17*mutants as compared to wild type. These mutants affect damage sensing and signal generation within the checkpoint network [39]. The significance of this observation remains to be analyzed.

Next, we compared transcript and protein abundance. Rad30 protein levels were readily detectable in unirradiated haploid and diploid yeast cells of all cell cycle stages as well as in stationary phase cells. Thus, budding yeast does not follow the *E. coli* paradigm where Y-type polymerases are part of the SOS system restricting protein expression to cells containing DNA damage [1]. In contrast, no significant fluctuations of Rad30 protein were determined in response to UV irradiation and to other stresses, such as hydroxyurea or camptothecin. Unlike Rev1 [40], the protein level also does not vary during cell cycle progression (independently shown in [40]).

Thus, the protein level does not mirror the changes in transcript abundance. The protein (studied as a Myc-tagged version) is characterized by a long half-life since protein synthesis inhibition does not affect its level throughout several hours of incubation. There was no difference between UV-irradiated or unirradiated cells. The transcript, however,

appears to be rather short-lived and even during the few minutes of preparation of UV-irradiated cell samples, a notable decrease in *RAD30* steady-state mRNA signal is found in relation to control transcripts. The subsequent increase exceeds the level of the unirradiated control but one wonders if this observation reflects a general regulation that temporarily stabilizes short-lived transcripts following RNA synthesis inhibition (e.g., by UV) rather than UV-specific increased de novo synthesis. An influence of the checkpoint system on such a more general transcription phenomenon is not unlikely. A recent survey of all proteins whose phosphorylation is mediated by mammalian checkpoint kinases has revealed a wide variety of targets, including general transcription control proteins [41]. Absence of correlation between transcript and protein levels is not without precedence among yeast genes involved in DNA damage responses. For example, transcript levels of *S. cerevisiae RAD50* fluctuate with the stages of meiosis whereas protein levels stay constant [42]. In general terms, it is well established that higher abundance of a transcript following a DNA damaging treatment does not predict any role in the cellular defense against the same agent [43, 44]. A recent study on global changes in yeast protein expression following MMS treatment [45] found many examples of proteins showing no significant change in abundance (<3-fold) whose transcripts had previously been described as highly inducible [46]. In mammalian cells, polymerase η provides another example [47]. If common, the disconnection between a "damage-inducible" transcript and its protein argues against reliance on transcript profiling instead of protein expression data, e.g. in modeling of regulatory networks or the design of cancer therapies.

Our data are not in agreement with a recently published study on TAP-tagged Rad30 protein levels that were described as unstable and subject to stabilization following UV treatment [35]. Initially, we did not favor the use of the TAP-tagged protein because of indications of compromised function. However, even when using the identical strain and identical high concentrations of cycloheximide, the described Rad30 half-life of 20 min in untreated cells and its stabilization after UV with maximum levels around 2h after treatment were not confirmed. Our results also differ when Myc-tagged Rad30 was used (expressed, however, on a plasmid from a heterologous promoter [35]). We do not know the reason for these discrepancies but we noted their use of a protein extraction method without bead disruption that in our hands resulted in low and unreliable protein yields. Other investigators have obtained results similar to ours for protein A-tagged Rad30 (R. Woodruff, M.E. Wiltrout and G.C. Walker, personal communication).

Next, we turned to covalent modifications and interactions of the Rad30 protein as a more likely means of regulating its activity or access to a damaged template. Several studies have concluded that human polymerase η has a higher affinity for monoubiquitinated PCNA that emerges after UV radiation than for the non-modified version and that this interaction may be critical for recruiting this and other translesion polymerases to the sites of lesions [20, 22, 24, 26]. In human Pol η, missense mutations (D652A, H654A) within a newly characterized ubiquitin-binding domain (UBZ) were found to abolish ubiquitin interaction, to reduce the number of damage induced foci or to interfere with the protein's ability to rescue the UV sensitivity of XP-V cells [23, 26].

The conserved UBZ domain has affinity for ubiquitinated substrates but may also be subject to ubiquitination [23]. Indeed, we found a fraction of Rad30 to be monoubiquitinated. Ubiquitination was still detectable in deletion mutants of E2/E3 enzyme complex Rad6/Rad18, of Rad5 or of Doa1 that are all required for PCNA monoubiquitination or polyubiquitination (Rad5) following UV or MMS treatment [1, 2, 19, 48]. (We have recently confirmed a defect in UV mutagenesis for *doa1* mutants [J. Gong and W.Siede, in preparation].) This does not exclude possible quantitative differences of ubiquitination or Rad30 levels e.g. in *rad6* mutants (Fig. 6) that are currently under investigation. However, these differences may be difficult to interpret due to cell cycle stage effects (see below). Consistent with our data, it has been suggested that Rad30 may be subject to E2/E3-independent self-ubiquitination [36].

We observed an increase of ubiquitination during G1 synchronizing treatment and, as expected, this ubiquitination level diminishes during S-phase reentry. Interestingly, this decrease is accelerated if cells are UV-irradiated just before release from G1 arrest. This is a UV-specific regulation that cannot be explained by any cell cycle effects since UV will even introduce a transient G1 arrest that should otherwise extend the state of high ubiquitination. Ubiquitination of the UBZ domain will presumably preclude interaction with ubiquitinated PCNA [23], so this regulation appears to enable Pol η recruitment during the cell cycle stage where translesion synthesis is indeed required. One should, however, not necessarily conclude that the only role of ubiquitination in G1 is to prevent PCNA/Pol η interactions. Circumstantial evidence links a large fraction of UV mutations in budding yeast to pre-replicative damage processing [49–51] and Pol η may also play a

pre-replicative role [52], possibly undergoing protein interactions that do not involve ubiquitinated partners. Localization to certain cellular compartments or organelles [53] may be yet another role of monoubiquitination.

We have characterized a mutant of the most C-terminal conserved UBZ residue (L577Q) (Fig. 8). This residue maps to the border of a conserved α-helical domain that interacts with ubiquitin. This mutation eliminated interaction with ubiquitinated substrates as shown in ubiquitin agarose pull-down experiments. In contrast to the effect of the D570A mutation [36] Rad30 ubiquitination was not completely abolished, however, the degree of Rad30 ubiquitination in unsynchronized cultures was generally lower in the mutant than in the wild type. During G1 synchronizing treatment, wild-type levels of ubiquitination were still achieved after some delay. Compared to the wild type, deubiquitination was accelerated during cell cycle reentry. A balance of ubiquitination and deubiquitination processes may result in the observed steady-state level of Rad30 ubiquitination. The Ubz* mutation may inhibit the former process but not the latter that is active during cell cycle reentry and further activated in UV-treated cells. Rad30 deubiquitination enzymes have not yet been identified.

Within the variability of the assays, the UV sensitivity and mutability phenotype conferred by Rad30-Ubz* (L577Q) is largely indistinguishable from that of a complete deletion. As predicted, inactivation of the Rad5-mediated error-free tolerance pathway was required to delineate this effect more clearly [13]. In agreement with current models, this result indicates that this domain and its interaction with ubiquitinated PCNA is critical for the overall enhancement of UV survival and the avoidance of mutations at

photoproduct sites by Pol η activity, most likely by counteracting a recruitment of more error-prone polymerases such as Pol η.

While this manuscript was in preparation, ubiquitination of yeast Rad30 was demonstrated in an independent study [36]. One discrepancy concerns the apparent absence of influence of UV irradiation on Rad30 ubiquitination, however, it should be noted that cell synchronization is required to detect any effect (data not shown). Overall, using a different UBZ-domain mutation (D570A) and a different mutational system, the authors reached conclusions very similar to ours.

Figure 1. Effect of UV irradiation on *RAD30* transcript abundance.

A) *RAD30* transcript levels detected by Northern blotting in logarithmic phase diploid wild-type, *rad9* or *rad17* mutant *S. cerevisiae* cells (BY4743). Samples were withdrawn and frozen immediately (5 min handling time) or at the different time intervals indicated after 254 nm UV treatment (180 J/m2). Equal loading was verified by ethidium bromide staining of the RNA gel (not shown). **B)** *RAD30* transcript levels in diploid wild-type cells synchronized and continuously arrested in M-phase with nocodazole (10μg/ml), with or without UV treatment (240 J/m2). **C)** *RAD30* transcript levels in a haploid wild type (SX46A) with or without UV treatment (80 J/m2), as compared to a non-fluctuating control transcript (*PDA1*). Note the drop in *RAD30* transcript levels at 5 min after UV (A, B)

Figure 1.

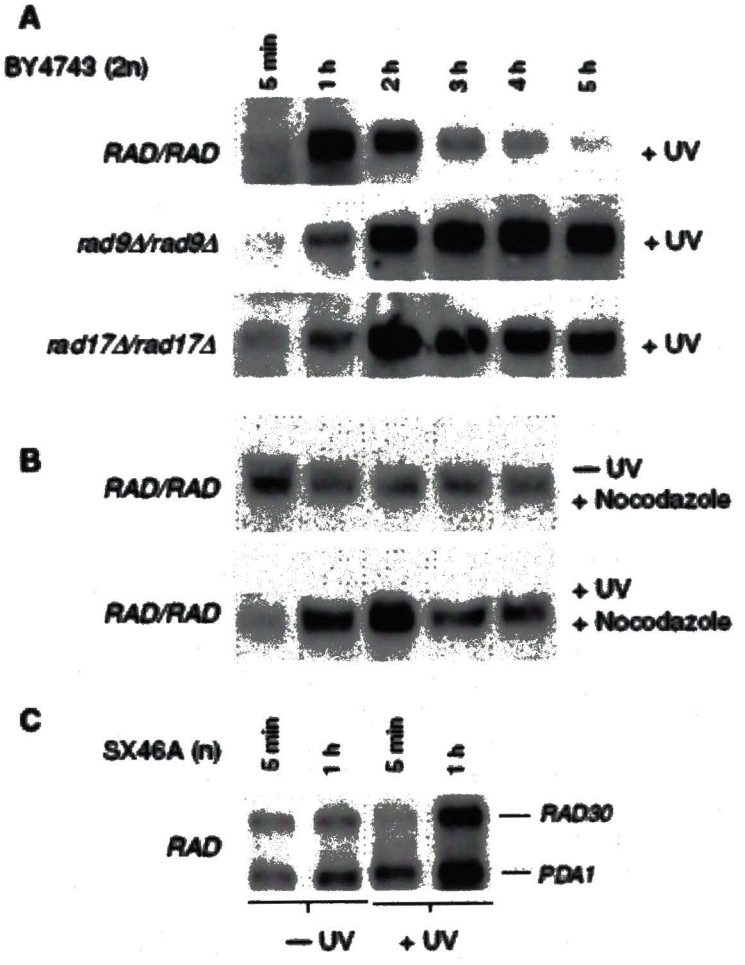

A
BY4743 (2n)
5 min
1 h
2 h
3 h
4 h
5 h
RAD/RAD
+ UV
rad9Δ/rad9Δ
+ UV
rad17Δ/rad17Δ
+ UV
B
RAD/RAD
— UV
+ Nocodazole
RAD/RAD
+ UV
+ Nocodazole
C
SX46A (n)
5 min
1 h
5 min
1 h
RAD
RAD30
PDA1
— UV
+ UV

Figure 2. Fraction of colony-forming cells as a function of UV-dose.

Logarithmic-phase cells of wild-type (BY4741) (*filled square*) and isogenic strains containing Rad-30-Myc (*multi sign*) and Rad30-TAP (*open circle*) were treated. Symbols represent individual measurements of 3–4 independent experimental series carried out for each strain; the average survival values are connected by *lines.*

Figure 2.

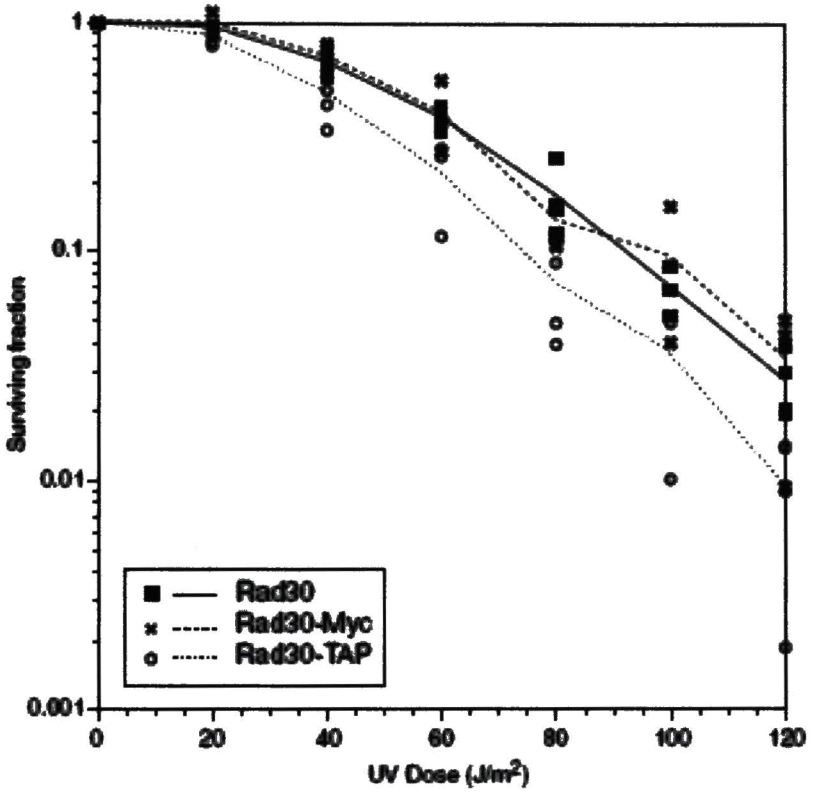

1
0.1
0.01
0.001
Surviving fraction
0
20
40
60
80
100
120
UV Dose (J/m²)
Rad30
Rad30-Myc
Rad30-TAP

Figure 3. Absence of influence of UV irradiation and cycloheximide on Rad30 protein levels

A) Time course of steady-state levels of Myc-epitope tagged Rad30 in logarithmic phase diploid cells (BY4743), with or without UV treatment (180 J/m2), with or without addition of cycloheximide (100 μg/ml, *CYH*) (*lower panels*), as determined by Western blotting. No signal was detected if a *RAD30* deletion mutant was used (not shown). *Act1* (actin) served as a loading control. **B)** *Rad30-Myc* levels detected in stationary phase diploid cells, with or without UV treatment (180 J/m2).

Figure 3.

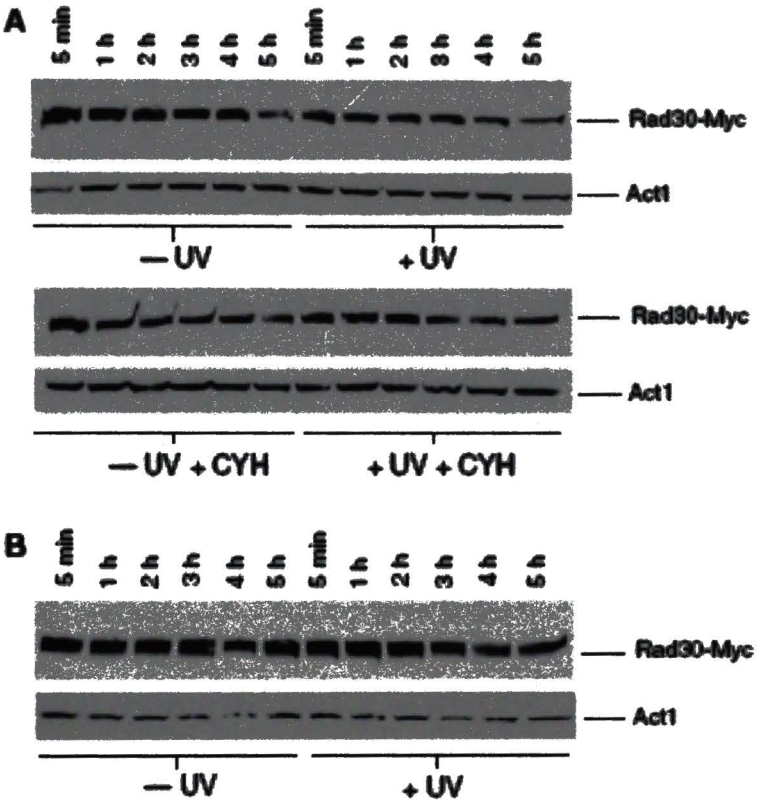

A
5 min
1 h
2 h
3 h
4 h
5 h
5 min
1 h
2 h
3 h
4 h
5 h
Rad30-Myc
Act1
— UV
+ UV
Rad30-Myc
Act1
— UV + CYH
+ UV + CYH
B
5 min
1 h
2 h
3 h
4 h
5 h
5 min
1 h
2 h
3 h
4 h
5 h
Rad30-Myc
Act1
— UV
+ UV

Figure 4. Absence of influence of cell-cycle position, hydroxyurea or camptothecin on Rad30 protein levels

A) Rad30-Myc levels in haploid cells (BY4741) synchronized in G1 by α- factor (α), then treated with 0 or 80 J/m2 UV just before release into fresh YPD medium, resulting in synchronous resumption of cell cycle progression. *Pgk1* (phosphoglycerate kinase) was used as a loading control.

B) *Rad30-Myc* levels in haploid logarithmic-phase cells during treatment with hyroxyurea (30 mg/ml, HU). **C)** *Rad30-Myc* levels in haploid logarithmic-phase cells that had been synchronized in G1 and released into fresh medium containing camptothecin (170 μg/ml, *CPT*) or DMSO as solvent control.

Figure 4

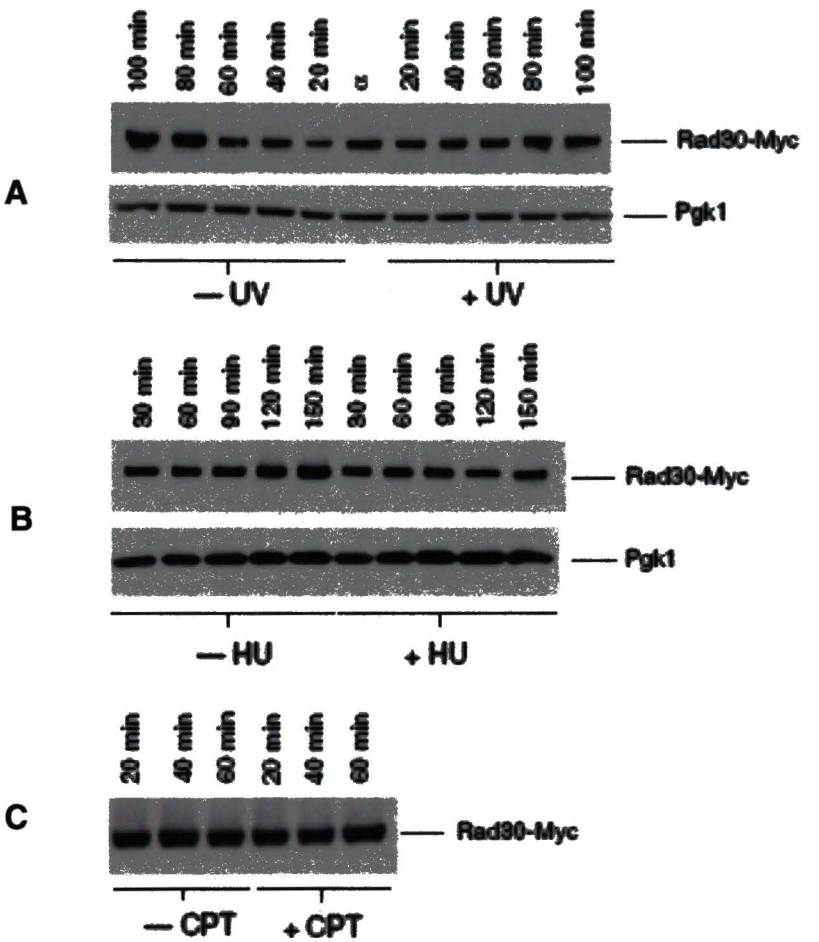

A
100 min
80 min
60 min
40 min
20 min
α
20 min
40 min
60 min
80 min
100 min
Rad30-Myc
Pgk1
— UV
+ UV
B
30 min
60 min
90 min
120 min
150 min
30 min
60 min
90 min
120 min
150 min
Rad30-Myc
Pgk1
— HU
+ HU
C
20 min
40 min
60 min
20 min
40 min
60 min
Rad30-Myc
— CPT
+ CPT

Figure 5. Characterization of Rad30-TAP post-irradiation levels and half-life.

A) A portion of a logarithmic-phase culture (BY4741) was UV-treated (50 J/m2) and Rad30-TAP levels were detected by Western Blotting during incubation following treatment. *Pgk1* was used as a loading control. **B)** Rad30-TAP was detected in unirradiated cultures treated with 500 μg/ml cycloheximide (*CYH*), added at 0 min. Twenty microgram protein was loaded per lane.

Figure 5.

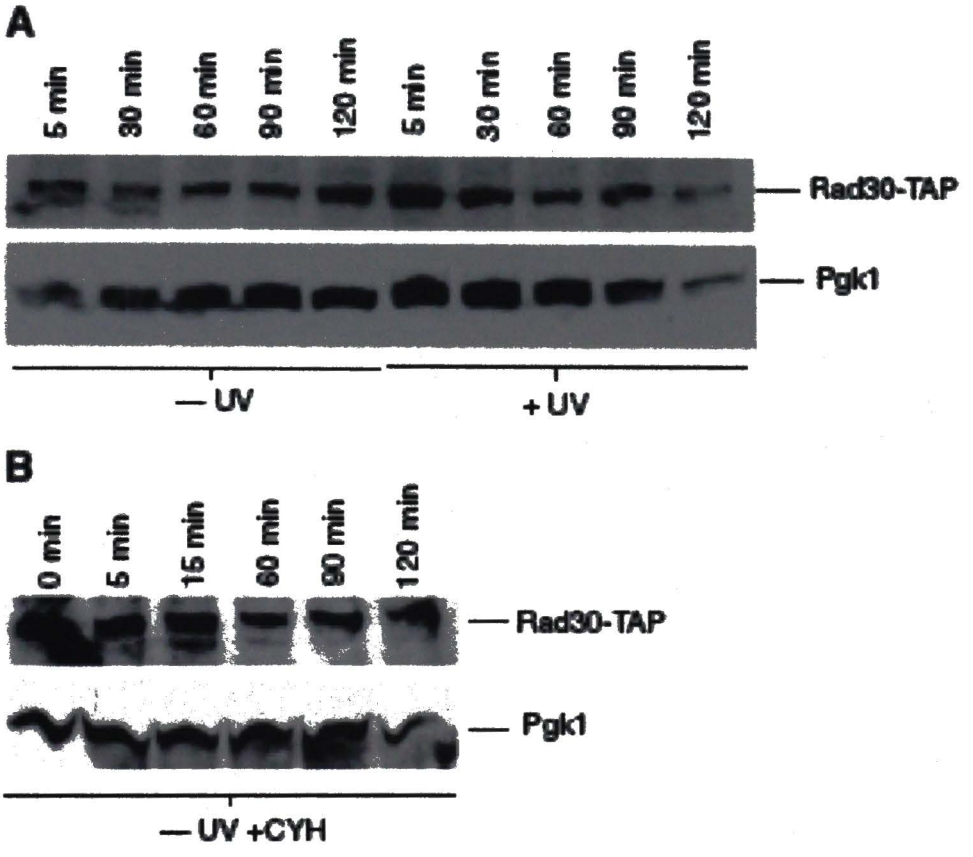

A
5 min
30 min
60 min
90 min
120 min
5 min
30 min
60 min
90 min
120 min
Rad30-TAP
Pgk1
— UV
+ UV
B
0 min
5 min
15 min
60 min
90 min
120 min
Rad30-TAP
Pgk1
— UV +CYH

Figure 6. Ubiquitination of Rad30.

Ubiquinated proteins were immunoprecipitated from logarithmic-phase wild type and isogenic deletion mutants of *RAD5*, *RAD6*, *RAD18* and *DOA1*. Rad30-Myc was detected among the precipitated proteins. This level was compared to the total level of Rad30-Myc, with the signal shown representing 15% of input used for immunoprecipitation (*lower panel*). No signal was detected if a *RAD30* deletion mutant was used (not shown).

Figure 6.

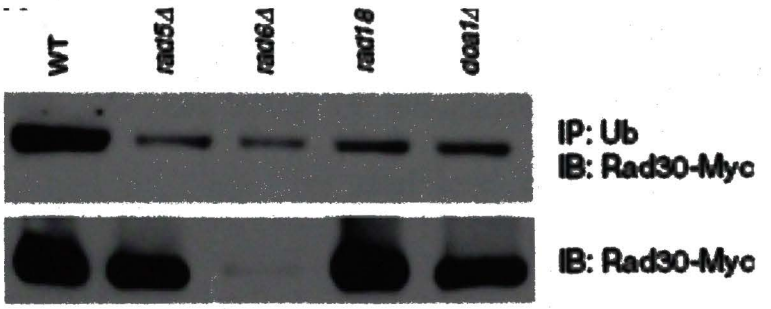


Figure 7. Influence of cell-cycle position and UV treatment on Rad30 ubiquitination

A) Level of ubiquitinated Rad30-Myc during synchronization treatment with α factor (G1 arrest) compared to total level of *Rad30-Myc*. **B)** Level of ubiquitinated Rad30-Myc following release from α factor arrest and resumption of cell cycle progression. An aliquot of the culture was subjected to UV irradiation (80 J/m2) before dilution into fresh YPD. Immunoprecipitation with IgG instead of anti-ubiquitin antibody represents a negative control to confirm specificity. The total level of Rad30-Myc was also determined (lower panel).

Figure 7.

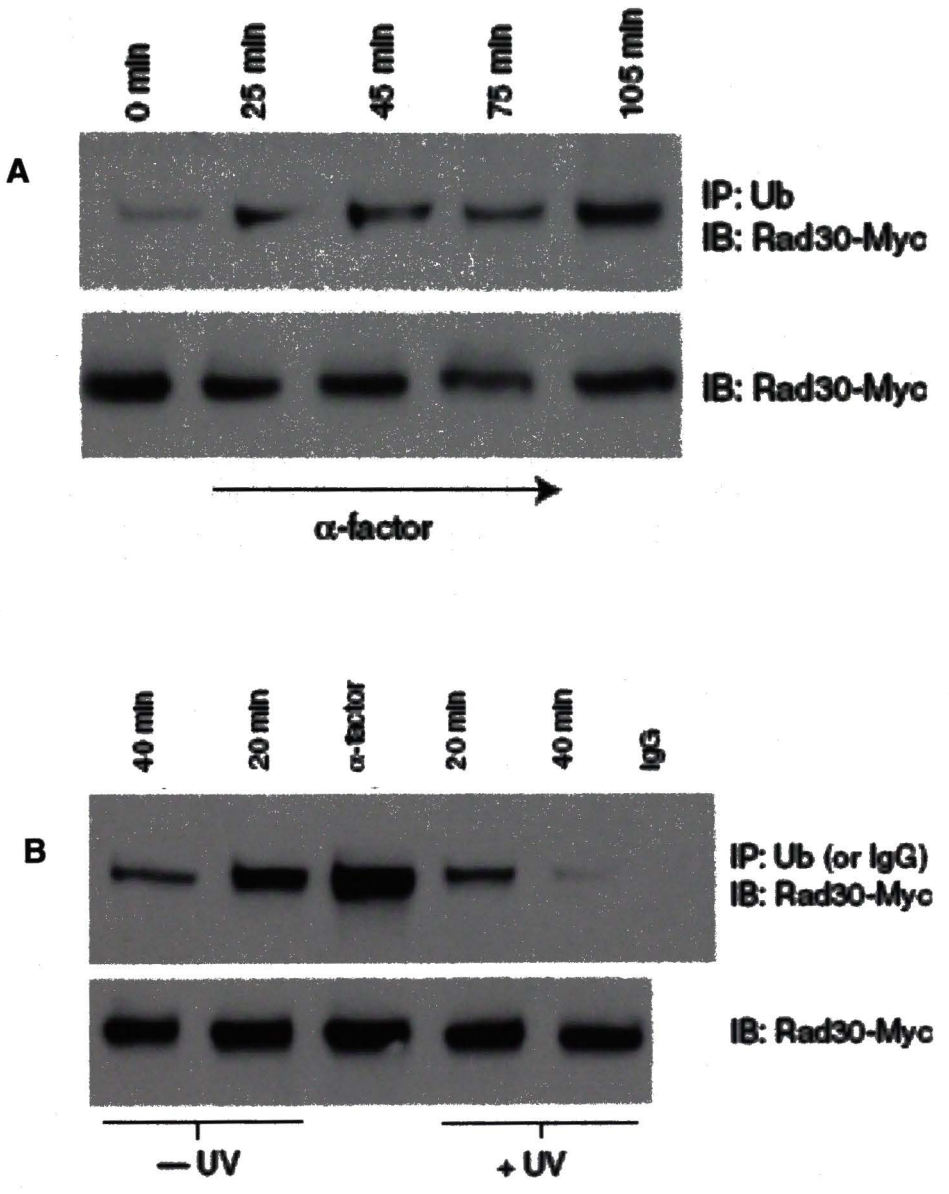
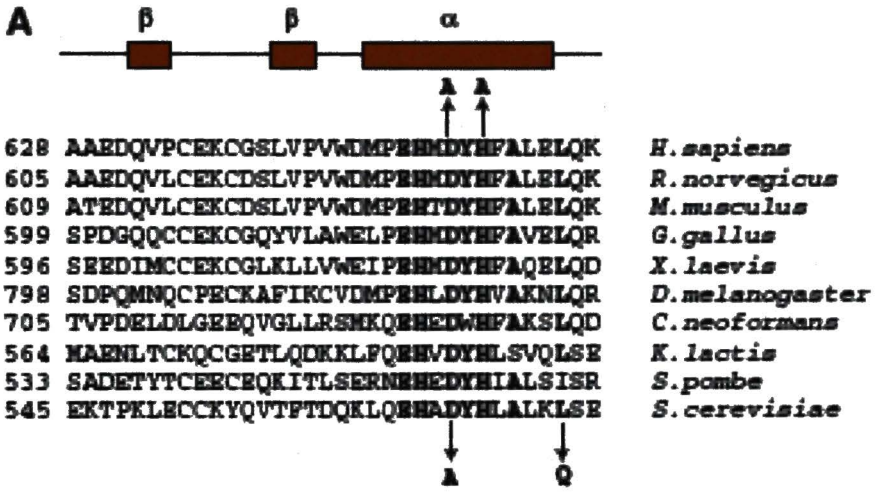

A
0 min
25 min
45 min
75 min
105 min
IP: Ub
IB: Rad30-Myc
IB: Rad30-Myc
α-factor
B
40 min
20 min
α-factor
20 min
40 min
IgG
IP: Ub (or IgG)
IB: Rad30-Myc
IB: Rad30-Myc
— UV
+ UV

Figure 8. Conservation of the UBZ domain of polymerase η.

A) Pol η UBZ domains [23] of various organisms are compared and aligned with structural features derived from human Pol η as described [54]. Published mutations of conserved residues that have been studied are indicated: D652A and H654A of *H. sapiens*, D570A and L577Q of *S.cerevisiae* Pol η [23, 26, 36], this study). **B)** Predicted three-dimensional structure of UBZ domain (*left*)–ubiquitin (*right*) interaction [54]. Figure kindly provided by P.Zhou

Figure 8.


A
β
β
α
A A
628 AAEDQVPCEKCGSLVPVWDMPEHMDYHFALELQK H.sapiens
605 AAEDQVLCEKCDSLVPVWDMPEHMDYHFALELQK R.norvegicus
609 ATEDQVLCEKCDSLVPVWDMPEHTDYHFALELQK M.musculus
599 SPDGQQCCEKCGQYVLAWELPEHMDYHFAVELQR G.gallus
596 SEEDIMCCEKCGLKLLVWEIPEHMDYHFAQELQD X.laevis
798 SDPQMNQCPECKAFIKCVDMPEHLDYHVAKNLQR D.melanogaster
705 TVPDELDLGEEQVGLLRSMKQEHEDWHFAKSLQD C.neoformans
564 MAENLTCKQCGETLQDKKLFQEHVDYHLSVQLSE K.lactis
533 SADETYTCEECEQKITLSERNEHEDYHIALSISR S.pombe
545 EKTPKLECCKYQVTFTDQKLQEHADYHLALKLSE S.cerevisiae
A Q

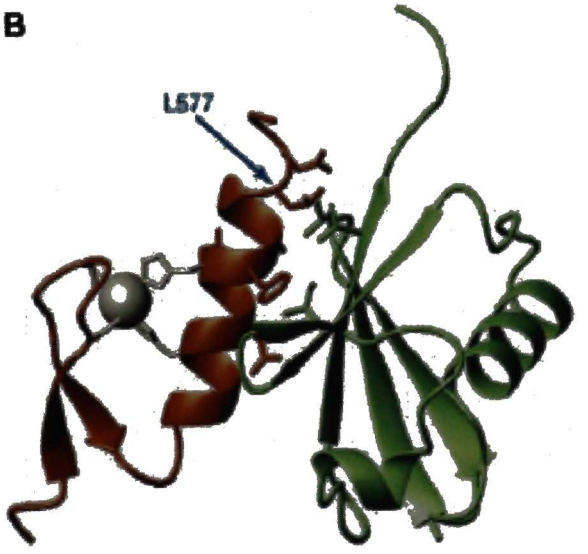

B
L577

Figure 9. Biochemical characterization of Ubz* (=L577Q) mutant of Rad30.

A) Level of ubiquitinated Rad30-Ubz*-Myc during synchronization treatment with α- factor (G1 arrest) compared to total level of Rad30-Ubz*-Myc (*lower panel*). **B)** Relative amounts of ubiquitinated Rad30 (WT) and Rad30-Ubz* (Mut) signal during α-factor treatment normalized by total level of Rad30 and Rad30-Ubz*, respectively (see Figs. 5B, 7A). The highest relative ubiquitination level achieved in the wild type was set to 1. **C)** Level of ubiquitinated Rad30-Ubz*-Myc at 20 and 40 min following release from α- factor arrest. An aliquot of the culture was subjected to UV irradiation (80 J/m2) before dilution into fresh YPD. The total level of Rad30-Ubz*-Myc was also determined (*lower panel*). **D)** Amount of ubiquitinated Rad30 (*WT*) and Rad30-Ubz* (*Mut*) signal, at 20 and 40 min following release from α-factor arrest of UV-treated and non-treated cells, normalized by total level of Rad30 and Rad30-Ubz* (see Figs. 5C, 7C). The highest relative ubiquitination level achieved in the wild type (=G1 arrested) was set to 1 (same as in Fig. 5B).

Figure 9.

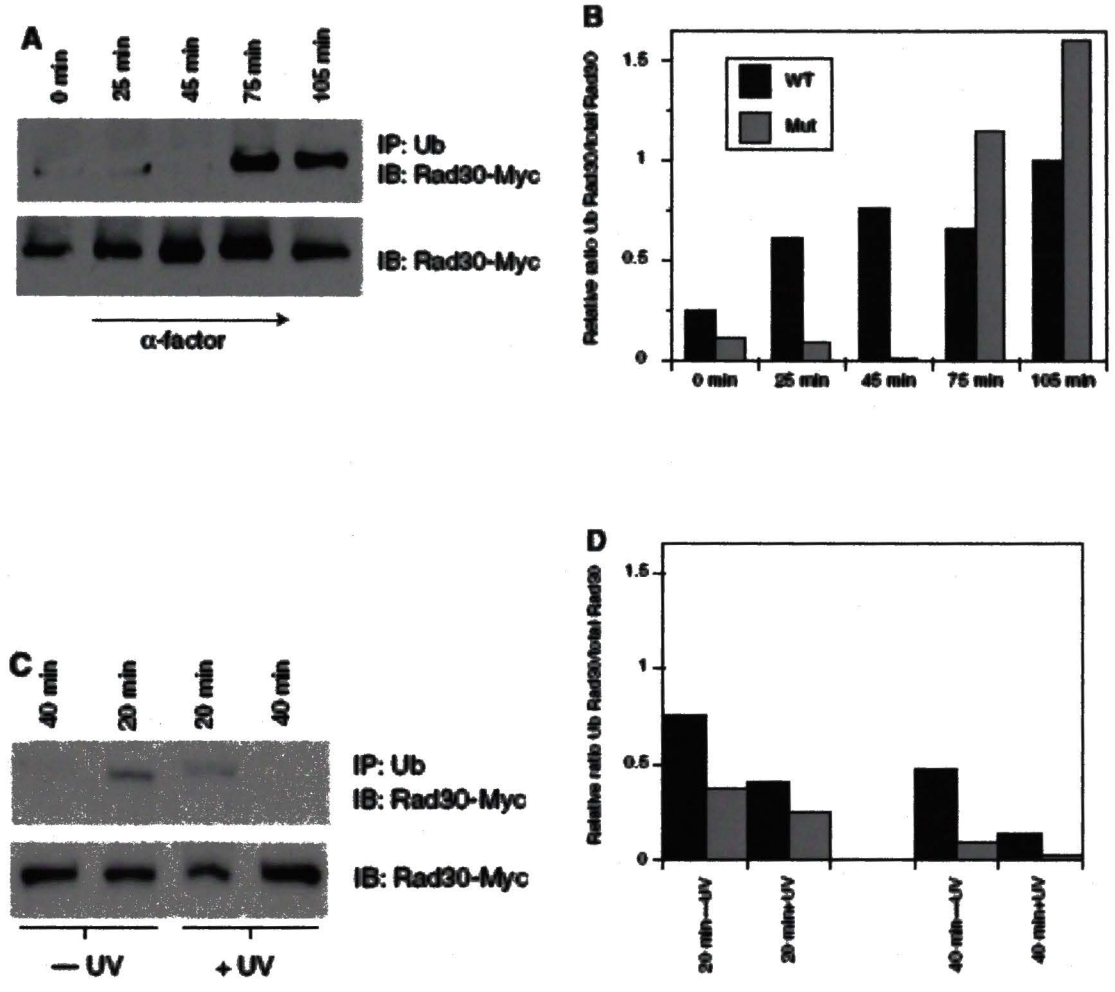

A
0 min
25 min
45 min
75 min
105 min
IP: Ub
IB: Rad30-Myc
IB: Rad30-Myc
α-factor
B
WT
Mut
0 min
25 min
45 min
75 min
105 min
C
40 min
20 min
20 min
40 min
IP: Ub
IB: Rad30-Myc
IB: Rad30-Myc
— UV
+ UV
D

Figure 10. Interaction with ubiquitinated substrates

Ubiquitin agarose pull down of Rad30 (*WT*) or Rad30-Ubz* (*Mut*), followed by Western blotting with anti-Myc antibody. Protein G-agarose was used as a negative control to demonstrate specificity. Total extract levels of Rad30 (*WT*) and Rad30-Ubz* were also determined (15% of input, *lower panel*). No signal was detected if a *RAD30* deletion mutant was used (not shown).

Figure 10.

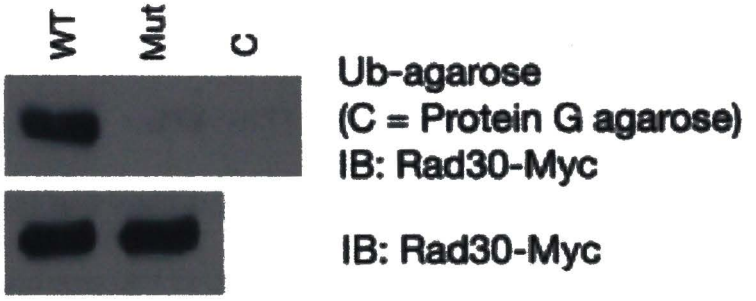
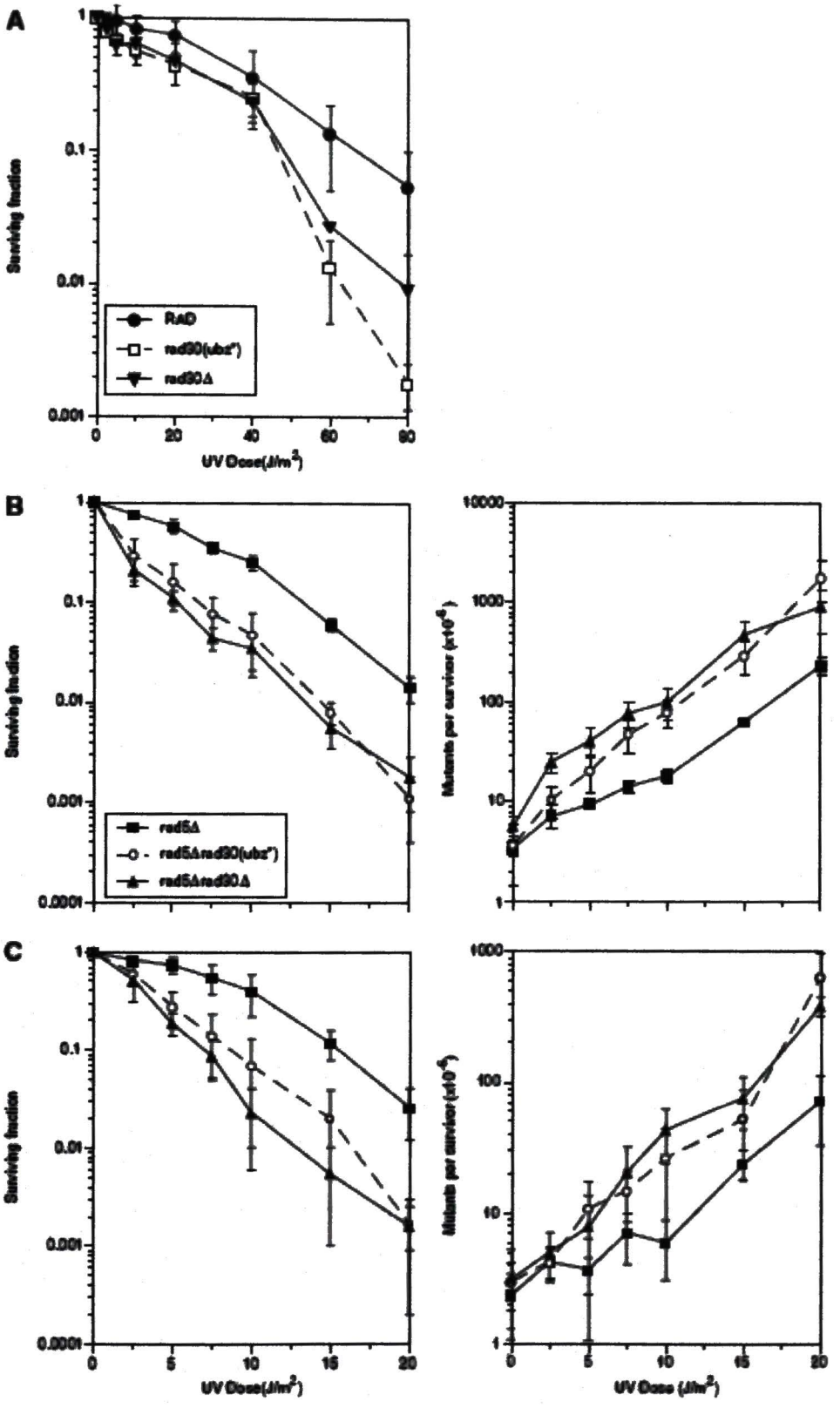

WT
Mut
C
Ub-agarose
(C = Protein G agarose)
IB: Rad30-Myc
IB: Rad30-Myc

Figure 11. Influence of *rad30-ubz(=L577Q) mutation on UV survival and mutagenesis.**

A) Fraction of colony forming cells as a function of UV dose. Stationary-phase cells of wild type (Y300 background) (filled circle), *rad30-ubz*::KanMX4* (open square) and *rad30Δ::KanMX4* (filled inverted triangle) were treated. **B,C)** Survival and *trp1-1* revertant frequencies as a function of UV dose of logarithmic-phase cells (**B**) and stationary-phase cells (**C**) of *rad5Δ::HIS3* (filled square), r*ad5Δ::HIS3rad30-ubz*::KanMX4* (open circle) and *rad5Δ::HIS3rad30Δ::KanMX4* (filled triangle). Data shown represent averages and standard deviation of 3–6 independent experiments.

Figure 11


A
Surviving fraction
RAD
rad90(ubx*)
rad90Δ
UV Dose(J/m²)
B
Surviving fraction
rad5Δ
rad5Δrad90(ubx*)
rad5Δrad90Δ
Mutants per survivor (x10⁻⁶)
C
Surviving fraction
UV Dose(J/m²)
Mutants per survivor (x10⁻⁶)
UV Dose (J/m²)

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Monoubiquitination and its implications:

The highly complex and interrelated processes of DNA replication and repair are regulated by the assembly or disassembly of large protein complexes. Many of the protein molecules involved are stable and in some cases very abundant. Recent studies have made it quite evident that post-translational modification plays an essential role in their regulation. The deployment of a DNA-repair pathway *in vivo* requires multiple layers of regulation. For example, a typical DNA-repair pathway might involve DNA-lesion detection, signaling to recruit DNA-repair factors, the activation of DNA-repair enzymes, and the disassembly or degradation of DNA repair factors after the initial damage is fixed [1]. Although some DNA-repair processes are generally constitutively active, some are activated at the precise times in a cell cycle, or in response to specific types of DNA lesions [2]. Phosphorylation, ubiquitination and other post-translational modification events can ensure a timely and efficient activation of DNA-repair or lesion bypass enzymes, recruit factors to the sites of lesions and regulate cell-cycle checkpoints.

76-amino-acid protein, Ubiquitin (Ub) is conserved from yeast to humans. In a reversible manner, ubiquitin is attached to the side chain of a lysine residue in the substrate by a three step enzyme pathway consisting of a Ub-activating enzyme (E1), a ubiquitin conjugating enzyme (E2) and a Ub-protein ligase (E3). Proteins are targeted for proteasome-dependent degradation through attachment of a polyubiquitin chain in which the first ubiquitin is linked to the substrate and each subsequent Ub is attached to a specific lysine residue, Lys 48, in the preceeding Ub of the chain. In contrast, a common

feature of many non-proteasomal functions of Ub is that the substrate protein is modified by only a single Ub (monoubiquitination), several single ubiquitin molecules (multi-ubiquitination) or a polyubiquitin chain with linkage at lysine residues other than Lys 48. Ubiquitination reactions are reversed by the action of deubiquitinating enzymes (DUBs) of which there are many types [3].

Ubiquitin-binding domains:

Ubiquitin- binding domains (UBDs) are a collection of modular protein domains that non-covalently bind to ubiquitin. Ubiquitin-binding proteins generally have small (25-150 amino acid), independently folded UBDs that can interact directly with monoubiquitin and/or polyubiquitin chains. Proteins having a UBD(s) can interact with ubiquitin or a ubiquitinated substrate and might be regulated by ubiquitination. In addition, several UBD families have members that do not interact with ubiquitin at all [4].

Recently, two types of UBDs were discovered in Y-family TLS polymerases in humans. Bioinformatic analysis of C-terminal of Pol ι and Rev1 recognized two copies of Ubiquitin-binding motif (UBM). Ubiquitin-binding Zn finger domain (UBZ) were identified in Pol η and Pol κ. UBZ-family Zn fingers are clearly distinct from DNA binding Zn fingers. Thus, in humans, all Y-family TLS polymerases contain UBDs in their C-termini [5]. Pol η has only one copy of UBZ domain versus two copies of UBZ domain in Pol κ. These UBM and UBZ domains are highly conserved from *Saccharomyces cerevisiae* to humans [5]. UBZ domain of Pol η represents a novel member of C_2H_2 zinc finger family that interacts with ubiquitin to regulate translesion

synthesis [6]. In humans, UBD domains enable Y-family polymerases Pol η and Pol ι to interact with monoubiquitinated substrates and undergo monoubiquitination *in vivo* [5]. Also mouse Rev1, a member of Y-family polymerases, can physically interact with ubiquitin via two copies of UBMs located at its C terminus and undergoes monoubiquitination itself *in* vivo [7]. UBDs contribute to moderate binding affinity between ubiquitinated PCNA generated at the stalled replication fork and Pol η. The PCNA interacting peptide (PIP) box provides the specificity for the interaction. Together with C-terminal PIP box, the UBZ domain enhances the specificity and binding of the polymerase to monoubiquitinated PCNA, allowing timely recruitment of Pol η to the stalled replication fork. The moderate affinity for this interaction and deubiquitination of PCNA ensure subsequent dissociation of the TLS polymerase from the replication fork beyond the DNA lesion to restore high-fidelity genomic replication [6]. In contrast, there are studies that indicate that capacity to bind to and hence control the functions of ubiquitinated targets is inhibited by monoubiquitination of ubiquitin binding proteins *in vitro* [8] and *in vivo* [9].

Like its mammalian homolog, *Saccharomyces cerevisiae* Pol η is also monoubiquitinated in a manner dependent on its ubiquitin-binding domain [10, **11**= this study]. In budding yeast, however, UBZ domain interacts weakly with free ubiquitin or ubiquitinated PCNA. Therefore, ubiquitin-binding domain of Pol η enhances affinity for the ubiquitinated form of PCNA only in conjunction with basal affinity for the unmodified PCNA, mediated by PIP [10].Nevertheless, various studies involving point mutations of the conserved residues of UBZ domain of Pol η in humans and

Saccharomyces cerevisiae emphasize the essential role of ubiquitin-binding domain for *in vivo* function of the polymerase. Mutations of the conserved residues in UBZ domain resulted in a decrease in UV resistance along with an increase in UV-induced mutagenesis [5, 10, 12].

By enhancing affinity for monoubiquitinated PCNA, UBZ domain is required for accumulation of Pol η at the stalled replication forks in response to DNA damage *in vivo* in humans [5, 12]. Pol η and Pol ι harbouring point mutations in UBZ domain are defective in their interaction with Ub or ubiquitinated PCNA and exhibit significant reduction in accumulation of polymerases at the replication foci after DNA damage [12]. In cells exposed to UV radiation, the association of Rev1 with replication foci is dependent on functional UBM [7]. In other words, an ability to bind Ub is a prerequisite for targeting DNA polymerases η, and ι and Rev1 to the sites of cellular damage. Recent studies showed that the conserved aspartate residue (D652 in humans and D570 in yeast) in the α-helix portion of UBZ domain is responsible for targeting Pol η to replication foci in humans and confers considerable increase in UV sensitivity and UV mutability in yeast [5, 10]. Our studies in the previous chapter demonstrated that a point mutation of a conserved residue present at the border of α-helix of the UBZ domain (L577Q) resulted in an increased UV sensitivity and mutability [11].

Not only the α-helix but C_2H_2 Zn-finger domain of the UBZ motif is also important for mediating polymerase's interaction with ubiquitinated substrates. A recent study demonstrated that H654A mutation of UBZ domain in Pol η resulted in significantly reduced accumulation of the polymerase at the lesion site due to its inability to interact

with ubiquitinated PCNA. Such a mutant, nevertheless, could interact with unmodified PCNA due to its intact PCNA interacting peptide (PIP). Hence some of the mutant protein could still be targeted to replication foci [12]. There is one study which showed that point mutations in C_2H_2 motif of the UBZ domain in Pol η did not affect the accumulation of polymerase at the replication foci. A C_2H_2 mutant is unable to bind zinc which is required to interact with Ub. This would mean that binding of ubiquitin to PCNA via UBZ domain is dispensable for a polymerase's ability to access PCNA at the stalled replication fork [13].

Interestingly, Rad18, a ubiquitin ligase responsible for PCNA monoubiquitination, associates constitutively with Pol η through domains at their C-terminal regions. Rad18p is crucial for recruitment of Pol η to the damaged site through protein-protein interaction and PCNA monoubiquitination. In humans, Pol η does not accumulate at replication foci in RAD18 (-/-) cells after UV irradiation. Furthermore, Rad18p itself undergoes intra-nuclear translocation to colocalize with PCNA at the replication fork after UV irradiation. This relocalization of Rad18p is via direct or indirect post-translational modification [14].

An important and unanswered question is why do UBD containing proteins tend to be monoubiquitinated rather than polyubiquitinated? Is this because UBDs in complex with ubiquitin mask the lysines important for ubiquitin chain formation? Indeed , an intramolecular interaction between the UIM (Ubiquitin Interacting Motif) and covalently attached ubiquitin has been shown to restrict ubiquitin chain extension on the transcription factor Met4 by shielding the terminal ubiquitin molecule in the chain [15, 16]. However, it is not yet fully understood whether and how monoubiquitination of

ubiquitin-binding proteins may contribute to the regulation of their function *in vivo*. Although the exact role of monoubiquitination of the polymerases is not established yet, it is tempting to speculate that Pol η may be regulated by its compartmentalization in or out of nucleus and/or change in subcellular localization during different phases of the cell cycle as well as in response to UV damage. This distribution of the polymerase may be directly dependent on the degree of ubiquitination in *Saccharomyces cerevisiae*.

Monoubiquitination: a signal for intracellular trafficking

In a recent issue of Science, Li *et al* reported that p53 may face two different fates depending on the extent of ubiquitination. Polyubiquitination signals degradation of p53 in the nucleus whereas monoubiquitination serves as a nuclear export signal in non-stressed cells. In response to DNA damage, cytoplasmic p53 may undergo deubiquitination, reenter the nucleus and act as a first line of defense [17]. This would mean that harmful effects of p53 in the nucleus are avoided in non-stressed cells by its monoubiquitination which inactivates p53. But, the simple notion that p53 monoubiquitination is a nuclear export trigger hence merely an inactivation device is challenged by other recent data. Monoubiquitinated form of p53 in the cytoplasm is a stable form and provides a trafficking signal that redirects it from a fate of degradation and inactivation in unstressed cells to mitochondrial translocation and activation early during the stress response [18]. The authors showed that nuclear export is not required for mitochondrial translocation upon DNA damage. Instead, distinct nuclear and cytoplasmic

p53 pools become simultaneously and rapidly stabilized after genotoxic stress indicating that mitochondrially translocated p53 arises from a distinct cytoplasmic pool.

Thus, monoubiquitination can serve as a signal for intracellular trafficking between compartments [19, 20]. In the absence of mitochondrial translocation motif within p53 and the fact that phosphorylation / acetylation modifications play no major role in the targeting of p53 [21], monoubiquitination directs cytoplasmic p53 to the mitochondria [17].

Another interesting example is of Human T-cell leukemia virus type I (HTLV-I) oncoprotein Tax. In non-stressed cells, Tax is sumoylated and predominantly a nuclear protein that localizes to nuclear foci known as Tax Speckled Structures (TSS). DNA damage induces monoubiquitination of Tax and triggers its export from nucleus into the cytoplasm. Monoubiquitination facilitates the dissociation of Tax from nuclear foci and hence regulates the localization of Tax and its interaction with cellular proteins [22].

Mitochondrial DNA repair pathways

The yeast mitochondrial genome is known as the *rho* factor, represented by the Greek letter ρ. Mitochondrial DNA (mtDNA) amounts on average to ~15% of the DNA content of *Saccharomyces cerevisiae* which is equivalent to ~50 copies of the ~75 kb genome per haploid cell. mtDNA molecules occur in small clusters called "nucleoids" that vary widely in size and number in response to physiological conditions [23]. The DNA polymerase responsible for replication of mtDNA, Pol γ, is encoded by the nuclear genes MIP1 in yeast and POLG in humans [24, 25].The current detailed understanding of the

genetic and biochemical activities of mitochondria is owed to the unique ability of *S.cerevisiae* to survive without respiration. Notable differences in mtDNA structure and replication in yeasts and humans include copy number (~10^2-10^4 copies in human cells versus ~20-100 copies in yeast cells), genome size (16.6 kb in humans versus 85.8 kb in yeasts), genome structure (predominantly high molecular weight linear forms in yeast versus circular genomes in human cells) and mechanisms of mtDNA replication. The catalytic subunit of the human mtDNA polymerase shares high homology with the *Saccharomyces cerevisiae* Pol γ, but yeast differs by the absence of an associated processivity factor, p55, which is present in humans [23, 26, 28].

Maintenance of mitochondrial genome is critical for maintaining respiratory capacity of the cell. Higher eukaryotic cells lose viability with loss of respiratory function, making efficient repair and replication of mtDNA crucial for cell survival. Unlike nuclear DNA, mtDNA is continuously replicated, even in terminally differentiated cells, such as nerve cells and cardiomycetes. Consistently, the accumulation of mutations in mtDNA is ~ 10-fold greater than nuclear DNA, due to mitochondria being the power house of the cells, produce large amounts of reactive oxygen species (ROS) during the process of oxidative phosphorylation and lack of protective histones [29]. Fidelity of mtDNA replication can be achieved by three mechanisms: selection of correct nucleotide, which results from the intrinsic discrimination capacity of the polymerase γ ; 3'-5' exonucleolytic editing by the polymerase which removes mismatches at the 3' end of the growing DNA chain, and mismatch repair system. Proofreading and mismatch repair deficiencies in *Saccharomyces cerevisiae* are lethal for mitochondria [30].Mice harboring a proof-

reading deficient version of catalytic subunit of Pol γ (PolgA) in a homozygous fashion show premature aging phenotype. These mitochondrial mutator mice carry extensive point mutations and mtDNA deletions [31].

Over the past two decades, mitochondrial defects have been implicated in a wide variety of neurological and muscular degenerative diseases, aging and cancer. The essential role of mitochondrial oxidative phosphorylation for producing cellular energy, the generation of reactive oxygen species, and the initiation of apoptosis are three important aspects responsible for mitochondrial pathogenesis [32]. Mutations in nuclear genes can also exert their phenotypic effects by indirectly inactivating oxidative phosphorylation or destabilizing the mtDNA.

For a long time it was thought that mitochondria lacked DNA repair systems as there are multiple copies of mtDNA and damaged molecules could merely be degraded or lost during cell division. Nearly 40 years ago, mitochondrial myopathy, first indication that mitochondria may play role in pathogenesis was reported. The first mitochondrial diseases to be understood at the molecular level are the maternally inherited Leber's hereditary optic neuropathy (LHON), resulting from mtDNA missense mutation, and a spontaneously occurring group of neuromuscular diseases, now classified as chronic progressive external ophthalmopelia (CPEO) and the Kearns-Sayre Syndrome (KSS), resulting from mtDNA deletions [32]. It is clear from the literature that repair in mitochondria exists and that mtDNA is subject to repair by multiple repair pathways. There is enough evidence in literature to show that base excision repair (BER) is the primary repair pathway responsible for repairing oxidative damage in nuclear and

mitochondrial genomes. In yeast mitochondria, three N-glycosylases have been identified so far, Ntg1p, Ogg1p and Ung1p. Ntg1p has the widest substrate specificity excising oxidized pyrimidines, thymine glycols and cytosine photoproducts generated by UV irradiation [33]. In *Saccharomyces cerevisiae*, direct damage reversal pathway via *PHR1* encoded photolyase is also present but absent in mammalian cells. Also mismatch repair and recombinational repair mechanisms in mitochondria of both yeast and mammals have been documented [34]. However, mitochondria are devoid of nucleotide excision repair in mammals and in *S. cerevisiae*. Therefore, the classic NER substrates such as UV-induced thymidine dimers and cisplatin intrastrand cross-links remain unrepaired in mammalian cells.

Translesion Synthesis in Mitochondria

Translesion synthesis is an important damage bypass mechanism known to operate in the nucleus, however, its role in bypass of mtDNA damage *in vivo* remains to be shown. Although proteomic analysis of *S. cerevisiae* mitochondria identified the presence of Rad18, which is involved in nuclear TLS, however a role for this protein in mitochondrial TLS is not shown yet [34]. Pol ζ and Rev1p are responsible for error-prone TLS in nucleus. Together these enzymes are responsible for both UV-induced and spontaneous mutagenesis in the nucleus [35].

To date, DNA polymerase γ was the only polymerase described in the mitochondria [24]. Recently, a study provided evidence that yeast TLS proteins Pol ζ and Rev1p localize to mitochondria. These proteins contain putative mitochondrial targeting signal

(MTS) at their N-termini. The authors also demonstrated that inactivation of *REV3* and *REV7* encoding Pol ζ, as well as of *REV1* genes led to suppression of frameshift mutations in mtDNA [36]. However, the authors failed to see the localization of human homologs of Pol ζ and Rev1p to the mitochondria. Consistent with these observations, another group demonstrated that Rev1p and Pol ζ are responsible for the majority of spontaneous and UV-induced mitochondrial frameshifts in *S. cerevisiae*. In contrast, deleting the translesion polymerases Pol ζ and Rev1p results in a dramatic increase in UV-induced mtDNA point mutations [37]. This means that there is an alternative damage tolerance pathway that is more mutagenic than Rev1p and Pol ζ, which is responsible for generating mitochondrial point mutations in their absence.

We hypothesize that similar to other TLS polymerases Pol ζ and Rev1p, Pol η also localizes to mitochondria in *Saccharomyces cerevisiae*. UV-induced mtDNA damage bypass via Pol η forms an alternative TLS pathway essential for mitochondrial genome stability.

The following study was carried out to determine if Pol η localizes to mitochondria. If so, its subcellular distribution may depend on degree of monoubiquitination. Are there two separate pools of Pol η, cytoplasmic and/or mitochondrial and nuclear or does the same pool of the polymerase redistributes itself depending on the cell cycle position or in response to UV damage?

CHAPTER III

IS SUBCELLULAR DISTRIBUTION OF DNA POLYMERASE ETA REGULATED BY MONOUBIQUITINATION?

PREFACE

The process of ubiquitination is best known for its role in targeting proteins for degradation by the proteasome. Recent studies have unraveled various non-traditional roles of ubiquitination which include but are not limited to changing the molecular landscape of a protein and influencing protein-protein interactions, regulating many biological processes like DNA repair, endocytosis, signal transduction, chromatin remodelling and activation of protein kinases. DNA repair and DNA-damage response pathways involve the monoubiquitination of key DNA-repair proteins to help them modulate the assembly or disassembly of complexes, change the subcellular localization and the regulation of enzymatic activity in a timely manner. In our previous study, we have shown that DNA polymerase eta is constitutively monoubiquitinated in *Saccharomyces cerevisiae*. However, degree of the modification varies with cell-cycle position as well as in response to DNA damage. Thus the objective of the work in the following section was to determine how monoubiquitination regulates DNA polymerase eta in *Saccharomyces cerevisiae*.

TRANSLESION POLYMERASE ETA LOCALIZES TO MITOCHONDRIA IN *SACCHAROMYCES CEREVISIAE*

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Running Title: DNA polymerase eta localizes to mitochondria

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Acknowledgements: This study was supported by grant ES011163 from the National Institutes of Health.

Key words: Pol η, Mitochondria

[To be submitted to FEMS Yeast Research]

Analogous to phosphorylation, signaling through monoubiquitination has emerged as a major regulatory function in eukaryotic cells. Phosphorylation, ubiquitination and other post-translational modification events can ensure a timely and efficient activation of DNA-repair or lesion bypass enzymes, recruit factors to the sites of lesions and regulate cell-cycle checkpoints. Monoubiquitination can serve as a signal for intracellular trafficking between compartments [19, 20]. In the absence of mitochondrial translocation motif within p53 and the fact that phosphorylation / acetylation modifications play no major role in the targeting of p53 [21], monoubiquitination directs cytoplasmic p53 to the mitochondria [17].

Maintenance of mitochondrial genome is critical for maintaining respiratory capacity of the cell. A number of human diseases, including cancer, have been attributed to pathogenic mutations of mtDNA. For a long time it was thought that mitochondria lacked DNA repair systems as there are multiple copies of mtDNA and damaged molecules could merely be degraded or lost during cell division. BER is the primary repair pathway responsible for repairing oxidative damage in nuclear and mitochondrial genomes. In *Saccharomyces cerevisiae*, direct damage reversal pathway via *PHR1* encoded photolyase is also present but absent in mammalian cells. Also mismatch repair and recombinational repair mechanisms in mitochondria of both yeast and mammals have been documented [34]. However, mitochondria are devoid of nucleotide excision repair in mammals and in *S. cerevisiae*. Therefore, the classic NER substrates such as UV-

induced thymidine dimers and cisplatin intrastrand cross-links remain unrepaired in mammalian cells.

Translesion synthesis is an important damage bypass mechanism known to operate in the nucleus, however, its role in bypass of mtDNA damage *in vivo* remains to be shown. Although proteomic analysis of *S. cerevisiae* mitochondria identified the presence of Rad18, which is involved in nuclear TLS, however a role for this protein in mitochondrial TLS is not shown yet [34].

Recently, a study provided evidence that yeast TLS polymerases Pol ζ and Rev1p localize to mitochondria. These proteins contain putative mitochondrial targeting signal (MTS) at their N-termini. The authors also demonstrated that inactivation of *REV3* and *REV7* encoding Pol ζ, as well as of *REV1* genes led to suppression of frameshift mutations in mtDNA [36]. However, the authors failed to see the localization of human homologs of Pol ζ and Rev1p to the mitochondria. Consistent with these observations, another group demonstrated that Rev1p and Pol ζ are responsible for the majority of spontaneous and UV-induced mitochondrial frameshifts in *S. cerevisiae*. In contrast, deleting the translesion polymerases Pol ζ and Rev1p results in a dramatic increase in UV-induced mtDNA point mutations [37]. This means that there is an alternative damage tolerance pathway that is more mutagenic than Rev1p and Pol ζ, which is responsible for generating mitochondrial point mutations in their absence.

In this study, we provide evidence that yeast TLS polymerase η localizes to mitochondria. Furthermore, we demonstrate that the localization is not influenced by cell-cycle position and after UV damage.

MATERIALS AND METHODS

Yeast strains

The yeast strains used in this study were derived from BY4741 (*MATa his*3Δ *leu*2Δ *met*15Δ *ura*3Δ). Rad30 protein was epitope tagged with 13xMyc at its C-terminus following transformation and microhomology-mediated recombination at its chromosomal location with a PCR product, using a plasmid-borne, *KanMX4*-marked module [38]. The same strain containing a GFP-tagged Rad30 version was purchased from Open Biosystems. Yeast transformation techniques, general growth conditions and media recipes can be found elsewhere [39].

Intracellular localization of Rad30-Gfp

To study localization of Pol η, GFP tagged version of Rad30p was overexpressed by placing full-length polymerase under control of the inducible GAL1 promoter [38].Briefly, promoter of *GAL1* was PCR amplified from a plasmid module with a Kanamycin resistance marker for selection. The amplified PCR product was transformed and P_{GAL1} integrated into the chromosome so that RAD30-GFP was placed immediately downstream of the promoter due to homologous recombination at the chromosomal location with the upstream and downstream sequences of the PCR product.

Cells from a single colony were grown overnight in 5 ml of YPG (1% Yeast extract, 2% Bacto peptone and 3% v/v glycerol) at 30°C. Cells were spun down and resuspended in a larger volume of YPG (~25 mls) and incubated for 1-2 days to give a titer of 1-2X10^7 cells/ml. ~5 ml was drawn as an uninduced control sample and then 20% galactose was

added to a final concentration of 0.5 to 2% to the remaining culture for overexpression of Pol η-GFP (Protocol taken from www.bio.brandeis.edu/haberlab/jehsite/protocol.html).

To study the dependence of localization on cell-cycle position, asynchronous logarithmic-phase haploid cells expressing Rad30-GFP were synchronized in G1 by adding yeast mating factor α (US Biological, dissolved in water) in two aliquots separated by 1.25 h of incubation, up to a final concentration of 10μg/ml. Simultaneously, galactose was added at various concentrations and the cells were incubated for a total of ~ 2 h. Samples were drawn in G1 phase, washed and resuspended in water for UV treatment at 80 J/m^2 . The UV treated cells and the untreated control cells were resuspended in fresh YPG + Galactose medium for subsequent samples at 20', 40' and 60' after G1 arrest.

For visualization, cells were resuspended in 500 μl of 1 μg/ml 4', 6-diamidino-2-phenylindole (DAPI) (Research Organics) in water as a marker for the nuclear and mitochondrial DNA. The cells were incubated for 10 minutes in the dark, pelleted, washed twice with water and once with 1X PBS. Fluorescence was examined using a green fluorescent protein (GFP) optimized filter and a DAPI optimized filter using a Olympus AX70 upright microscope equipped with Olympus DP 70 didgital camera under 100X magnification.

Subcellular fractionation

Around 5 litres of yeast culture in YPD (1% Yeast extract, 2% Bacto peptone and 2% dextrose), yielding ~ 30g of wet yeast cell weight was collected. A crude mitochondrial pellet was prepared as described previously [40]. Briefly, cells were suspended in

Spheroplast buffer (1.2 M sorbitol, 20mM phosphate buffer, pH 7.4, 0.5% β-mercaptoethanol) and treated with Zymolyase 20T (2.5 mg/g of cells) (US Biological) for 45 minutes at 30 ° with gentle shaking. Hereafter, all the procedures were carried out at 4°C. After centrifugation, the pellet was suspended in Spheroplast lysis buffer (0.6M sorbitol, 20mM MES-KOH, pH 6.0) supplemented with 1.5% fungal protease inhibitor cocktail (Sigma) followed by disruption in a tight-fitting Dounce homogenizer by using 15 strokes of the pestle. After differential centrifugations at 1500g for 5 minutes twice to separate cell debris and nuclear extract followed by 12,000g for 10 minutes, a crude fraction of mitochondria was produced. The crude mitochondrial pellet was suspended in 20 ml of Iodixanol (40% w/v) (OptiPrep^{TM}) resulting in a solution of density (ρ) 1.225 g/ml. In 38 ml tubes for the swinging-bucket rotor (Beckman SW28), 10 ml of the mitochondrial suspension was layered followed by 14 ml each of the ρ = 1.16 and 1.10 g/ml solutions of Iodixanol to form a discontinuos gradient. The tubes were centrifuged at 80,000g for 3 h and the band of mitochondria at the interface of the solutions of ρ = 1.10 and 1.16 g/ml was collected and diluted in Mitochondria suspension buffer (0.6 M sorbitol, 20mM Hepes-KOH, pH 7.4, 1.5% protease inhibitor cocktail). The purified mitochondria were harvested at 10,000g for 10 minutes.

(Protocol taken from http://www.axis-shield.com/densityhome/optiprep/S27.pdf).

Western Blot Analysis

Protein concentration of samples of the purified mitochondrial suspension was determined (Bio-Rad assay), 1X SDS-loading buffer was added and boiled for 5 minutes. Proteins were analyzed by SDS-PAGE and Western blotting using standard techniques

(Current Protocols Online; http://www.mrw2.interscience.wiley.com/cponline). Mouse monoclonal anti-Porin antibody (Molecular Probes) was used at a dilution of 1:1000 and incubated overnight at 4°C. Anti-Myc antibody (Covance) was used at a dilution of 1:1000 and incubated for 2h at room temperature. Anti-mouse IgG-HRP was used as a secondary antibody at a dilution of 1:2000 for 1h at room temperature.

RESULTS

Yeast polymerase η localizes to mitochondria

BY4741 strain expressing Rad30p with green fluorescent protein (GFP) tagged at the C-terminus of Rad30, purchased from Open Biosystems, was used for the study. In this strain GFP is inserted in-frame immediately preceding the stop codon of RAD30 in the chromosome and the fusion protein is expressed from *RAD30* native promoter. With this strategy wild-type levels and patterns of protein expression are minimally perturbed [41]. DAPI was used as a control to determine the nuclear and mitochondrial compartments. Wild-type strain was compared with ρ^0 strain which is devoid of mitochondrial DNA. ρ^0 cells did not show any extra-nuclear DAPI staining, hence, reaffirming the established use of DAPI staining for nuclear and mitochondrial DNA (Figure 1).When live yeast cells expressing the fusion protein were examined by fluorescence microscopy, we found that Pol η localized to mitochondria as well as nucleus. However, Pol η being a very low abundance protein (1860 molecules/cell, Source: Saccharomyces Genome Database), the fluorescence signal was weak and unreliable.

For better visualization, we overexpressed Rad30-GFP by inserting galactose inducible promoter P_{GAL1} immediately upstream of *RAD30* ORF. For induction, we tried different concentrations of galactose in the range of 0.5% to 2%. Our findings were confirmed as the overexpressed Polη-GFP fusion protein localized to both DNA containing organelles, nucleus and mitochondria (Figure 2). To further substantiate our finding, we carried out western blot analysis of purified mitochondrial extract prepared from strain BY4741

expressing Rad30-Myc fusion protein. Rad30p was visualized by probing with anti-Myc antibody. As a positive control, same membrane was probed with an antibody against Porin (VDAC channel), an authentic mitochondrial protein (Figure 3). Together, these studies demonstrate that Pol η indeed localizes to mitochondria (and to the nucleus).

Although at lower concentrations (0.5, 1.0 and 1.5%) of galactose, the fusion protein was observed in both the compartments (Figure 4), however, at 2% galactose the fusion protein was largely nuclear (Figure 5).

Localization of Pol η is not affected by cell-cycle position and after UV damage

Yeast strain expressing Rad30-GFP was simultaneously treated with α-factor to arrest the cells in G1 phase, and 0.5% galactose to overexpress the fusion protein. Samples were taken in G1. Remaining culture was washed and resuspended in fresh YPG + 0.5% galactose. Subsequent samples were drawn at 20' and 40' post G1 release . To study the effect of UV, cells were suspended in water and treated with UV at 80 J/m^2 after removal of the pheromone followed by resuspension in fresh YPG+ 0.5% galactose. Cells in G1 phase could be identified by their peculiar "schmoo" shape and 20' and 40' samples represented early S phase and late S phase cells, respectively, confirmed by their budding pattern under the light phase microscope. Rad30-GFP localized to mitochondria and nucleus in G1 and S phase (Figure 6). A time course until 80' post G1 release did not show any change in localization (data not shown). After UV treatment, no change in the localization of Pol η in S phase was seen (Figure 7).

DISCUSSION

This study was carried out to investigate the role of monoubiquitination of Rad30/Polη as a means of regulating its subcellular localization and/or a signal for targeting it to mitochondria [17]. Recently, Rev1 and Pol ζ, translesion polymerases in *S. cerevisiae*, were reported to localize to mitochondria [36]. We show that Pol η, a nuclear translesion polymerase, also localizes to mitochondria. Pol η was observed in mitochondria in all the phases of cell cycle and the localization did not seem to be affected by UV damage. Using the PSORT II (http://psort.nibb.ac.jp) software designed to identify mitochondria targeting signal (MTS) in a protein, probability of mitochondrial localization of Pol η was only 21.7%. Interestingly, mitochondrial prediction for the known mitochondrial protein Cox2 (subunit II of cytochrome C oxidase) was only 11.1% by the same software. Surprisingly, when expression of Polη-GFP was induced at a higher concentration of galactose (2%), hardly any fusion protein could be located in the mitochondria. There are some instances in literature where overexpression of a protein resulted in its mislocalization [42]. Our observation is, however, unique where overinduction of protein results in loss of mitochondrial signal. Induction at 2% galactose resulted in almost 20 fold increase in fusion protein than control (without induction). At such high levels, translocation or import of the protein may be impeded via receptor or cytoplasmic chaperons. It is possible that localization of human Pol η to mitochondria could not be detected due to its overinduction. Our observation argues against reliance on

overexpression of target proteins to study localization and may warrant reevaluation of data.

In the past few years, there has been a growing amount of evidence of ubiquitination influence on intracellular localization of the proteins. Involvement of ubiquitination in targeting of nuclear-encoded preproteins to mitochondria was shown [43]. Also, mitochondrial targeting of phosphatidylserine was shown to be regulated by ubiquitination [44]. In the absence of a known putative mitochondria targeting signal in Pol η, monoubiquitination may be the signal for mitochondrial localization. However, further study is required to investigate the role of Polη in mitochondrial mutagenesis. It will be interesting to determine, if any, mitochondrial phenotype resulting from the loss of Polη. It has been reported that a 20-fold increase in UV-induced mitochondrial DNA point mutations are observed in Rev1 and Pol ζ deficient strains, implicating an alternative damage tolerance pathway specific to the mitochondrial compartment [37]. Pol η may be the one carrying out the alternative damage tolerance besides Pol ζ and Rev1.

We also found that the polymerase localizes to nucleus in G1 and S phases of the cell-cycle in UV irradiated as well as unirradiated cells. We had expected to locate Polη mainly in mitochondria in G1 phase when most of the protein is monoubiquitinated. As the cells enter S phase, deubiquitination of the protein is enhanced, signaling translocation of the protein to the nucleus. In contrast, there seems to be separate pools of the polymerase, cytoplasmic (mitochondrial) and nuclear. Although, the possibility of intranuclear translocation from nuclear matrix to replication fork in response to increased

deubiquitination in S phase can not be ruled out. Further studies may be required to determine chromatin bound fractions of Pol η in S phase as well after UV damage. Since the polymerase functions in a time efficient manner, it is more likely that the protein is always in the nucleus and the difference in ubiquitination levels may be regulating the actual recruitment of the polymerase to the replication fork.

Varying degree of monoubiquitination may be involved in either (a) facilitating new or stronger interactions with protein(s) responsible for recruiting the polymerase to the site of DNA replication or (b) inhibiting Polη interaction with protein(s) which may prevent its access to undamaged template.

For instance, Pol κ, a translesion polymerase in humans, was shown to have an unexpected role in mammalian NER [45]. It may be true for Pol η also which may be involved in NER during G1 phase. A high degree of monoubiquitination in G1 phase may enable Pol η to interact with a different partner(s) than S phase.

Figure1: DAPI as a marker for nuclear and mitochondrial compartments

Top panel: Wild-type strain BY4741 without GFP fusion at the C-terminus of Rad30p was incubated with DAPI. The cells were examined by fluorescence microscopy (using green fluorescent protein (GFP) optimized filter and DAPI optimized filter) and differential interference contrast (DIC) optics.

Bottom panel: Wild-type (WT) strain and rho0 strain (lacking mtDNA) were incubated with DAPI and examined under fluorescence microscope. DAPI stained nuclear as well as mitochondrial nucleoids (shown by arrows) as seen in WT cells. ρ0 cells showed only nuclear staining by DAPI.

Figure1.

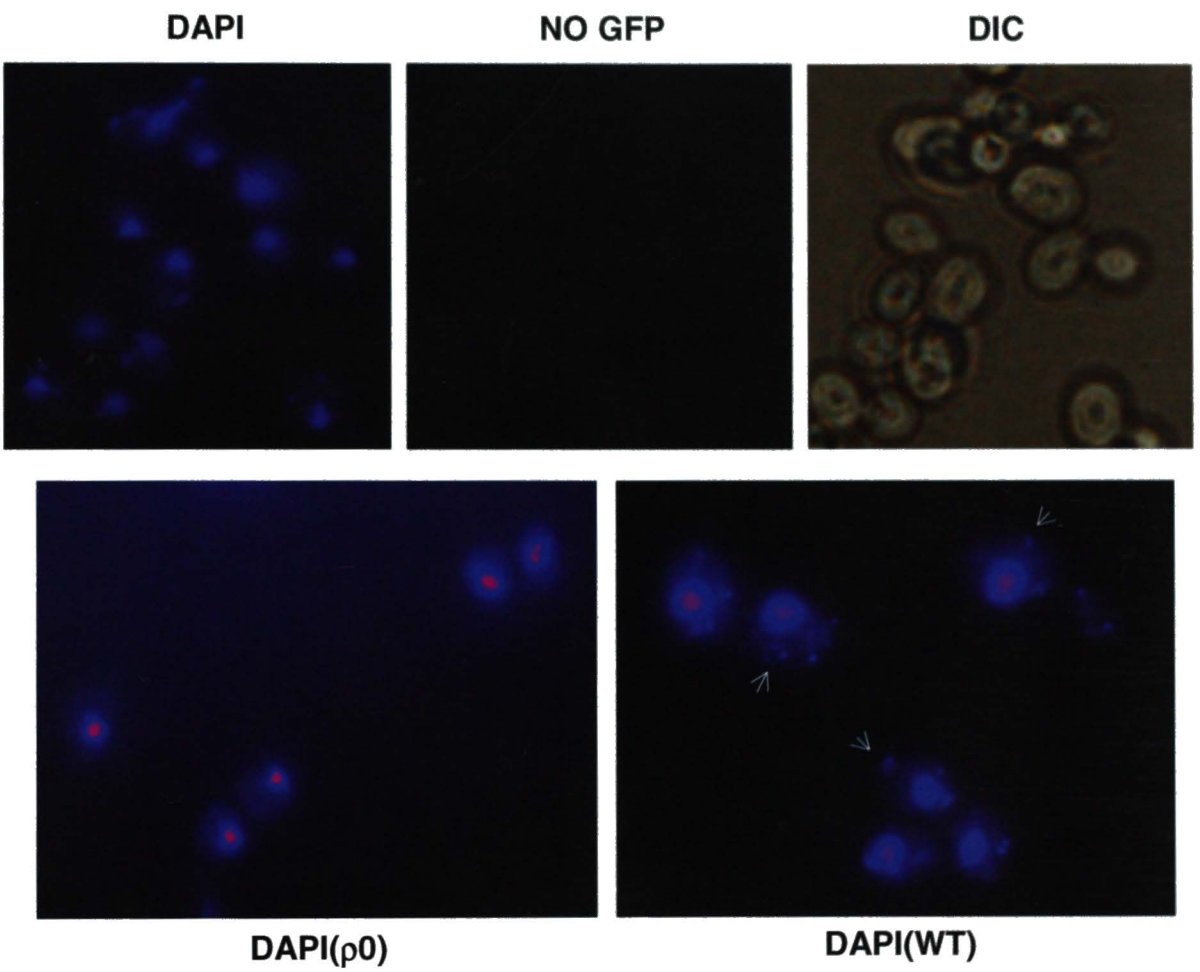


Figure 2. Polη localizes to mitochondria (in addition to nucleus)

The yeast strain BY4741 expressing Polη-GFP fusion protein through P_{GAL1} promoter inserted chromosomally was grown in YPG. When the titers reached ~$1X10^7$ cells/ml, galactose was added at the final concentration of 0.5% and samples were taken at 1.5h, 2h and 3h after addition of galactose. At the time points, the protein localized to mitochondria and nucleus. With live cells it was difficult to get nuclear and mitochondrial signal together in the same focus. Representative images are shown.

Figure 2.

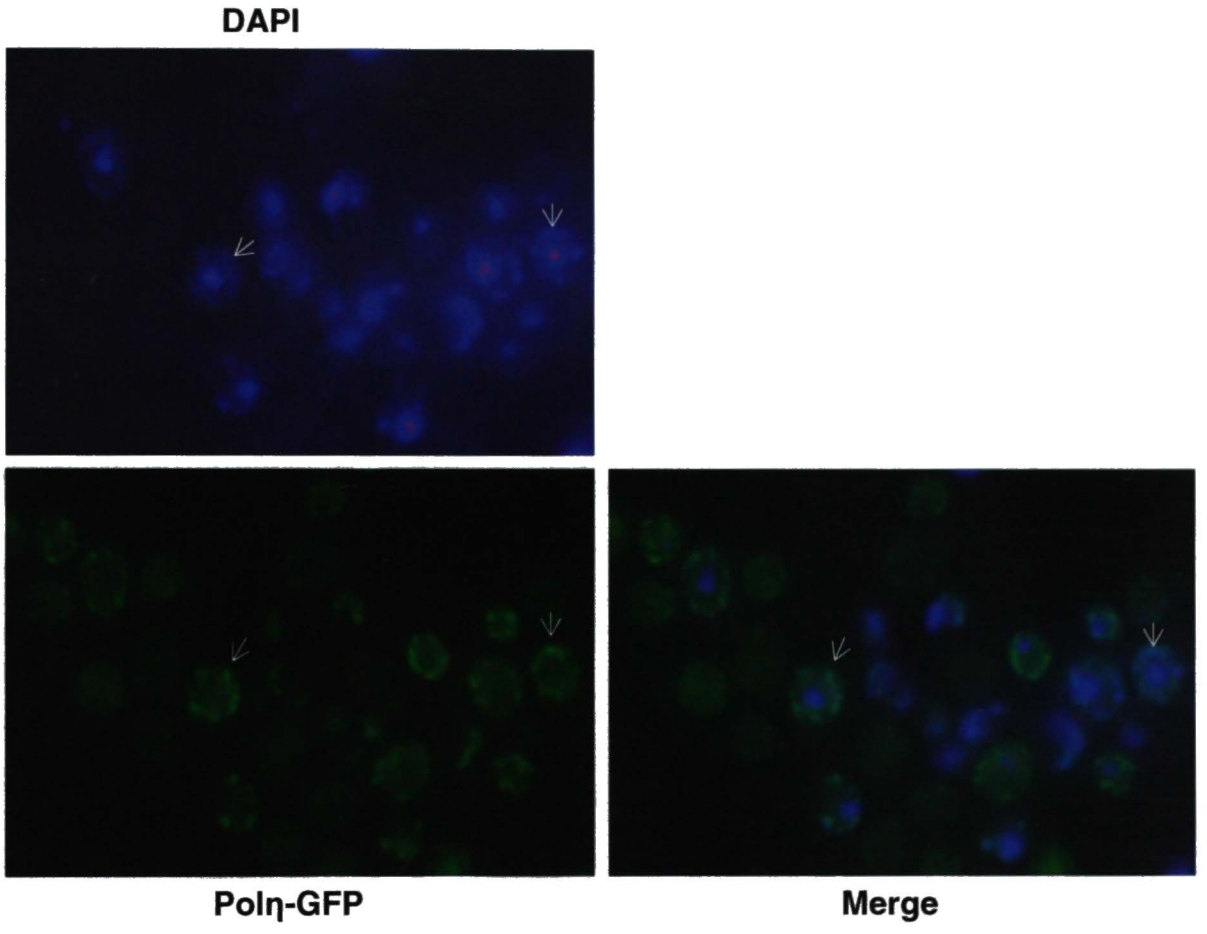


Figure 3. Western blot analysis confirms the presence of Polη in pure mitochondrial extracts A crude mitochondrial pellet was obtained after spheroplasting, homogenizing and differential centrifugations. This crude extract was layered in iodixanol discontinuos density gradient (Optiprep^{TM}) and centrifuged at 80,000g for 3h to yield pure mitochondria. Pol η and Porin (authentic mitochondrial protein) were identified in the purified mitochondrial extract by anti-Myc and anti-Porin antibodies, respectively.

Figure 3.

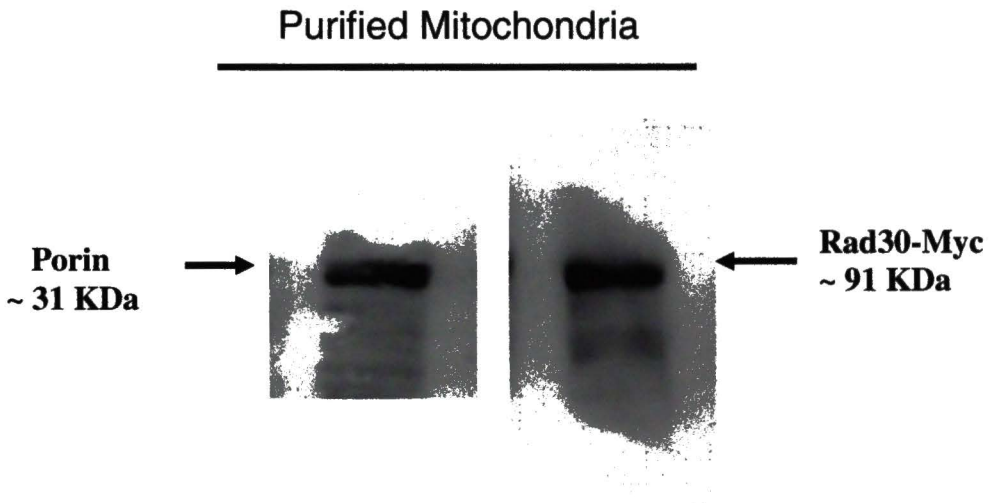


Figure 4. Induction of Polη-GFP at galactose concentrations of 0.5-1.5% Various concentrations (0.5, 1.0, 1.5) of galactose for various time points (1.5h, 2h, 3h) were tried to induce the expression of the fusion protein. At 0.5-1.5% final concentration of galactose, the fusion protein localized to both mitochondria (shown by arrow) and nucleus at the time points.

Figure 4.

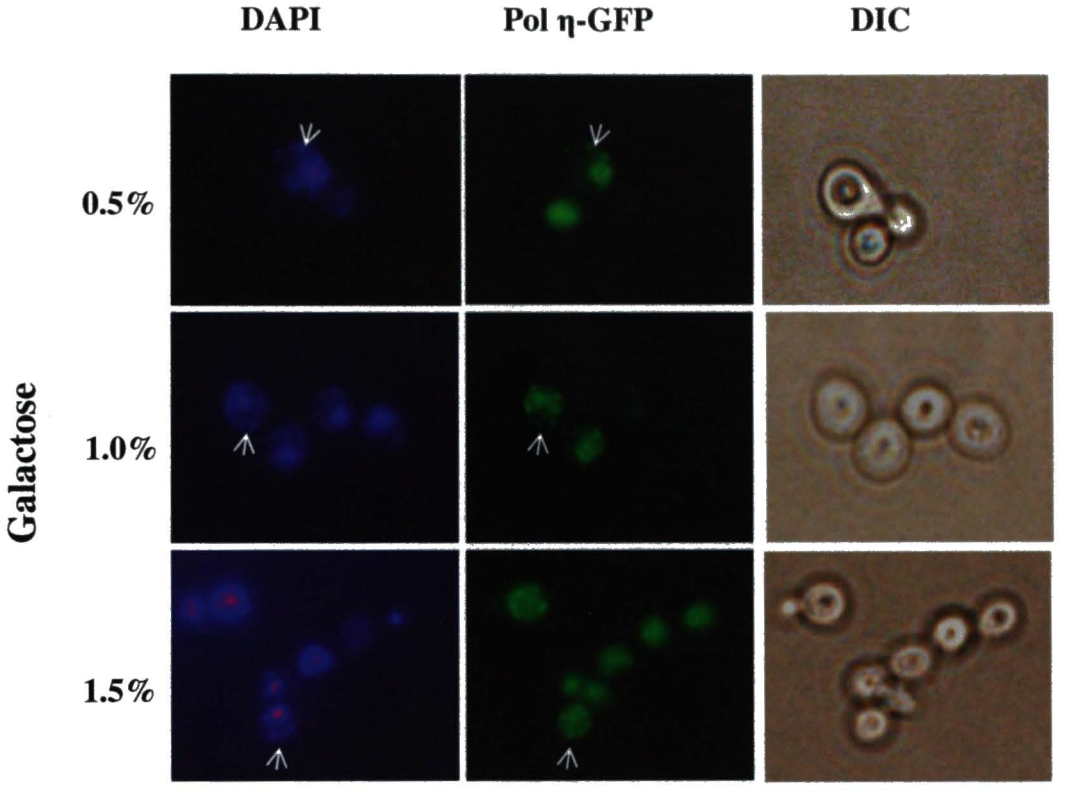


Figure 5. Induction of Pol η-GFP at 2% galactose

At 2% galactose induction, Polη-GFP was predominantly in nucleus. Almost none protein was located in the mitochondria.

Figure 5.

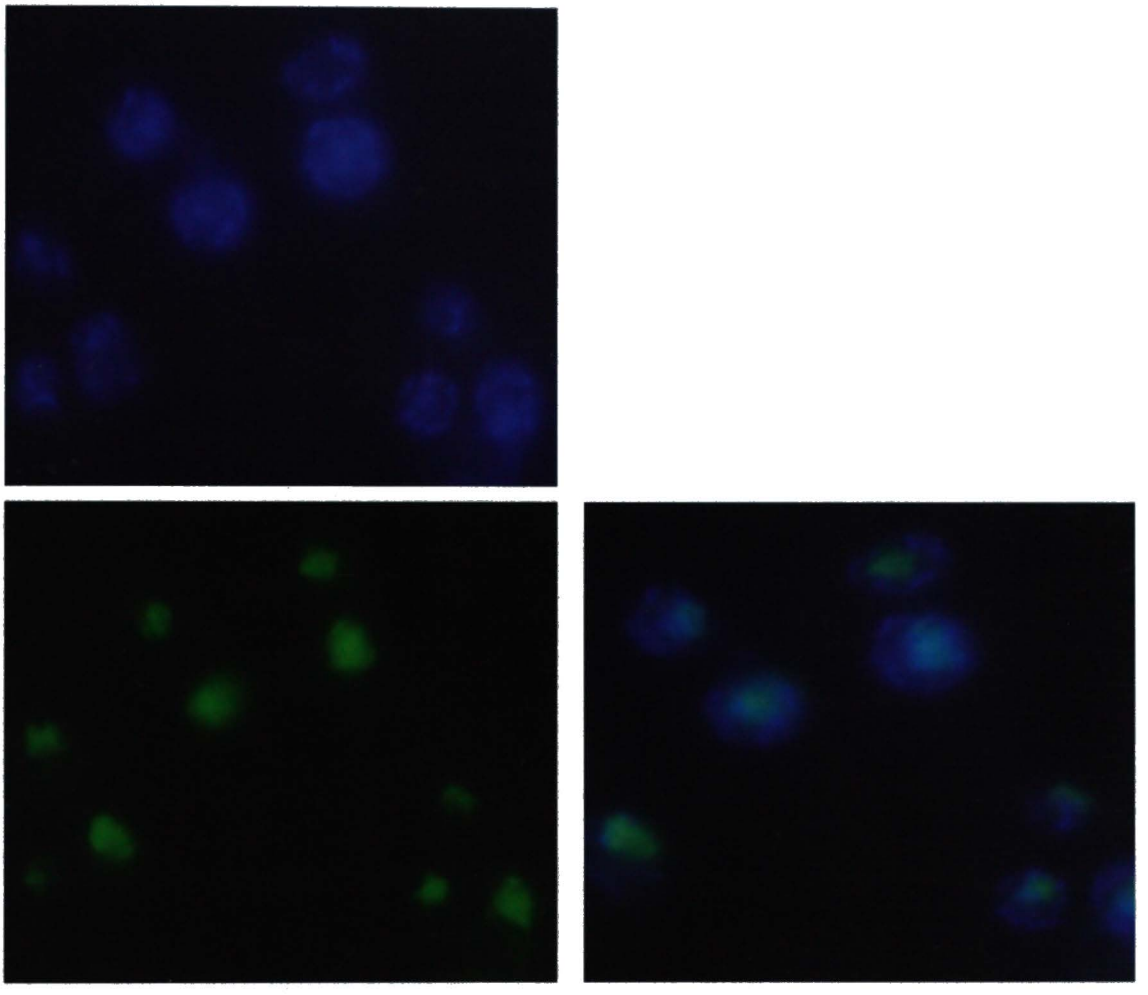


Figure 6. Polη-GFP localizes to both mitochondria and nucleus in G1, early S (20') and late S (40') phases of the cell-cycle. BY4741 strain expressing Polη-GFP was incubated in YPG to reach a titer of $1X10^7$ cells/ml. Cells were treated with α-factor (two aliquots of 5μg/ml each) and 0.5% galactose simultaneously for 2h. G1 arrested samples were taken and remaining culture was washed and resuspended in YPG+0.5% galactose to allow the cells enter S phase in a synchronous manner. Samples were taken at 20' and 40' post G1 release. Representative images are shown.

Figure 6.

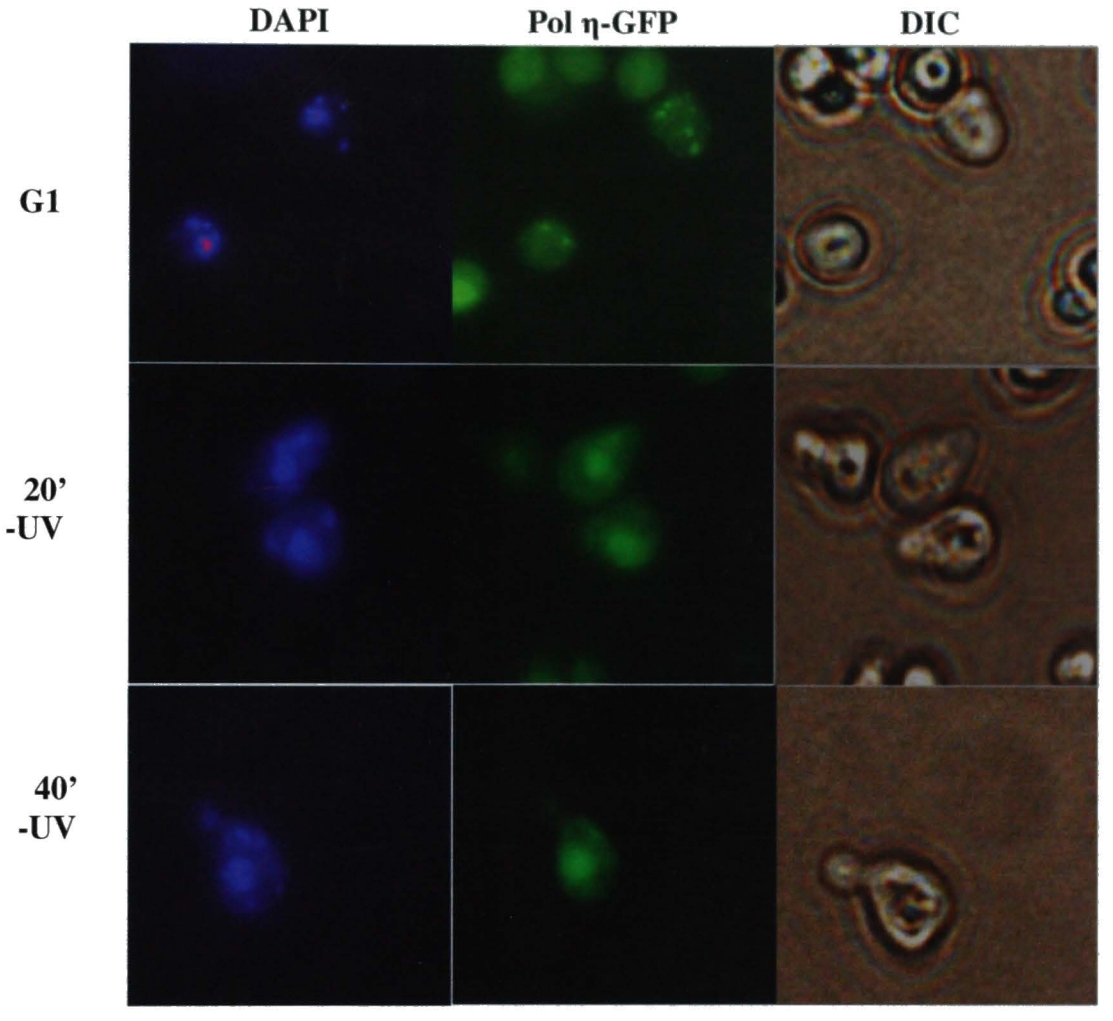
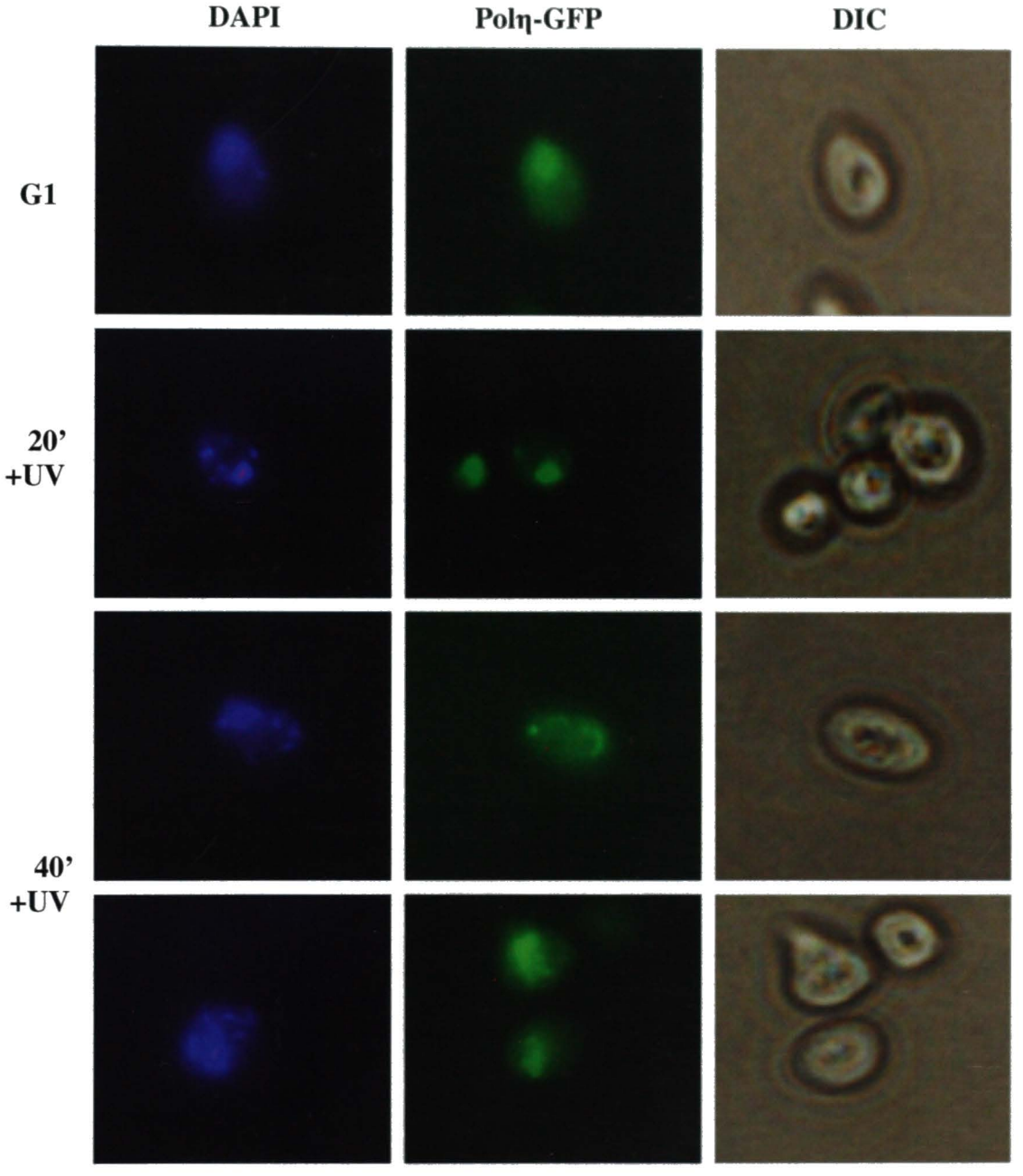


Figure 7. Localization of Polη-GFP is not influenced by UV damage.

BY 4741 strain expressing Polη-GFP was incubated in YPG till a titer of $1X10^7$cells/ml was obtained. Cells were treated with α-factor (two aliquots of 5μg/ml each) and 0.5% galactose for 2h. G1 arrested samples were drawn before washing and suspending the cell pellet in distilled water to UV irradiate at $80J/m^2$.Cells were pelleted and resuspended in fresh YPG + 0.5% galactose. Subsequent samples were taken at 20' and 40' post G1 release and UV treatment. Representative images are shown.

Figure 7.



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CHAPTER IV

CONCLUSIONS

The variant form of Xeroderma pigmentosum (XP-V) results from mutations in hRAD30A or XP-V gene which encodes for human Pol η. XP-V patients are highly sensitive to sunlight and suffer from high incidence of skin cancers. Of the eukaryotic DNA polymerases, only human Pol eta (hPolη) and its yeast counterpart (Rad30) have the unique ability to replicate DNA containing a cis-syn thymine-thymine (T-T) dimer. Importantly, opposite an undamaged template, both human and yeast polymerase η misincorporate at a frequency as high as 1/100 to 1/1000. This low fidelity of the polymerase derives from its flexible active site that renders the enzyme more tolerant of geometric distortions in DNA and enables it to synthesize DNA past a T-T dimer. The purpose of this dissertation is to study how error-prone DNA polymerase Pol η is regulated in *Saccharomyces cerevisiae.* Since TLS polymerases are highly conserved from prokaryotes to mammals, such a study may give insight on regulation of its human counterpart.

We started out by analyzing the published UV inducibility of the *RAD30* transcript in more details. We found that RAD30 transcript increased after UV damage. This upregulation of the message was observed even in rad9 and rad17 mutants which affect damage sensing and signal generation, respectively, within the checkpoint network.

Next, we compared if the protein levels mirrored the changes in transcript abundance. In the absence of reliable commercial antibodies against Rad30p, we tagged chromosomal RAD30 C-terminally with the 13xMyc-epitope. It was ensured that the tagged version of the protein was fully functional like wild-type protein. Although, Rad30 protein levels were readily detectable in unirradiated yeast cells in all cell cycle stages as well as in stationary phase cells, there was no change in protein levels in response to UV induced DNA damage and to other stresses such as hydroxyurea or camptothecin. Also, the protein level does not vary during cell cycle progression. We reasoned that protein turnover rate may have accelerated following UV irradiation and an elevated transcript level may be required to maintain a constant protein level. However, after inhibiting *de novo* protein synthesis by cycloheximide, there was no reduction in the protein levels after UV treatment. The protein appeared to be very stable and the results were also verified by using Rad30-TAP, another tagged version.

We hypothesized that in the absence of *de novo* protein synthesis, covalent modifications of Polη may be enabling it to act in a time efficient manner by either influencing its interaction with protein partner(s) or controlling its access to template. As a matter of fact, we found a fraction of Rad30 to be monoubiquitinated. Interestingly, ubiquitination was still detectable in deletion mutants of Rad6, Rad18, Rad5 and Doa1, proteins that contribute to PCNA monoubiquitination or polyubiquitination (Rad5) following UV or MMS treatment.

We observed an increase of ubiquitination during G1 and, as expected, this ubiquitination level diminished during S phase reentry. Importantly, this decrease in

modification is accelerated after UV treatment, a UV specific regulation. A balance of ubiquitination and deubiquitination processes may result in the observed steady-state level of Rad30 ubiquitination. We think that a high state of ubiquitination in G1 probably precludes interaction of Polη with ubiquitinated PCNA due to its lower affinity for the latter. As cells enter S phase, deubiquitination is stimulated resulting in reduced amounts of ubiquitinated polymerase and enabling recruitment of Polη when translesion synthesis is actually required.

The last part of chapter II involves characterization of a mutant Rad30p, defective in ubiquitination. This mutant allele of *RAD30* had a mutation in the most C-terminal conserved UBZ residue (L577Q). As a result of this mutation, the extent of Rad30 ubiquitination in asynchronized cultures was lower than the wild-type levels, however, during G1 synchronization, wild-type levels of ubiquitination were still achieved with some delay. Deubiquitination was further accelerated during S phase reentry as compared to wild-type protein. We concluded that mutation in UBZ domain may inhibit ubiquitination but not deubiquitination that is active during cell-cycle reentry and further activated in UV-treated cells. Moreover, such a mutant protein showed a complete loss of its ability to interact with ubiquitinated substrates. As expected, compared to wild-type protein, the mutant allele rendered the strain more UV sensitive and mutagenic. The degree of UV sensitivity and mutagenicity were almost comparable to a complete deletion mutant. This result emphasizes the importance of UBZ domain in enabling Polη interaction with ubiquitinated PCNA or unknown protein partners and its critical role for better UV survival and avoidance of mutations.

Now, we knew that Polη gets monoubiquitinated, we were interested to investigate if it was a signal for localization to mitochondria and, furthermore, if varying degree of ubiquitination influenced the localization of the polymerase in a cell-cycle dependent manner. As described in chapter III, Polη studied as Polη-GFP protein was indeed located in mitochondria by immunofluorescence studies. This result was confirmed by detecting Pol η protein in purified mitochondrial extract by Western blot analysis. The mitochondrial localization of the polymerase was not affected by cell-cycle stage or UV treatment. Within the scope of this study, we found that the fusion protein localized to mitochondria in addition to nucleus in G1, early S and late S phases of the cell-cycle in the presence or absence of UV irradiation.

FUTURE DIRECTIONS

For this dissertation work, I studied Polη protein regulation in great details. However, why do cells bother to increase *RAD30* message after UV if it does not get translated? It will be interesting to study *RAD30* transcript regulation. Is an increase in transcript after UV a direct result of increased *de novo* transcription or message stability or a combination of both. This can be determined by using a transcription inhibitor such as lomofungin.. If the transcript is induced by UV, it will be important to determine UV-inducible sequence(s) in the promoter region.

We demonstrated that the balance between ubiquitination and deubiquitination activities determine the state of ubiquitination of Polη in G1 and S phases. It turned out that the polymerase may be self-ubiquitinated. Future studies may be carried out to look for deubiquitinating enzymes. For *S.cerevisiae*, a knock out library of non-essential genes is commercially available which can be used to this end.

During our studies with the mutant allele of Polη, we found that mutant protein had a dominant negative effect when crossed with wild-type protein in a diploid strain. How the mutant allele affects the activity of the wild-type Polη is worth exploring. Are there different interacting partners for both versions of the protein? A yeast two hybrid system would be an ideal system to look for interacting partners and the findings can be further confirmed by co-immunoprecipitation.

We did not see any difference in localization of fusion protein Polη-GFP in various phases of the cell-cycle and after UV treatment. However, it is still possible that there is

an increase in chromatin bound fractions of the polymerase in S phase and/or after UV treatment. This experiment would require separation of pure nuclei. Chromatin fractions can be extracted by treatment of pure nuclear extract with 0.01% (v/v) Triton X-100.

Is localization of the mutant protein (missense mutation in UBZ domain) different from the wild-type protein since the mutant protein can not interact with ubiquitinated PCNA. There is a possibility that the mutant version may still localize to the nucleus and chromatin due to its intact PIP motif. The mitochondrial localization of the protein may not be affected by this mutation.

It would be really interesting to determine, if any, mitochondrial phenotype from loss of Polη. There are various mutation systems designed to determine different types of mutations in mtDNA like frameshift or point mutations, and assessing respiration deficient or petites colonies resulting due to major deletions in mtDNA.

Last but not the least, we can determine the status of ubiquitination of Rad30 in mitochondria and nucleus by immunoprecipitating the proteins with Ubiquitin antibody from pure mitochondrial and nuclear fractions followed by Western blotting with anti-Myc antibody.

This dissertation work was limited to a simple yet sophisticated unicellular eukaryote, Saccharomyces cerevisiae. Future studies in human cell lines should corroborate these findings.

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