Role of TLS Polymerases in Replication of DNA Lesions in Human Cells

by

Juan Antonio Conde

2016

The Role of TLS Polymerases in Replication of DNA Lesions in Human Cells

Committee for Juan Conde Certifies that this is the approved version of the following dissertation:

Role of TLS Polymerases in Replication of DNA Lesions in Human Cells

Committee:

Satya Prakash, Ph.D.

Louise Prakash, Ph.D.

James Lee, Ph.D.

John Papaconstantinou, Ph.D.

Robert Fox, Ph.D.

Maurice Willis, M.D.

Dean, Graduate School

Role of TLS Polymerases in Replication of DNA Lesions in Human Cells

by

Juan Antonio Conde, B.S., M.S.

Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas Medical Branch

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas Medical Branch

May, 2016

Dedication

To

The loving memory of my mother Hilda Guadalupe Fabela

And my father Jose Antonio Conde

Acknowledgements

I would like to thank my mentors, Louise and Satya Prakash, for welcoming into their lab and providing strong support and guidance during my training. I would like to thank Satya, his genius and creativity enlightened me and allowed me to expand upon my knowledge of DNA repair. I would like to express my gratitude towards Louise, her drive for perfection and persistence for quality has taught me the importance for structure and detail in research. Her kindness for accepting a nontraditional student and the help that Louise and Satya provided with all the obstacles during my PhD training will not be forgotten. I would like to thank Hoon for his support, he has been an invaluable friend and my unofficial mentor, his help in the laboratory went far and beyond what was required for a postdoc. I would like to thank the entire Prakash lab, Jayati, Debashree, Jason, Bob, Lora, Roland, and Karan for their support and friendship.

I would like to thank the members of my dissertation committee: James Lee, Robert Fox, John Papaconstantinou, Maurice Willis, and past member Sarita Sastry, for their support and suggestions during my research years.

I would like to thank my friends who have been with me throughout this journey. Matt, thank you for being there during my entire research years, you have been my rock of support and my invaluable friend. Stephan and Zina, thank you my friends for all the fun times that took my mind from research. Brian thank you for what you taught me about myself, all the time in the world would not be enough to share with you. Aaron, my favorite Sith lord and Pokemon genius, you have been the best of friends and partner in crime these last few years. Dan, my “bro”, your friendship and puns will never be

forgotten. And to my other friends, Tyler and Ashley, Jordan and Bethany, thank you for making my doctoral years an unforgettable experience.

Finally, I would not have been able to accomplish my PhD without the help and love from my family. Rosy, Luis, Daisy, and Teto thank you for making Galveston a second home for me. To my father, who has worked so hard to support us, my mother, who cares from me from the other side, my sister and her family, and to my younger brother, who is always there for me, this thesis is as much yours as it is mine.

Role of TLS Polymerases in Replication of DNA Lesions in Human Cells

Publication No._____

Juan Antonio Conde, Ph.D.

The University of Texas Medical Branch, 2016

Supervisors: Louise Prakash and Satya Prakash

Cells are constantly exposed to numerous genotoxic lesions that inhibit replication. The inability of cells to correctly repair these lesions results in mutations or chromosomal aberrations that threaten the integrity of the genome. DNA damage tolerance mechanisms, including translesion synthesis (TLS), alleviate this block at the expense of increasing mutagenesis.

Minor groove DNA lesions result from lipid peroxidation or exposure to environmental pollutants. Prevalent among these lesions are those produced by tobacco products, particularly benzo(a)pyrene-diolepoxide (BPDE), a polycyclic aromatic hydrocarbon strongly associated with carcinogenesis, particularly lung cancer. Alkylating lesions result from exposures to endogenous methylating agents and naturally occurring methyl halides. They might interfere with base pairing and are cytotoxic. The long-term goal is to understand the mechanisms by which replication through such ubiquitous lesions occurs in human cells.

Tumorigenesis is a multistep process associated with accumulation of mutations. Understanding of the biochemical basis of lesion bypass and the role of TLS polymerases will result in insights on how human cells handle exposure to environmental carcinogens and how the TLS processes contribute to cancer avoidance or to cancer risk.

TABLE OF CONTENTS

List of Tables

List of Figures

List of Abbreviations

[1-MeA	1-methyl adenine
3-MeA	3-methyl adenine
AP	apurinic site
ATR	Ataxia Telangiectasia and Rad3 related
BαP	Benzo(α)pyrene
BBMEF	big blue mouse embryonic fibroblast
BER	base excision repair
BPDE	benzo(α)pyrene diol epoxide
Cat	catalytic
CPD	cyclobutane pyrimidine dimer
DMEM	Dulbecco's modified eagle medium
DPBS	Dulbecco's phosphate buffered saline
DSB	double stranded break
FBS	fetal bovine serum
GSBS	Graduate School of Biomedical Science
HBSS	Hank's buffered saline solution
HR	homologous recombination
Kras	Kirsten's rat sarcoma viral oncogene homolog
MMS	methyl methanesulfonate
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N^2-γHoPdG	Hydroxy-1,N2-propano-2 deoxyguanosine

NER	nucleotide excision repair
NHEJ	non-homologous end joining
ORF	Open Reading Frame
PAD	polymerase associated domain
PAH	polycyclic aromatic hydrocarbon
PCNA	proliferating nuclear cell antigen
PKC	protein kinase C
PRR	post replicative repair
RPA	replication protein A
SAM	S-adenosyl methionine
Tg	thymine glycol
TDC	Thesis and Dissertation Coordinator
TLS	translesion DNA synthesis
UBM/UBZ	Ubiquitin binding motif
UTMB	University of Texas Medical Branch
UV	ultraviolet
XPV	Xeroderma pigmentosum variant]

Chapter 1 Introduction

TRANSLESION DNA SYNTHESIS

Introduction

DNA repair processes are crucial for maintaining the integrity of the genome. The consequence of non-functional repair systems can result in mutagenesis, carcinogenesis, and cell death. Cells are constantly exposed to genotoxic lesions that inhibit replication. DNA is highly reactive and is under constant attack by endogenous and exogenous sources resulting in ~100,000 different DNA lesions per day (70, 71). This high number of lesions means that the cell will at some point encounter a situation where its DNA repair processes have not removed a DNA replication block, which can have dire consequences to the cell. The inability to replicate across a lesion leads to accumulation of stalled replication forks, which can generate double stranded breaks (DSBs) and lead to genomic instability. The presence of DSBs increases the level of chromosomal translocations in cells (64, 198). DNA damage tolerance mechanisms, including translesion synthesis (TLS) allow for replication of DNA containing lesions and alleviate this block, but do so at the expense of increasing mutagenesis (15, 16, 17).

Experiments with excision defective mutants (uvrA, uvrB, or uvrC) of *Escherichia* coli exposed to ultraviolet (UV) irradiation have shown that UV delays DNA replication, but cells were eventually able to replicate DNA even in the presence of UV induced cyclobutane pyrimidine dimers (CPDs) (200). This replication across UV damage was referred to as post replication repair (PRR) and its mechanism was poorly understood at the time. It was believed that recombination played an important role in PRR since excision (uvrA) and recombination (recA) defective mutants were more sensitive than excision defective mutants alone and were killed by a single pyrimidine lesion in the cell

(200). However, later studies demonstrated that protein synthesis was required for PRR and the inhibition of protein synthesis by chloramphenicol in bacterial cells (199) or hydroxyurea in human lymphoma cells resulted in inhibition of PRR and mutagenesis (201). Furthermore, labeling of DNA with bromouracil revealed that PRR in lymphoma cells required de novo DNA synthesis (201). The induction of this repair process required the recA and lexA genes and UV mutability was eliminated by mutations in either of these genes. However, although recombination as demonstrated by Howard-Flanders was an important contributor to PRR in an error-free manner, a portion of mutagenic PRR was independent of recombination as demonstrated from recombination defective mutants ($recA^{+}recB^{-}recF^{-}$) (199). Experiments with the ϕ174 by Radman, demonstrated the inability of the phage to replicate after infection of wt *E. coli* but able to replicate after infection on *E. coli* that were irradiated with UV and induced for a PRR response (199). However, the failure to purify a protein with polymerase activity led to the conclusion that an inducible factor was modifying the activity of the DNA replicative polymerases.

In 1999, it was discovered that one of these inducible mutagenesis proteins, Rad30, a protein involved in UV mutagenesis in *Saccharomyces cerevisiae*, was a novel DNA polymerase, which could perform accurate lesion bypass across a CPD (16). This study served to illuminate several aspects of mutagenesis and the rationale for mutagenesis changed from the idea that mutagenesis proteins regulate the activities of the replicative DNA polymerases to the novel concept that these mutagenesis proteins were themselves in fact actual DNA polymerases (16,43). These TLS polymerases have recently been characterized and proof has emerged that they are not error-prone when replicating a respective cognate lesion, in other words they have distinct biological properties that allows error-free lesion bypass. A paradigm has emerged that these polymerases are regulated during specific DNA damage and their deregulation is implicated with

mutations, genomic instability, and cancer (35). The idea is that lesion bypass by TLS polymerases, on a non-cognate DNA lesion caused by a carcinogen, results in a particular mutation signature. Carcinogenesis studies have revealed that there exists a specific mutation fingerprint for each carcinogen (14, 22, 24, 33, 34). The better characterized models are that of UV's link with skin cancer and that of tobacco smoke's link with lung cancer. Studies have revealed that deficiency of polymerase η is associated with the xeroderma pigmentosum variant (XPV) and is correlated with skin cancer (16, 45-48). Importantly, biochemical studies have revealed that lack of polymerase η results in switching to error-prone polymerases resulting in the mutation signature that is associated with UV carcinogenesis (49).

General Mechanism

The cell has strategies to tolerate DNA replication blocking lesions. At sites of DNA damage the cell utilizes a number of PRR mechanisms, also known as damage tolerance or damage avoidance mechanisms, to replicate DNA. The cell can directly avoid the damaged site by utilizing the nascent daughter strand as the template for replication or could utilize homologous recombination to repair a gap in the newly synthesized strand (Figure 1). These damage avoidance mechanisms utilize the alternate undamaged DNA strand and are error-free, however they can be disruptive and time consuming and a mistake might lead to a high probability of gene rearrangements (57).

TLS on the other hand utilizes specialized polymerases to replicate across the damaged site. When the replication fork stalls, specialized polymerases are recruited to the lesion site, a polymerase switch between the replicative and TLS pols occurs which allows insertion across the damage site. Depending on the lesion, the TLS pol that is recruited might extend various nucleotides after initially inserting across the lesion, or a second

TLS Pol might be recruited to do the extension. Once replication has occurred past the lesion, a switch back to the replicative Pol occurs and replication continue (72).

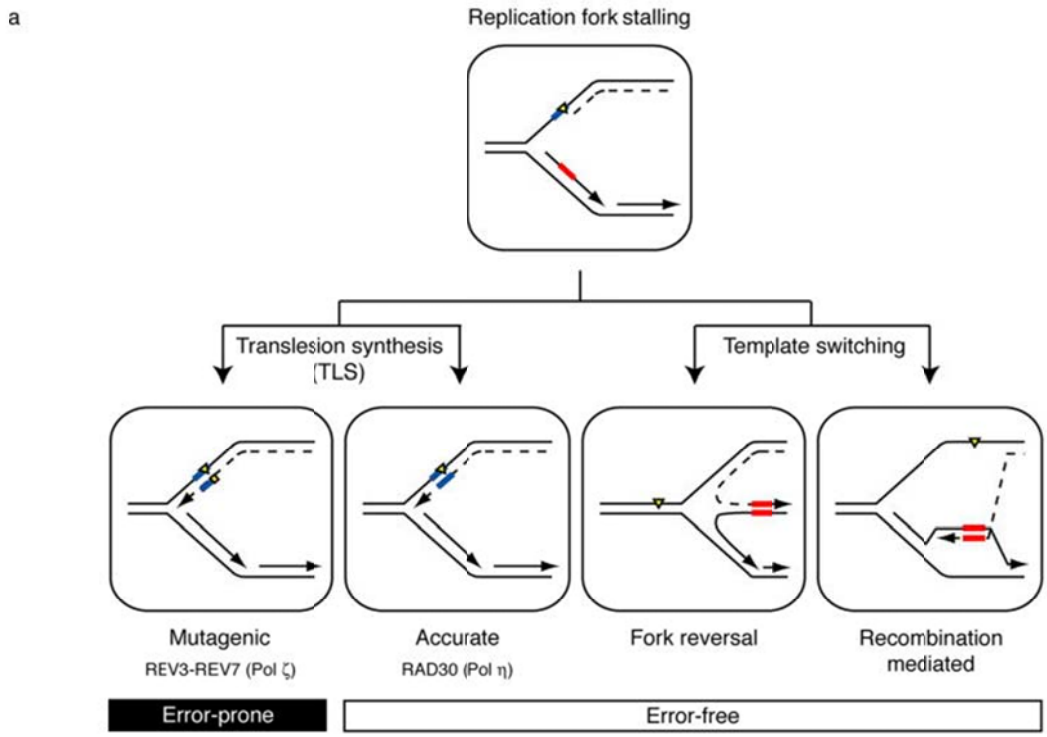


Figure 1. Overview of DNA damage tolerance pathways

Lesions in the DNA template (yellow triangle) block processive DNA replication (dashed line). DNA damage tolerance mechanisms allow bypass of replication-blocking lesions by replicating over the damaged DNA (translesion synthesis, left) or using the undamaged sister chromatid (template switching, right). Template switching involves a structural rearrangement of the replication fork for which two models have been proposed. Fork reversal involves the formation of a four-way junction or "chicken-foot" intermediate (left) while recombination-mediated template switching involves D-loop formation and strand invasion (right). Templates used to bypass lesions and their complimentary sequences are boxed in blue for translesion synthesis and red for template switching. The mutagenic nature of the process is indicated.

TLS Polymerases

The human genome is known to encode 14 different DNA polymerases, nine of which are specialized and involved in TLS. The largest DNA polymerase family is the Y-family containing Pols η, ι κ, and Rev1. Other Pols that have been shown to be involved

in TLS are Pol ζ from the B family, Pols λ and μ from the X family, and Pols θ and ν from the A family (73, 74). Although they possess a similar basic structure, they have a large degree of functional divergence and they play different roles depending on the type of lesion encountered. They possess some functional similarities, they are error prone when copying undamaged DNA, have low catalytic efficiency, and are non-processive compared with the replicative pols. They also possess the remarkable ability to bypass different types of DNA lesions (35, 74). The conservation of these TLS polymerases in all domains of life and expansion in higher organisms points to their critical role in the maintenance of the genome (75).

Crystal structures of the various TLS polymerases reveal similar structural features. The TLS Pols maintain the overall right handed topology as the replicative polymerases with a palm, fingers, and thumb subdomains. The palm domain is the catalytic center and contains invariant acidic residues necessary for catalysis indicative of a conserved catalytic mechanism (35). The fingers and thumb subdomain are strikingly different from the replicative pols. In the replicative pols two α-helices form a large tight closing structure with the incoming nucleotide resulting in the proper geometric alignment of the catalytic residues for the nucleotidyl transfer reaction. In contrast the TLS pols contain small, stubby finger and thumb subdomains. The TLS pols, because of this, have lower processivities and fidelities. TLS pols do not possess 3' → 5' exonuclease activity. In addition, they also contain a polymerase associated domain (PAD) which increases the potential DNA binding surface area from ~600-700 Å to ~1000-1100 Å comparable to the replicative pols (35). The active sites of the TLS pols vary tremendously from the replicative pols. They are more open and sterically less constrained to help accommodate various DNA lesions. Additionally, the TLS pols possess unique structural features suggestive of their evolutionary specialization to bypass various types of DNA damage (35).

ALKYLATING AGENTS

Reactivity of Alkylating Agents

Alkylating agents are an ubiquitous family of reactive electrophiles that react with the ring nitrogens and exocyclic oxygen atoms of nucleic acid bases to generate a variety of covalent adducts. The types of DNA lesions generated by these agents depend on several factors, among these are the type of alkylating group, the number of reactive sites within the alkylating agent, the reaction mechanism, and the type of nucleic acid substrate (Figure 2). Alkylating agents are affected by the nucleophicity of the ring substituent. Because of this, the major adduct formed by monofunctional agents is at the N^7 position of guanine accounting for ~60-80% of the total alkylation lesions in DNA (76, 77). The 7-Methyl guanine (7-MeG) lesion is relatively innocuous, it is not reactive, mutagenic, or cytotoxic by itself, but can result in spontaneous depurination of the base generating an apurinic site (AP), which is toxic and mutagenic. S_N1 alkylating groups react readily with oxygens to generate the lesion O-6Methyl guanine which mispairs with thymine during replication and causes mutagenic and cytotoxic events. O-alkyl lesions, however, are generated far less frequently than N-alkyl lesions. Ethylating lesions are known to be more mutagenic than methylating lesions. Bifunctional alkylating groups react similarly to monofunctional agents, but because of their two reactive moieties, they can lead to the formation of interstrand crosslinks (76, 77).

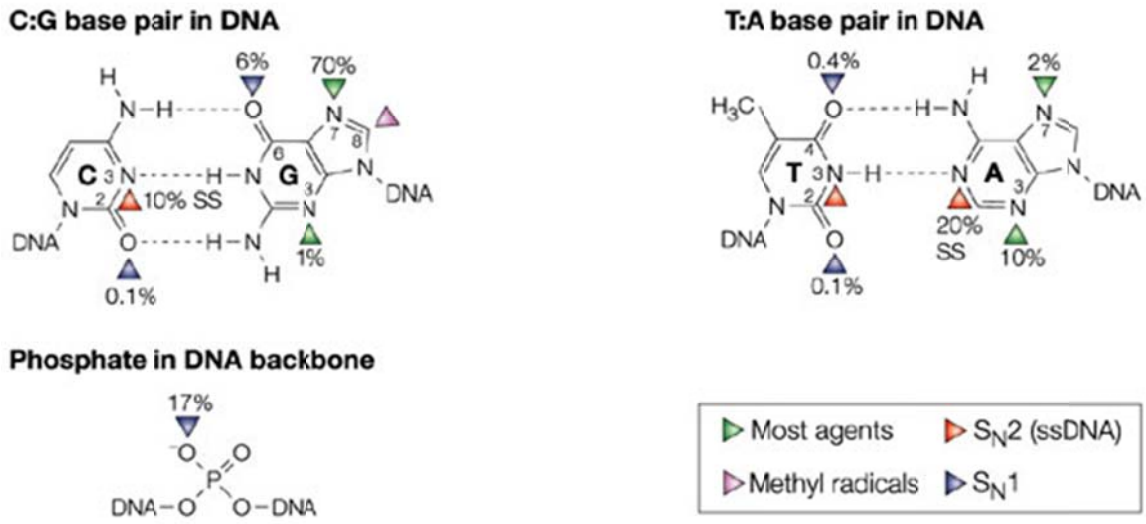


Figure 2. Sites of methylation on the bases and sugar–phosphate backbone of DNA

Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Molecular Cell Biology] (76), copyright (2004).

1-Methyl Adenine Lesion

S_N2 alkylating agents generate 7-MeG and additionally can react with the N^1 and N^3 group of adenine, as well as the N^3 group of cytosine. The 3-Methyl adenine (3-MeA) lesion accounts for ~10-20% of the total methyl adducts, is cytotoxic, and can inhibit DNA synthesis. The N^1 group of adenine is more nucleophilic than adenine's N^3 group, however, it is sterically hindered in double stranded DNA because the N^1 site is involved in Watson-Crick (WC) hydrogen bonding (78, 79). In single stranded DNA, the 1-Methyl adenine (1-MeA) lesion is readily observed and accounts for 15-20% of the total methyl adducts with 3-MeA accounting for only ~4% of adducts. The 1-MeA lesion strongly blocks replication but is weakly mutagenic, causing A→ T transversions in less than 1% of lesions (80). The non-reactivity of 7-MeG and the high amount of 1-MeA generated make the 1-MeA lesion important in ssDNA and in dsDNA when the N^1 site is transiently exposed during replication, recombination, or transcription.

The 1-MeA lesion prevents the N^1 site of adenine from WC hydrogen bonding and affects the base pair structure of the DNA double helix. The 1-MeA lesion switches the base orientation from the *anti* to a *syn* orientation with respect to their sugar rings. This forces 1-MeA to adopt Hoogsteen base pairing but retain pairing specificity with thymine and stacking interactions within the double helix, causing little perturbation in the sugar pucker or the DNA double helix structure. Because of this, 1-MeA is a very stable adduct comparable to adenine with very little of the expected Dimroth rearrangement occurring (80, 81).

The 1-MeA lesion can be generated by various endogenous and exogenous sources of alkylating agents and has been detected both *in vitro* (84-87) and *in vivo* (88-90, 81). S-adenosyl methionine (SAM) is a common cosubstrate of methyl group transfers. Because of its high transfer potential, it spontaneously methylates cellular nucleic acids in a similar manner as methyl methanesulfonate (MMS) through an S_N2 mechanism (91, 92). The intracellular concentration of $4x10^5$ M SAM is enough to create mutagenic adducts (91, 93) and it has been estimated that this concentration of SAM would be equivalent to the continuous exposure of a cell to ~20nM of MMS (91). Other metabolic products that create 1-MeA include betain or choline (94) and nitrosation products of cellular amines (95). The most abundant methylating agents are the alkyl halogens produced from decaying vegetation (96). Other exogenous sources include methyl nitrates from tobacco smoke, combustion products, and byproducts of industrial production of alkyl derivatives (77).

Repair of 1-MeA Lesion

Alkylating lesions have been deemed a threat to human health through their cytotoxic, teratogenic, and carcinogenic properties (97). The 1-MeA lesion is a strong inhibitor to

replication because it impedes WC hydrogen bonding and the action of replicative DNA polymerases. Estimates of overall lesion frequency are ~1.6 lesions/kilobase (82, 83). This has been demonstrated *in vitro* (85) and in reactivation experiments of a phage model system after MMS damage (98). Organisms possess several repair systems for alkylation damage. 1-MeA is directly removed by the action of a dioxygenase, AlkB in *E. Coli,* and ABH2/ABH3 in mammals. These enzymes utilize α-ketogluterate and Fe^{2+} for oxidative methyl transfer, resulting in the release of formaldehyde and the modified base (99). The importance of this direct reversal is demonstrated by the increase in sensitivity to MMS in $AlkB^-$ cells (100) as well as a small increase in mutagenesis (98). Similarly, mouse models lacking the AlkB homolog ABH2 demonstrated accumulated 1-MeA in liver genomic DNA and mouse embryonic fibroblasts derived from $ABH2^{-/-}$ mice are hypersensitive to MMS damage (101). A mutagenesis study of the individual lesions created by MMS again demonstrated that these lesions inhibit replication. Interestingly, activation of the SOS inducible repair system in *E. coli* relieved the block created by MMS, suggesting that TLS plays an important in role in alleviating methylation induced DNA damage (81). Mutational analysis of 1-MeA in $AlkB^-$ and SOS induced cells revealed low mutagenicity, suggesting that TLS is error free across 1-MeA (81). Further evidences for a role of TLS in alleviating the block of replication from alkylating damage are the degradation of HLTF and increase interaction of SHPRH with TLS polymerases by MMS treatment (102) and the increase in sensitivity to MMS by deletion of Rev3 (103-106). Deletion of Dot1 in *S. cerevisiae*, a protein involved in the checkpoint response, increases Rev1/Pol ζ mediated tolerance to MMS (107) and increases Rev1 replication foci (108).

The role of TLS polymerases in the bypass of 1-MeA has not been fully elucidated. Greater understanding of the biological factors activated in response to alkylation damage might lead to strategies for cancer prevention and novel therapeutics.

MINOR GROOVE LESIONS

Reactivity of Guanine

The electronic structures of the purine-pyrimidine rings of DNA confer their respective reactivity properties. Guanine and cytosine are very adept at complement stabilization due to their resonance energies. The purines are excellent electron donors with guanine having the highest nucleophilic reactivity of ring nitrogens. This makes guanine highly reactive to electrophiles. Reactions with the N^2 amino group of guanine are very favorable not only due to the electronic properties of the NH_2 group but also because any bulky adduct can then be accommodated into the minor groove of DNA allowing for minimal steric clashes and disruption of the DNA backbone (Figure 3; Figure 4) (4, 5). Minor groove DNA lesions at the N^2 position of guanine have been documented from endogenous sources such as lipid peroxidation (6-8), oxidation with glucose intermediates (9-11), oxyradicals (6-8) and estrogen (12-13); and exogenous sources such as polycyclic aromatic hydrocarbons (PAHs) (14).

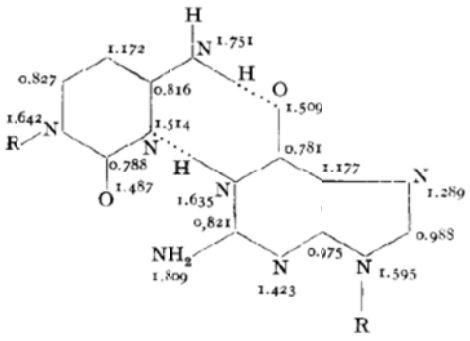


Figure 3. Electrical charges of guanine cytosine pair

Reprinted by permission from Macmillan Publishers Ltd: [Biochimica et Biophysica Acta] (4), copyright (1959).

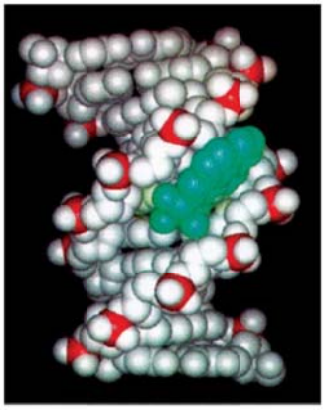


Figure 4. Minor groove lesion

Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Cancer] (63), copyright (2004).

Persistent Minor Groove DNA Lesions

NMR structural studies of a minor groove lesion with the (+) anti-benzo(α)pyrene diol epoxide-dG (BPDE) revealed minimal perturbation of the overall structure. The benzo(α)pyrene ring intercalates into the DNA helix with displacement of the pyrenyl ring into the minor groove of DNA with a plane parallel to the helix axis. The aromatic ring system provides base stacking interactions which increase the stability of the DNA adduct (111). The DNA helix remains β shaped with normal C2-endo sugar pucker. Importantly WC base pairing is unperturbed with overall deviation of only 0.68 Å (109, 110). This probably contributes to the resistance of BPDE to lesion removal by NER processes. NER recognizes specific conformational features created by the lesion with the DNA, such as base pair disruptions or DNA helical distortions. BPDE lesions, among other PAHs causing minor groove lesions, retain their base pairing by accommodating adducts on the major or minor groove of DNA and make for poor substrates for recognition by NER (111-113).

Minor groove DNA lesions occur commonly in response to many types of exogenous or endogenous damage. These types of lesions have important implications to human health. Polyunsaturated fatty acids are a major target of oxygen radicals. Lipid peroxidation can lead to formation of exocyclic adducts such as etheno or propano adducts. Elevated levels of etheno adducts have been observed in patients with familial adenomatous polyposis. Propano adducts are similarly elevated in tissues of smokers (7). Formation of minor groove adducts due to oxidation with glucose intermediates result in the formation of advanced glycation end products which are associated with aging, diabetes, and uremia. Women with tamoxifen therapy for breast cancer, have elevated levels of minor groove adducts and higher risks of developing endometrial cancer. Estrogens, form minor groove

adducts with DNA, and these adducts have been documented to increase cancer risk (12, 13).

Minor groove lesions are a potent inhibitor to replication (116). There are two main consequences of failure to correctly address minor groove DNA adducts, the inability to replicate across the lesions leads to accumulation of stalled replication forks which can generate DSBs, resulting in genomic instability. Importantly hereditary cancers have elevated levels of genomic instability and this instability is closely associated with defects in various DNA repair proteins (64, 65). A second consequence is incorrect replication of the lesion by DNA damage tolerance mechanisms, such as translesion synthesis (TLS), which alleviates the replication block but at the expense of increasing mutagenesis (15-17). Minor groove lesions can directly contribute to cancer risk either by increasing DSBs, leading to genomic instability, or by increasing mutagenesis.

Benzo(α)Pyrene: The Classic Model of Chemical Carcinogenesis

Studies in the 19th century revealed that certain occupational exposures, such as paraffin refining, shale oil work, and work with coal tar correlated with an increased incidence of cancer. Early attempts to introduce an experimental model of chemical carcinogenesis were unsuccessful until the induction of skin tumors by coal tar in the ears of rabbits (117, 5). This led to the hunt for the chemical responsible for induction of carcinogenesis by coal tar. Through organic synthesis and spectroscopic methods, E.L. Kenneway's group in the Research Institute of Cancer was able to synthesize teratogenic tars and identify aromatic compounds with increasing carcinogenic potential. This eventually led to the isolation of a polycyclic aromatic hydrocarbon (PAH), benzo(a)pyrene (BαP), as the principal carcinogen from 2 tons of coal tar (118-120).

Mutations and genomic instability have been associated with cancer (18). Epidemiological, occupational, and migration studies have demonstrated the major role of environmental factors as contributors to carcinogenesis (19). These links with cancer are especially strong among sunlight and skin cancer (33), tobacco and lung cancer (14, 22), and aflatoxin and liver cancer (34). Eighty percent of the estimated number of cancer deaths are due to environmental exposure, with tobacco accounting for ~30% of cancer deaths, making smoking one of the leading causes of preventable global cancer deaths (20). Tobacco is a mixture of chemical compounds that can be divided into a particulate and a vapor phase. Studies have demonstrated the carcinogenic potential of many of these compounds, which strongly implicate PAHs as causative agents in cancer development. Among PAHs, the most characterized compound, due to its high concentration in cigarette smoke and potential for mutagenicity upon metabolism, is BαP.

BαP is a procarcinogen that is formed from incomplete combustion of organic compounds. Enzymatic metabolism by the cytochrome p450 system is required for conversion of BαP into the reactive bay region diol epoxide. The reaction occurs in three steps: (1) BαP is oxidized by cytochrome p450 into BaP-7,8 epoxide; (2) Epoxide hydrolase opens the ring to produce BaP-7,8-dihydrodiol; (3) The cytochrome system oxidizes the ring to form 4 possible stereoisomers of BPDE (Figure 5). The (+)-trans-anti-BPDE is the most mutagenic metabolite of BαP.

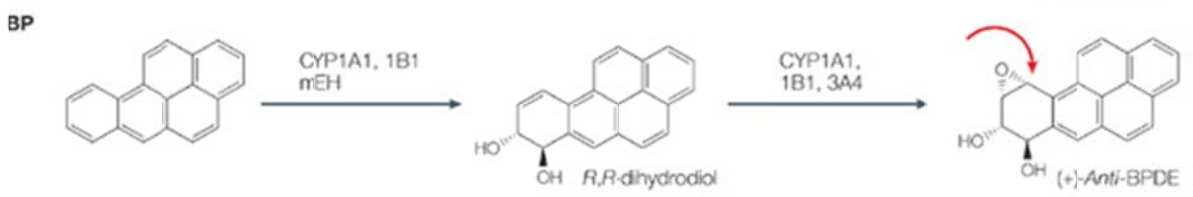


Figure 5. Metabolism and activation of BαP

BP is initially converted mainly by CYP1A1 or CYP1B1 into the 7,8-epoxide. This epoxide is a substrate of microsomal epoxide hydrolase (mEH), which produces the 7,8-dihydrodiol. Both reactions together stereoselectively form the R,R-dihydrodiol. Further epoxidation at the vicinal double bond catalysed by CYP1A1, CYP1B1 and CYP3A4 generates the ultimate genotoxic diol-epoxide of BP (BPDE). Of the four possible resulting diastereomers, the (+)-anti-BPDE is formed at the highest levels.

BPDE reacts with DNA to form covalent adducts with guanine, adenine, and to a far lesser extent cytosine (19). The reaction with the N^2 amino group of adenine is predominant and accounts for ~85-90 % of adducts, with the N^6 adenine lesion accounting for ~5 - 8% of cases and cytosine adducts accounting for <3% of the total. Studies with the p53 gene, which is heavily mutated in tobacco-related cancers, have correlated the mutation load with the amount of tobacco smoke and PAH present. Importantly, the sites of mutations within the p53 gene correlate with the site of minor groove BPDE adduct formation. These sites are predominantly at methylated CpG islands on the non-transcribed strand and have a specific pattern of mutations of G → T transversions. Studies with the Kirsten rat sarcoma viral oncogene homolog (KRas) in human tumors, as well as the *cII* and *LacI* reporter genes in mouse cells revealed a similar pattern of mutations after exposure to BPDE (5, 14, 21-27).

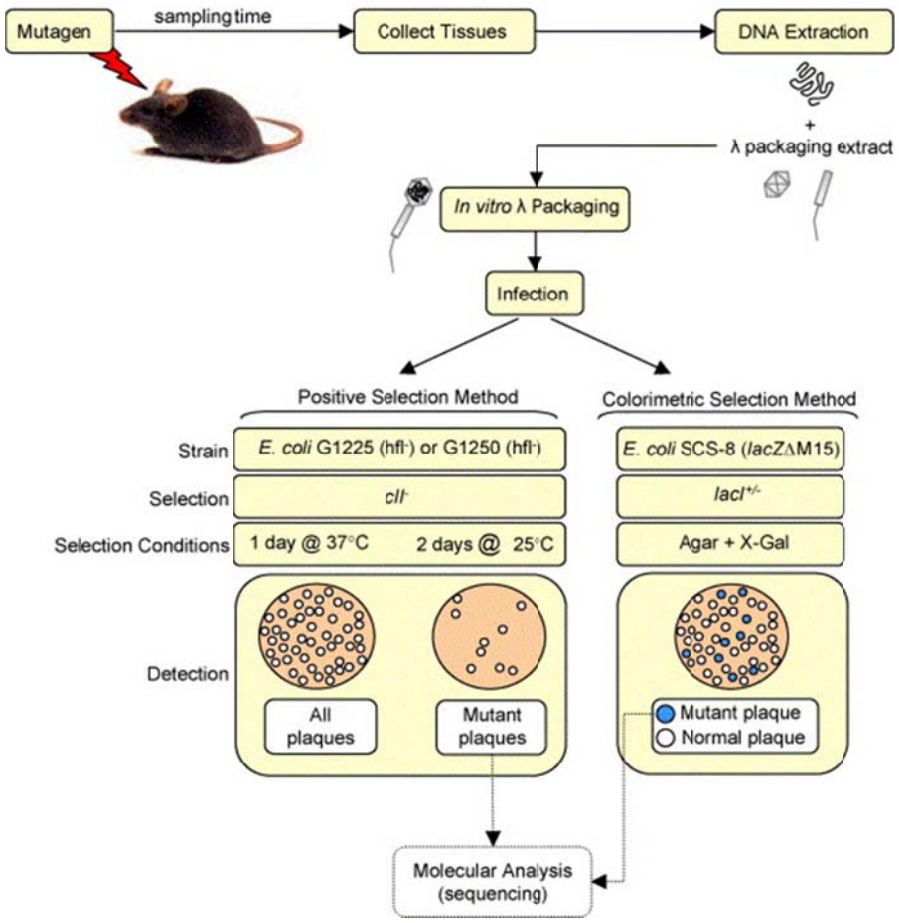
BPDE minor groove lesions are generated by combustion of organic products. Production of BαP in the US is ~1300 tons/year (121, 122). Exposure to PAHs alone has a strong correlation to lung carcinogenesis. An epidemiological study in a rural area of China with low percentage of tobacco smoking but high exposure of PAHs by wooden stoves

revealed similar patterns of mutations as well as elevated levels of lung cancer (123). Another important observation is that changing the design of cigarettes to decrease the amount of BαP decreased small cell lung carcinomas characteristic of BPDE carcinogenesis, however, the increasing amount of nitrosamines led to a corresponding increase of adenocarcinoma cases which are correlated with nitrosamine exposure (124). These studies provide strong evidence of BPDE as the principal chemical carcinogen in tobacco smoke and as a causative agent in cancer development.

Recent studies of TLS across minor groove lesions have revealed insight into the mechanisms of lesion bypass (28-32,36-42). However these studies have been performed in *in vitro* settings leading to contradictory information and have not clarified the physiological roles of TLS polymerases in the extension and insertion activities across minor groove adducts. Many of these studies utilized gapped plasmids transfected into host cells, which do not provide information of the role of TLS polymerases during replication. The innovation of our shuttle vector approach is the fact that our heteroduplex vector can accurately measure and distinguish the contribution of each of the DNA damage tolerance mechanisms. More importantly, unlike gapped plasmids, our shuttle vector provides information on the role of TLS polymerases during replication. Additionally, by utilizing NER deficient cell lines, we can ensure that the lesions introduced into our plasmid system will not be repaired before lesion bypass mechanisms are activated. The in vivo positive mutation screening mouse system we use provides an excellent model for measuring the contributions of each TLS polymerase to lesion bypass in a chromosomal gene. These methods coupled with the powerful technology of siRNA knockdown provide a powerful rationale for identifying the molecular basis of lesion bypass across the minor groove N^2-BPDE-dG lesion. This knowledge may reveal insights into the etiology of cancer and provide for novel therapeutics to be developed.

Chapter 2 Materials and Methods

cII Mutational Assay in Mouse Cells



Reprinted by permission from Macmillan Publishers Ltd: [Reviews in Mutation Research] (206), copyright (2005).

The big blue mouse system is a powerful method for assessing the genotoxicity and mutagenesis of test compounds (Stratagene). The big blue mice have integrated into their genomes the λLIZ shuttle vector, which contain two reporter genes, a cI repressor, and a lacI repressor. The cI repressor target was utilized due to its small size, which makes it simple to analyze and sequence. The system utilizes the bacteriophage system of regulation of its lysogenic and lytic cycle. The bacteriophage's cII protein is involved in

the commitment of the phage to either the lytic or lysogenic cycle. The cII protein induces transcription of several genes required for the lysogenic cycle and establishes transcription of the cI repressor protein. The cI repressor inhibits transcription of various genes required for the lytic cycle. The bacterial host strain carries mutations that facilitate the bacteriophage's lysogenic response by increasing the stability of the cII protein. Mutations of the cII gene result in a nonfunctional cII and the phage will undergo the lytic cycle. In order to correctly quantify mutation events, it is necessary to quantify the number of bacteria infected by the phage, therefore, a titer is plated at selective conditions 37 °C, under which the time sensitive mutation cI857 will always force the bacteriophage to undergo the lytic cycle, allowing the correct determination of the total number of bacteria infected (Figure 6, 62).

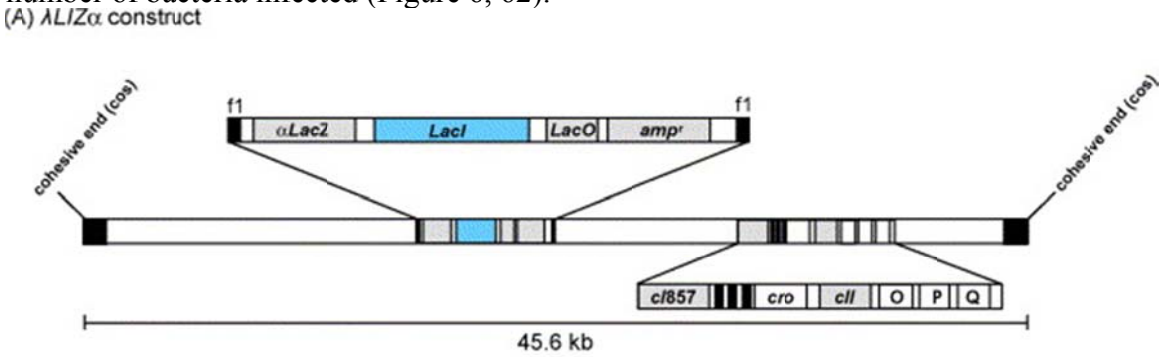


Figure 7. Target chromosomal transgene inserted in the mouse cells

The cII protein commits the λ phage to either the lysogenic or the lytic cycle. The cI(1857), is a temperature-sensitive cI repressor protein.

siRNA Knockdown in Mammalian Cells

The big blue transgenic mouse embryonic fibroblasts (BBMEFs) were grown in Dulbecco's modified eagle medium (DMEM) containing 10% Fetal Bovine Serum (FBS) and 1X of antibiotic/antimycotic (GIBCO). Transfection was carried out by lipofection with Lipofectamine 2000 (ThermoFisher). Fifty μM of Lipofectamine 2000 were incubated in 750 μM of Opti-MEM (Thermo Fisher) for 5 minute at room temperature.

The Lipofectamine mixture was then added to 750 μM Opti-MEM containing 50 pmoles of a corresponding siRNA and incubated for 20 minutes at room temperature. BBMEFs cells were collected by removing the media from the plate, washing once with 5 ml of Dulbecco's phosphate-buffered saline (DPBS), and incubating the cells with 1.5 ml of 0.25% Trypsin-EDTA for 5 minutes. The cells were collected with 5 ml of DMEM, centrifuged for 5 minutes at 200 G, and resuspended in 5 ml of DMEM. Approximately 5 x 10^6 cells were plated on 100 mm plates and the transfection media containing 500 pmoles of synthetic duplex siRNA and 50 μg of the Lipofectamine 2000 was added to the plates. The cells were incubated with the lipofection siRNA mixture for 4 hours in minimal media. After 4 hours the media was changed with regular media. BPDE treatment was performed after 48 hours. The media was removed and the cells were treated with 500 nM of BPDE in Hank's buffered saline solution (HBSS) for 30 min at 37 ^{o}C. A second siRNA knockdown was performed after 24 hours of the BPDE treatment. The cells were allowed to grow for mutation fixation for 5 days following BPDE treatment. siRNA knockdown has been confirmed by western blotting and RT-PCR, previously in our lab.

Genomic DNA Isolation

The cells were collected by trypsinization, spun down and washed with DPBS 5 days after the treatment with BPDE. Genomic DNA was extracted utilizing the Blood and Cell culture genomic kit (QIAGEN). Briefly, the cell pellet was resuspended in 8 ml of ice cold Buffer C1 1X (Blood and Cell culture genomic kit - QIAGEN). Lysis was performed for 10 min on ice, the sample was inverted several times until the suspension became clear. Sample was spun at 4 ^{o}C for 15 minutes at 1,300 g. The pellet was resuspended by vortexing in 8 ml ice cold Buffer C1 1X and spun again at 4 ^{o}C for 15 minutes at 1,300 g. The supernatant was discarded and the white pellet resupended in 5 ml of Buffer G2 (QIAGEN). Protein was removed from the samples by incubating the

samples for 1 hour at 37 °C with 100 µl of 10 mg/ml proteinase K. The genomic DNA was then purified by a gravity flow anion-exchange column (QIAGEN). The DNA was precipitated by addition of 3.5 ml of 100% isopropanol, spool to precipitate the DNA and then the DNA was transferred to a 1.5 ml eppendorf tube. The DNA was spun at 13,000 rpm for 5 minutes, the supernatant was discarded and then the DNA was washed with 750 µl of 70 % ethanol, spun a second time at 13,000 rpm for 5 minutes, and then the supernatant was discarded and the DNA resuspended in 50 µl of TE buffer pH 8.0.

cII Mutation Assay

G1250 (Stratagene) *E. coli* cells were grown overnight at 30 °C in 30 ml TB1 media (1.0% Peptone, 0.5% Sodium Chloride, 1.2% Agar, 0.0001% Thiamine HCl, 50µg/mL Kanamycin) supplemented with 300 µl of 20% maltose-1 M $MgSO_4$. The cells were collected after 24 hours by centrifugation at 1,500 g for 15 minutes. The supernatant was discarded and the cell pellet resuspended in 10 ml of ice cold 10 mM $MgSO_4$ solution. The cell suspension was adjusted to $_{OD}$ ~0.500 spectrophotometer reading. Eight µl of genomic DNA were utilized to reassemble a lambda phage for infection and transformation of the G1250. Eight µl of genomic DNA was added to the orange tube transpackaging reaction (Stratagene cII Transpack Kit). Mix by pipetting and incubated for 90 minutes in a 30 °C water bath. Following this, 12 µl of the blue tube transpackaging reaction (Stratagene cII Transpack Kit) was added to the genomic DNA mix and incubated for 90 minutes in a 30 °C water bath. One ml of SM buffer (100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris-HCl, and 0.01% gelatin) was added to each sample. Dilution titers were prepared by adding 10 µl of the sample mix in 990 µl of SM buffer. Six samples were prepared by adding 250 µl of the G1250 cell culture in $MgSO_4$ to a bacterial tube and adding 160 µl of the genomic transpack reaction mixture. Separately 3 dilution titers per sample were prepared by adding 100 µl of the genomic transpack

reaction titer to 250 µl of the G1250 cell culture in $MgSO_4$. The samples were incubated for 30 minutes at room temperature. Four ml of TB1 top agar (1.0% Peptone, 0.5% Sodium Chloride,0.7% Agar, 0.0001% Thiamine HCl) was added to the samples and the samples were plated in TB1 Kan^+ plates and grown at 30 ^oC for 48 hours. Separately 3 plates of 1/1000 dilution titers were prepared in the same way in TB1 Kan^+ plates and grown at 37 ^oC overnight to calculate total number of infected cells. The dilution titers were counted after 24 hours and the samples plates were counted 48 hours after plating and the mutation rate was calculated from the total infected cells.

Mutational Spectra

Mutant plaques obtained from the *cII* mutation assays were utilized as the template for amplification of their *cII* gene utilizing the *cII* specific primers LP2177 (5'-CCAGCCCTGAAAAAGGG-3') and LP 2178 (5'-CCTCTGCCGAAGTTGAG-3'). The DNA was then purified by a silica column (QIAGEN) and sequenced by the Sealy Center for Molecular Medicine Molecular Genomics Core at UTMB. Spectra were constructed from the data.

BPDE SENSITIVITY ASSAY

MTT Assay

Normal human fibroblasts were transfected with 10 pmoles of siRNA and 10 µg of Lipofectamine 2000. Ten µM of Lipofectamine 2000 were incubated in 250 µM of Opti-MEM (Thermo Fisher) for 5 minute at room temperature. The Lipofectamine mixture was then added to 250 µM Opti-MEM containing 10 pmoles of a corresponding siRNA and incubated for 20 minutes at room temperature. Three hundred thousand cells were plated in a multi well 6 well and depletion of the target gene was performed by incubating the cells for 4 hours with the siRNA lipofectamine mixture in minimal media.

After 4 hours the media was replaced with regular DMEM media. Forty eight hours after transfection the cells were treated with 500 nM BPDE at 37 ^{o}C in HBSS buffer for 1 hour. The cells were allowed to grow for 2 days and then a colorimetric assay utilizing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was performed. Briefly, the cell media was removed and 1 ml of MTT 1X solution added to each well. The cells were incubated for 30 min at 37 ^{o}C. The media was collected and analyzed by spectrophotometer.

DNA REPLICATION FOCI

Fluorescent Constructs

The Open Reading Frame (ORF) for TLS Pols ι, κ, and Rev1 were cloned into an eukaryotic expression vector containing the GFP gene (pGFP-N1 Clonetech) or the RFP gene (pcDNA3mRFP Addgene). Briefly, the ORF from the TLS pols was cut from CMV eukaryotic expression vectors by digestion with BamHI and ligated into the BamHI site of pGFP-N1 or the pcDNA3-mRFP. The vectors were sequenced to confirm correct construction and the protein expression was further confirmed by western blotting and fluorescence microscopy.

DNA Replication Foci Assay

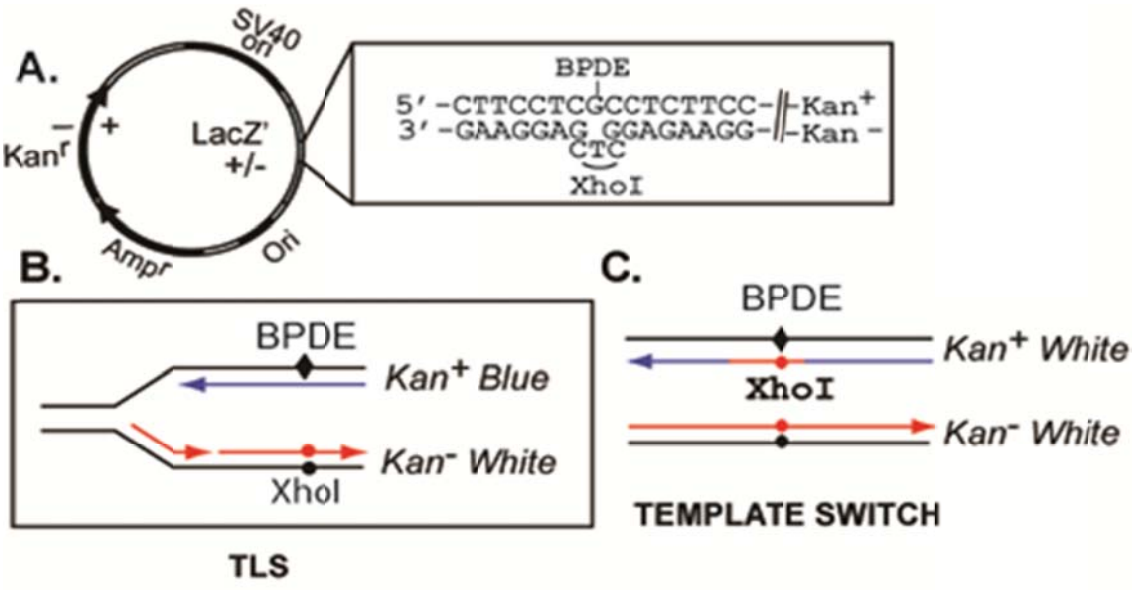
Normal human fibroblasts were seeded in multiwell 6 plates at 300,000 x 10^{6} cells/well. Ten μM of Lipofectamine 2000 were incubated in 250 μM of Opti-MEM for 5 minute at room temperature. The Lipofectamine mixture was then added to 250 μM Opti-MEM containing 1 μg of DNA of the fluorescence tagged TLS Pols DNA and incubated for 20 minutes at room temperature. The transfection mixture was added to the multiwell 6 containing the human fibroblasts and incubated for 4 hours in minimal media. After 4

hours, the media was changed to regular DMEM media. Forty eight hours after transfection the cells were treated with 500 nM BPDE in HBSS buffer for 1 hr at 37 ^{o}C. Six hours after BPDE treatment the cells were fixed with 4 % formaldehyde for 15 minutes and stained with 0.2 μg/ml of 4',6-diamidino-2-phenylindole (DAPI). The cells were mounted on a microscope slide with mounting solution (ProLong Gold LifeTechnologies). Slides were examined under a fluorescence microscope and DNA replication foci were quantified.

TLS PLASMID ASSAY

Construction of Plasmid Vectors Containing Lesion

Oligonucleotides containing either 1MeA or N^2-BPDE-dG lesion were purchased from Trilink Biotechnologies (SantaCruz, CA) were utilized to create a heteroduplex plasmid containing an inframe target LacZ gene containing either the 1-MeA lesion and harboring an MfeI restriction site and encoding for a functional β-galactosidase and on the opposite strand harboring an SpeI site and containing an out of frame +1 β-galactosidase. The lesion strand contains a Kan^+ resistance cassette while the opposite strand is Kan^-. Similarly the N^2-BPDE-dG lesion plasmid encodes for a functional β-galactosidase with no MfeI restriction site and with an opposite strand harboring a XhoI site.



(A) The plasmid has the SV40 origin for replication in human cells and the ampicillin (*Amp*^{r}) and kanamycin resistance (*Kan+*) genes for selection in *E. coli.* The plasmid carries the wild type *Kan+* gene on the lesion-containing strand and the kanamycin sensitive (*Kan-)* gene on the other DNA strand. **(B)** TLS through the BPDE adduct results in the formation of *Kan+* blue colonies. Replication of the opposite strand, which has no DNA lesion, results in *Kan-* white colonies. **(C)** Template switch results in continuing replication of the *Kan+* strand by using as a template the *lacZ'* strand harboring the XhoI sequence. This process generates white colonies that are *Kan+.*

We utilized a heteroduplex plasmid system previously developed in our laboratory to study TLS (49). The system is based on a modified plasmid pBluescript vector (Stratagene) containing a target sequence with a single DNA lesion, a lacZ' reporter, and an SV40 replication origin for bidirectional replication in SV40 transformed mammalian cells (Figure 7A). The rationale for the plasmid assay is as follows: replication of a plasmid containing a single lesion of 1MeA or N^2-BPDE-dG could occur either by TLS or template switch, and in most studies, it is difficult to determine which damage tolerance process is utilized (57). However, the heteroduplex TLS plasmid assay functions in an elegant way to differentiate between TLS and template switch. The strand with the lesion has an in frame lacZ' gene while the opposite strand has a lacZ gene that is out frame (Figure 7A). TLS produces a lacZ' gene which is in frame and

generates blue colonies (Figure 7B) whereas replication through the lesion by template switching results in an out of frame lacZ' gene and white colonies on x-gal plates (Figure 7C). TLS levels are calculated from the number of blue colonies among the Kan^+ colonies. The TLS plasmid system allows for the determination of strand bias since the lesion can inserted into the leading or lagging strand.

In Vivo Translesion Synthesis Assay in Human Cells

Normal human fibroblasts were grown as described previously and ~3 $x10^5$ cells plated in a 6 well plate. The cells were transfected by lipofection as described previously with 100 pmole of siRNA. Forty-eight hours after transfection a second knockdown with 50 pmole of siRNA and co-transfection with 1 μg of the heteroduplex plasmid vector was performed by lipofection. The cells were incubated and 30 hours after co-transfection the plasmid DNA was isolated by the alkaline lysis method and digested with DpnI to remove unreplicated plasmid. The plasmid DNA was used for transformation of *E. coli* XL1Blue cells (Stratagene). The cells were plated in LB plates containing kanamycin and 1 μM isopropyl-1-thio-β-D-galactopyranoside (X-gal). Blue and white colonies were counted and TLS frequency calculated.

Mutational Analysis

Blue colonies from the TLS plasmid assay were utilized to amplify the LacZ target gene by PCR amplification with the primers LP2366 (5'-CGC CCA ATA CGC AAA CC-3') and LP 2367 (5'-AAC GTG GAC TCC AAC GTC-3'). The PCR products were purified by silica column and sent for sequencing to the Sealy Center for Molecular Medicine Molecular Genomics Core at UTMB. Analysis of the sequencing resulted in the mutation frequency and mutational changes incorporated.

GENERATION OF STABLE CELL LINES

Site directed mutagenesis was utilized to create deletion or single amino acid substitution mutants of the TLS polymerases. PCR mutagenesis was performed utilizing the Flag tag eukaryotic expression vectors containing the ORF of Pols ι, κ, and Rev1 with the following overlapping primer pairs: Pol ι, LP2780 (5'-GGGCTTATTGATGCTGCTTTAATGCCATCATTA-3') and LP2781 (5'-TAATGATGGCATTAAAGCAGCATCA ATAAGCCC-3'); LP2782 (5'-GAGGAGTATTATCTGCCGCCTCT AAAAAACAAATG-3') and LP2783 (5'-CATTTGTTTTTTAGAGGCGG CAGATAATACTCCTC-3'); LP2784 (5'-CTGAAGGTGTTGCCC AAGAAGTCTTC-3') and LP2785 (5'-GAAGACTTCTTGGGC AACACCTTCAG-3'); LP2786 (5'-GTCTTCAAGCAGGCAGC AGTAGATATTC-3') and LP2787 (5'-GAATATCTACTGCTGCC TGCTTGAAGAC-3'); LP 2788 (5'-CTTCTGACATTGCACCTC AAGTTTTC-3') and LP2789 (5-GAAAACTTGAGGTGCAAT GTCAGAAG-3'); and LP2790 (5'-GTTTTCTATGAAGCAGCAG AAGCAGTAC-3') LP2791 (5'-GTACTGCTTCTGCTGCTTC ATAGAAAAC-3'); LP2816 (5'- CTTCTTACATGg CTAGCCAAAAG-3'); and LP 2817 (5'- CTTTTGGCTAGCCATGTAAGAAG-3'); LP2818 (5'- GATGAACGAATAGCACAAGGACCTAAAG-3') and LP2819 (5'-CTTTAGGTCCTTGTGCTATTCGTTCATC-3'); Pol κ, LP2794 (5'-GCATCAGTCTGGAAGCCTTGAATAAAGCCGTAGATGAATGTCTTGATGGACC TTC-3') and LP2795 (5'- TGATTGAAGGTCCATCAAGACATTCATCT ACGGCTTTATTCAAGGCTTCCAGACTGATGC-3'); LP2796 (5'- GCATCAGTC TGGAAGCCTTGAATAAACATGTAGCCGAATGTCTTGATGGACCTTCAATC-3') and LP2797 (5'- CACTGATTGAAGGTCCATCAAGACATTCGGCTAC

ATGTTTATTCAAGGCTTCCAGACTGATGC-3'); LP2798 (5'- GACTTCAG ATCTAACCCTGTTCAATGTGGCTGTGGATGTTTGCTTAAATAAAAG-3') and LP2799 (5'- CTTTTATTTAAGCAAACATCCACAGCCACATTGAACAGG GTTAGATCTGAAG-3'); and LP2800 (5'- CAGATCTAACCCTGTTCAATGTGCAT GTGGCTGTTTGCTTAAATAAAAGTTTTATCC-3') and LP2801 (5'- GGATAAAAC TTTTATTTAAGCAAACAGCCACATGCACATTGAACAGGGTTAGATC-3'); and Rev1 LP2837 (5'- GTCCCGTCACCTGATCAGCTGGATCAG-3') and LP2838 (5'- CTGATCCAGCTGATCAGGTGACGGGAC-3'); LP 2839 (5'- GTCCCGTCACCT GAACAGCTGGATCAG-3') and LP2840 (5'- CTGATCCAGCTGTTCA GGTGACGGGAC-3'); LP 2841 (5'- CTTCCAGCATTTGACCAGGTGGACCC-3') and LP2842 (5'- GGGTCCACCTGGTCAAATGCTGGAAG-3'); and LP2843 (5'- CTTCCAGCATTTGAACAGGTGGACC-3') and LP2844 (5' GGTCCACCTGTTC AAATGCTGGAAG-3') . Mutagenesis was performed by two steps PCR introducing the mutation on the first step by amplifying the ORF with the complementary primers on two different reactions and the vector specific primers for the pMEV27 vector LP2113 (5'- GGACTTTCCAAAATGTCGT-3') and LP2114 (5'- TACAGGTTGTCTTCCCAACT-3'). The products of the PCR reaction then were utilized as the template on the second PCR with the pMEV27 specific primers LP2113 and LP 2114. The PCR product was then digested with BamHI and ligated into the eukaryotic expression vector. Transfection by lipofection of normal human fibroblasts was carried out with 1 μg of DNA of the mutant construct as described previously. Twenty-four hours after transfection selection was performed with 500 μg/ml of zeocin for 2 weeks. Protein expression was confirmed by western blotting and immunohistochemistry with anti-Flag antibodies.

Chapter 3 Replication Through 1-Methyl Adenine

Alkylating agents are an unavoidable class of chemicals that react with a broad range of biological molecules resulting in cytotoxicity and mutagenicity in living organisms. The broad range and ubiquitous presence of alkylating agents implicates them as an important threat to human health (96). The cell activates a variety of DNA repair mechanisms in a complex biological response to protect the integrity of the genome (76).

Methylating agents, in particular, can readily react with DNA and form mutagenic and replication inhibiting lesions. The importance of these lesions can be observed by the number of methods that the cell has evolved in order to handle these lesions (76, 99). Reactions with the N^1 group of adenine are preferable when the nucleic acid is single stranded, due to the nucleophicity of the nitrogen group. These lesions strongly inhibit replication due to their disruptions of WC bonding and are particularly important when DNA is transiently exposed such as during replication, recombination, or transcription. The cell has develop direct reversal methods of repair (76,99) to handle this lesion, however due to their abundance this lesion is often present during replication and the methods that the cell uses to repair or tolerate this lesion are not known. Greater understanding of the biological factors in response to 1-MeA damage is necessary in order to increase cancer prevention and develop new therapeutics.

ROLE OF TLS POLYMERASES IN REPLICATION THROUGH 1-MEA LESION

Role of TLS Polymerases in Replication Across a Single 1-MeA Lesion

In order to identify the TLS Pols responsible for replicating through a 1-MeA lesion carried on the leading strand of an SV40-based plasmid, TLS frequency was determined

after siRNA depletion in human fibroblasts (Table 1). TLS was assessed by a functional *lacZ* gene and blue/white screening with X-gal.

Table 1. Effects of siRNA knockdown of TLS polymerases on the replicative bypass of 1-MeA lesions

siRNA KO	Total Colonies	Blue Colonies	% TLS
NC (GFP) or (Luc)	423	276	65.2
Pol η	356	187	52.5
Pol ι	428	193	45.1
Rev 3 (Subunit of Pol ζ)	524	228	43.5
Rev 7 (Subunit of Pol ζ)	458	205	44.8
Pol θ	415	163	39.3
Pol ι + Pol θ	372	161	43.3
Pol η + Pol ι	302	93	30.8
Pol η + Pol θ	408	122	29.9
Pol η + Rev 3	350	100	28.6
Pol η + Rev 7	296	92	31.1
Pol ι + Rev 3	228	63	27.6
Pol ι + Rev 7	295	80	27.1

As seen in Table 1 the frequency of lesion bypass across the 1-MeA lesion in human fibroblasts treated with negative control was ~65%. Knockdown of Pols η, ι, θ, and the subunits of Pol ζ, Rev3 and Rev7, led to a significant reduction in the TLS frequency suggestive of their role in lesion bypass across 1-MeA lesions (Table 1). Simultaneous depletion of Pols η and ι, Pols η and θ, or Pols η and ζ resulted in additive effects on TLS frequency compared with single depletions (Table 1) suggesting that Pol η is involved in a pathway different from that of Pols ι, θ, or ζ. Simultaneous depletion of Pols ι and ζ resulted in additive effects compared to depletion of Pols ι or ζ alone, suggesting that Pol ι and ζ are involved in different bypass pathways across 1-MeA lesions. Finally simultaneous depletion of Pols ι and θ resulted in no additive effects in

the TLS frequency suggesting that Pols ι and θ function together to replicate across the 1-MeA lesion.

Table 2. Effects of siRNA knockdown of TLS polymerases on the replicative bypass of 1-MeA lesions in XPV human fibroblasts

siRNA KO	Total Colonies	Blue Colonies	% TLS
NC (GFP) or (Luc)	254	120	47.2
Pol ι	292	90	30.8
Pol θ	260	78	30.0
Rev 3 (Subunit of Pol ζ)	224	71	31.7
Rev 7 (Subunit of Pol ζ)	212	60	28.3
Pol ι + Pol θ	312	86	27.6
Pol ι + Rev 3	238	10	4.2
Pol θ + Rev 3	308	16	5.2

In order to further confirm the role of TLS Pols in lesion bypass across 1-MeA, TLS frequency was analyzed after depletion of TLS Pols in a human XPV (XP30R0) fibroblast cell line. These cells lack Pol η due to mutational inactivation and their TLS frequency is similar to normal human fibroblasts depleted of Pol η by siRNA (Table 2 compared with Table 1). As expected, depletion of either Pols ι, θ, or ζ resulted in a reduction in the TLS frequency similar to the simultaneous depletion with Pol η in normal human fibroblasts (Table 1 compared with Table 2), again confirming the independent functions of these TLS pols with Pol η. Simultaneous depletion of Pols ι and θ resulted in no additive effects in the TLS frequency confirming that these Pols are involved in the same bypass pathway across 1-MeA. Simultaneous depletion of Pols θ and ζ resulted in additive effects suggesting that they are involved in independent pathways of bypass of 1-MeA lesions. Finally, simultaneous depletion of Pols ι and ζ or

Pols θ and ζ resulted in a drastic reduction of TLS, statistically close to zero, confirming that across 1-MeA the cell utilizes 3 independent pathways for bypassing this lesion.

MUTAGENICITY OF TLS OPPOSITE 1-MEA

The blue mutant colonies from the TLS heteroduplex plasmid system assay were utilized for amplification of the *lacZ* gene by PCR and the samples were analyzed by restriction enzyme and sequencing to determine the types of mutations that occur after depletion of the TLS pols in response to 1-MeA damage.

Table 3. Effects of siRNA knockdowns of TLS Pols on the frequencies of nucleotides inserted oppostie 1-MeA

siRNA KO	No of Kan+ colonies	Nucleotide Inserted				Mutation Frequency
		A	G	C	T	%
NC (Luc)	384	2	2	0	380	1.0
Pol η	308	0	0	0	380	0.0
Pol ι	190	2	1	0	187	1.6
Pol θ	176	2	0	0	174	1.1
Rev 3 (Subunit of Pol ζ)	288	3	1	1	284	1.4
Rev 7 (Subunit of Pol ζ)	192	3	0	0	189	1.6

TLS across 1-MeA occurs in a predominantly error-free manner. In negative control cells mutations appear in only ~1% of the cases. Lesion bypass may function in an error-free or mutagenic manner. In order to further explore the roles of the TLS Pols in the bypass of 1-MeA lesions, depletion of the TLS Pols responsible for bypassing 1-MeA was performed. Interestingly, depletion of the TLS Pols had no effect on the mutation frequency across 1-MeA lesions (Table 3). Inactivation of a bypass pathway might result in an increase or decrease in overall mutagenesis depending on whether that pathway was mutagenic or error-free. The fact that mutations did not increase after inactivation of any one of the 3 pathways suggests that the other 2 functional pathways were themselves

error-free and that TLS across 1-MeA is not mutagenic with any of the 3 bypass pathways.

Together these genetic observations allow us to conclude that opposite a 1-MeA lesion TLS occurs through at least 3 independent pathways: One where Pol η alone or in combination with another Pol bypasses the 1-MeA lesion, a second one where Pol ι in combination with Pol θ bypasses the 1-MeA lesion, and a third one where Pol ζ alone or in combination with an unidentified Pol bypasses the 1-MeA lesion.

Chapter 4 Replication Through DNA Minor Groove Lesions

Reactions with the N^2 amino group of guanine are very favorable allowing the accommodation of bulky adducts into the minor groove of DNA resulting in minimal steric clashes and disruption to the DNA backbone (4,5). Minor groove DNA lesions at the N^2 position of guanine have been documented from endogenous (6-13) and exogenous sources (14). These lesions threaten the integrity of the genome by blocking replication. DNA damage tolerance mechanisms, such as translesion synthesis (TLS), lead to alleviation of the replication block but do so at the expense of increasing mutagenesis (15-17).

Genetic instability is one of the hallmarks of cancer (18). Studies have revealed the contribution by environmental factors to carcinogenesis (19). Among environmental factors strongly associated with cancer are those of the tobacco compounds. Tobacco use is one of the leading causes of preventable cancer deaths in the developed world (20). Studies have demonstrated the carcinogenic potential of tobacco compounds and implicated polycyclic aromatic hydrocarbons (PAHs) as causative agents in cancer development (5,14,21). Among PAHs one of the chemicals with the highest concentration in cigarette smoke and potential for mutagenicity is benzo(α)pyrene (BαP) (5). Studies with BPDE, the activated metabolite of B(α)P, have implicated it with minor groove adduct formation in the TP53 (tumor suppressor p53) gene, which is very frequently mutated in tobacco-associated human cancers (14,22). The mutations in TP53 correlate with the sites of PAH adduct formation and are positively correlated with tobacco usage (12). Importantly TP53 mutations in lung cancer tissues of smokers have a preferential mutation signature (14, 23-27).

Carcinogenesis is often a multistep process requiring multiple mutations (18). The mutational fingerprint of a carcinogen provides clues into the mechanisms by which cells repair replication fork stalling lesions created by exposure to tobacco or other carcinogens. Such mutational fingerprints are similar among minor groove adduct lesions by compounds other than PAHs suggesting a conserved mechanism by which the cell handles this ubiquitous lesion (28-32). Understanding the molecular actors in such lesion bypass is necessary for determining the biochemical basis of mutations which will provide insights for cancer risk and therapeutics. Identifying the Pols involved in the bypass of minor groove lesions might allow us to avoid the mutagenesis of tobacco smoke that leads to carcinogenesis.

ROLE OF TLS POLYMERASES IN REPLICATION THROUGH DNA MINOR GROOVE LESIONS

TLS Polymerase Involvement in DNA Minor Groove Bypass

In order to identify the TLS Pols that are required for replicating through the N^2-BPDE-dG minor groove lesion, siRNA depletion of various TLS Pols was carried out in mouse embryonic fibroblasts derived from the BBMEFs after treatment with BPDE. The genomic DNA was isolated, utilized to reassemble λ phage particles, and used to transform G1250 hf1-*E. coli*. Mutagenesis was then assessed by a functional *cII* gene and formation of plaques.

Table 4. Effect of TLS Pols on BPDE induced mutations in the *cII* gene

siRNA KO	Mutation Frequency (x 10^{-5})
No BPDE	11.4 ± 2.9
BPDE, 200 nM, 0.5 h	
NC (GFP) or (Luc)	41.7 ± 3.3
Pol η	45.3 ± 3.5
Pol ι	16.9 ± 3.1
Pol κ	22.3± 3.4
Pol θ	24.3 ± 1.7
Rev 1	22.6 ± 2.0
Rev 3 (Subunit of Pol ζ)	40.4 ± 1.8
Pol λ	39.0 ± 0.8
Pol ν	25.7 ± 2.8

BBMEF cells were treated with BPDE 200 nM for 30 min at 37 °C

As seen in Table 4, the spontaneous mutation frequency in untreated cells (DMSO) was ~11 x 10^5. This mutation frequency rose almost fourfold to ~42 x 10^5 in cells treated with BPDE for 30 min. siRNA depletion of Pols η, ζ, and λ had no significant effect on the frequency of BPDE induced mutations suggesting that these polymerases are not involved in lesion bypass of BPDE adducts. Knockdown of Pols ι, κ, θ, ν, and Rev1 led to a significant reduction in the frequency of BPDE induced mutations suggestive of their role in lesion bypass of BPDE adducts (Table 4).

Lesion bypass by TLS Pols might be carried out by a single Pol such as Pol η's bypass of CPD lesions or by the concerted action of two TLS Pols in which one Pol will insert across the lesion and a second one will extend from the lesion site such as in the case of Pols η and ζ bypass of (6-4) photoproducts (35). The role of the TLS Pols is influenced by the type of lesion. Structural and biochemical studies have offered evidence of the roles that the TLS Pols will play in either being an efficient extender or be prone to

participate in the insertion step of TLS. From these we can suggest that the actions of Pols κ and θ will be in the extension step while Pols ι, ν, and Rev1 will participate in the insertion step of lesion bypass of BPDE adducts.

Pol η has been demonstrated to be unable to extend across an N^2-BPDE-dG lesion *in vitro* (56) or in gapped plasmids (40, 130, 53). However Pol η has been demonstrated to be able to efficiently bypass a major groove N^6-BPDE-dA adduct (40, 169). Studies with nuclear foci formation revealed that Pol η does not form DNA replication foci in response to BPDE treatment (170), however slight sensitivity was found in MEF's that are Pol $\eta^{-/-}$ (171). This suggests that Pol η might act on major groove BPDE adducts which are formed in 5-8% of the cases after BPDE treatment vs 85-90% of N^2-BPDE minor groove adducts (19). However, in order to conclusively rule out a role for Pol η in the bypass of minor groove BPDE lesions, the mutational spectra of Pol η knockout in the *cII* gene as well as TLS measurements across a single BPDE lesion by a plasmid system was performed (Table 6).

Pol κ differs from other TLS pols in its higher rate of nucleotide incorporation, specificity, and ability to proficiently extend mispaired termini (35). Evidence for a role for Pol κ in bypass of minor groove lesions comes from studies *in vitro* from plasmid extension assays (38, 56, 42, 30, 36), gapped plasmid assays (40, 130), as well as functional studies in mouse embryonic fibroblasts (MEFs) of sensitivity (42) and DNA replication foci studies (160, 172, 42). Lesion bypass assays have revealed that Pol κ is unable to insert nucleotides opposite a variety of lesions such as *cis-syn* TT, (6-4) TT, 8-OxoG, thymine glycol (Tg), or BPDE adducts (35). However, it is proficient in extending from C opposite various minor groove lesions (173, 35). Structurally, Pol κ has a small active site that can accommodate only a single WC base pair and would probably be blocked from replication by a bulky adduct. It is referred to as an extender polymerase

because of its ability to efficiently extend mismatched primer termini (51). Pol κ has a structural component, the N-clasp, which augments the conventional right-handed grasp of the template-primer locking the thumb, finger, palm, and PAD subdomains around the DNA. This complete encirclement of the DNA by Pol κ might allow the misaligned 3'OH to acquire the proper alignment for nucleophilic attack and the polymerase action to occur (51).

Much less is known about Pol θ. Lesion bypass activity across an AP and Tg has been demonstrated *in vitro* by plasmid extension assays (174), as well as *in vivo* in which Pol θ performs both the insertion and extension step across a Tg lesion (175) but only the extension step across a 1-MeA lesion (176). It has a critical role for alternative Ku-independent end joining of double stranded breaks (177, 178) as well as being able to influence DNA replication timing (179). Interestingly biochemical experiments revealed the ability to efficiently extend from minimally paired primers (178) which would suggest a role as an extender polymerase in TLS. Structurally Pol θ utilizes a strategy similar to Pol κ for directly synthesizing from unmatched primer-termini (180). Pol θ utilizes positively charged residues to strongly bind the negatively charged phosphate backbone of DNA creating a primer-grip strategy where the specialized thumb subdomain establishes unique contacts and firmly grasps the DNA template (180) which, similar to Pol κ, might allow for alignment of the 3' OH and for the polymerase reaction to occur.

Pol ι is different from other TLS pols having increased efficiency and fidelity opposite templates purines rather than pyrimidines (35). Pol ι has been shown before to be able to carry out the insertion step in lesion bypass of AP, (6-4) TT, or N^2 adducted guanine (35). It has been demonstrated by plasmid extension assays that Pol ι is able to participate in the insertion step of minor groove adducts (181) or in combination with Pol κ to be able

to replicate past a minor groove lesion (182-184). This is due to the unique structure of Pol ι which utilizes amino acid residues from its finger domain to tilt and rotate the template DNA to adopt a *syn* conformation allowing the adduct to go into the major groove of DNA and utilizing Hoogsteen base pairing for DNA synthesis (50).

Pol ν, is an A family member TLS Pol closely related to Pol θ, it plays a role in lesion bypass of Tg lesions (185) and major groove adducts (186). The structure of Pol ν revealed that its Oa and Ob helices swing over the DNA duplex and occlude the DNA duplex from the template binding site requiring its finger domain to swing sideways to accommodate the nascent base pair (187) this would make it a poor enzyme for extension of mispaired primer termini. Interestingly, K679 of Pol ν binds with guanines and stabilize a T-G wobble mispair, conferring a unique mutation signature for Pol ν of G→T transversions (187). Interestingly, mutations of BPDE are predominantly G→T transversions (12, 14, 22-27) which might be expected if a TLS Pol prone for this type of mutation was active in bypassing BPDE adducts.

The role of Rev1 is more difficult to decipher. Rev1 has a peculiar and most extreme DNA Pol incorporation specificity; it preferentially inserts a dCTP opposite any template (188, 189). This specificity has a structural basis, both the yeast and human Rev1 utilize a radical protein-template replication strategy for their deoxycytidyl transferase activity (50, 190). A conserved region for Rev1, the N-digit, utilizes a Leu residue to evict the incoming template residue from the active site. The enzyme then utilizes an Arg residue as the template resulting in pairing and insertion of an incoming dCTP (50, 190). The templating guanine pairs with the G loop of Rev1 through its Hoogsteen edge N^7 and O^6 and in human Rev1 this template is further accommodated by the presence of a "flap" on its hydrophobic pocket. This "flap", a collection of 54 amino acid inserts in the catalytic core, is in position to accommodate the N^2 amino group of template G and might

accommodate bulky adducts at this position. The N^2 group is highly susceptible to chemical modifications and *in vitro* studies have revealed that Rev1 is capable of inserting a nucleotide opposite various minor groove lesions, such as Hydroxy-1,N2-propano-2 deoxyguanosine (N^2-γHoPdG) for yeast Rev1 (182, 50) or N^2 alkyl adducts for human Rev1 (191). Since Rev1 inserts only dCTP, this will result in error free bypass of minor groove adducts of guanine.

Rev1, in addition to insertion of dCTP, has a structural role in TLS. Rev1 is necessary for AP and UV mutagenesis (35, 202). However, its DNA synthetic activity is dispensable for UV mutagenesis (192) and for insertion across an AP site (193, 194). Furthermore, N^2 major groove adducts of acetyl aminofluorene (AAF)-dG require Rev1 but not its synthetic activity. The C terminal ~100 amino acids of Rev1 bind to Pols η, κ, ι, λ, and Rev7 (195-197) and competition experiments between Pols κ and Rev7 with Rev1 suggest that Rev1 can switch between TLS pols (195). These experiments suggest a possible structural role of Rev1 in assembly of other TLS pols in lesion bypass (35).

Epistasis Analysis of TLS Pols in Minor Groove Lesions

Table 5. Effect of knockdowns of combinations of TLS Pols on BPDE induced mutations in the *cII* gene integrated in the mouse genome

siRNA KO	Mutation Frequency ($X10^{-5}$)
No BPDE	11.4 ± 2.9
NC (GFP) or (Luc)	41.7 ± 3.3
Pol ι + Pol κ	16.0 ± 1.0
Pol ι + Pol θ	13.9 ± 1.6
Pol κ + Pol θ	15.8 ± 3.2
Pol ν + Pol θ	25.4 ± 3.6
Pol ν + Pol ι	13.4 ± 1.6
Pol ν + Pol κ	22.9 ± 4.1

BBMEF cells were treated with BPDE 200nm for 30 min at 37 °C

In order to determine whether these Pols function together, or independently of one another in replicating across the BPDE lesion, epistasis analysis after simultaneous knockdown of two TLS Pols was performed. Double knockdown of Pols κ and θ (Table 5) revealed additive effects compared with single knockdown (Table 4) suggesting at least 2 independent pathways of bypass across BPDE lesions. This is not surprising since, as mentioned before, both of these Pols are probably involved in the extension step across the BPDE lesion.

Depletion of Pols ι and κ or Pols ι and θ resulted in no additive effect on mutagenesis, suggesting that Pol ι inserts and extension is carried out by either Pol κ or Pol θ across the BPDE lesion (Table 5). Depletion of Pols ι and ν had an additive decrease in mutagenesis suggesting that Pol ι and Pol ν participate in pathways independent of each other (Table 5). Pols ι and ν are known as inserter polymerases so it is not surprising that they would be involved in 2 different pathways of BPDE lesion bypass. Furthermore, the mutation frequency obtained from the simultaneous depletion of Pols ι and ν is statistically the same as no treatment control suggesting that together these two polymerases are responsible for the mutagenesis of lesion bypass of BPDE adducts. Similarly, depletion of Pol ν and either Pols κ or θ had no additive effect suggesting that Pol ν utilizes either Pol κ or Pol θ for extension of minor groove lesions (Table 5). The role of Rev1 is difficult to ascertain from epistasis in the *cII* assay and these experiments will be performed with a plasmid system containing a single BPDE lesion and with a catalytic deficient Rev1 in the future.

ROLE OF TLS POLYMERASES IN REPLICATION ACROSS A SINGLE N^2-BPDE-DG ADDUCT

The big blue mice fibroblast cells offer an excellent model for mutagenesis studies in a chromosomal gene. Studying lesion bypass across a chromosomal gene allows for validation of our shuttle vector as a model for lesion bypass replication. The system however can be influenced by non-minor groove lesions such as those made by reaction of BPDE with N^6-dA BPDE (19). The mutational studies provide no indication of the relative contributions of TLS Pols to replication through the N^2-dG BPDE adduct. For this purpose, we have analyzed the contributions of TLS Pols to replicating through the N^2-dG BPDE adduct carried on a SV40-based duplex plasmid.

We utilized a heteroduplex plasmid system previously developed in our laboratory (49). The system is based on a modified plasmid pBluescript vector (Stratagene) containing a target sequence with a single DNA lesion, a lacZ' reporter, and an SV40 replication origin for bidirectional replication in SV40 transformed mammalian cells. The rationale for this plasmid assay is as follows: replication of a plasmid containing a single N^2-BPDE-dG adduct could occur either by TLS or template switch, and in most studies, it is difficult to determine which damage tolerance process is utilized (57). However, the heteroduplex TLS plasmid assay functions in an elegant way to differentiate between TLS and template switch. The strand with the N^2-BPDE-dG adduct has an in frame lacZ' gene while the opposite strand has a lacZ gene that is out frame (Figure 8 in Introduction). TLS produces a lacZ' gene which is in frame and generates blue colonies (Figure 8) whereas replication through the lesion by template switching results in an out of frame lacZ' gene and white colonies on x-gal plates (Figure 8). TLS levels are calculated from the number of blue colonies among the Kan^+ colonies. The TLS plasmid system allows for the determination of strand bias since the lesion can inserted into the leading or lagging strand.

Table 6. Effect of siRNA knockdown of TLS Pols on the Frequency of Replicative Bypass of N^2-BPDE-dG Lesions in Human Cells

siRNA KO	No of Kan^+ colonies	No blue colonies among Kan^+	% TLS
NC (GFP) or (Luc)	555	88	15.9
Pol η	452	69	15.3
Pol ι	466	40	8.6
Pol κ	656	43	6.6
Rev 1	774	37	4.8
Rev 3 (Subunit of Pol ζ)	564	97	17.2
Rev 7 (Subunit of Pol ζ)	250	38	15.2
Pol θ	335	39	11.6
Pol ν	230	26	11.3

The TLS frequency across a single N^2-BPDE-dG adduct after depletion of various TLS Pols closely mirrors the results obtained from the *cII* assay system (Table 6 compared with Table 5). Depletion of Pol ζ's subunits Rev3 or Rev7, or Pol η, had no effect on the TLS frequency across BPDE adducts. The lack of any discernible effect in TLS frequency after depletion of Pol η further suggests that Pol η's role in the cell after BPDE damage is in bypassing major groove N^6-BPDE-dA lesions and that Pol η is dispensable for bypassing minor groove N^2-BPDE-dG lesions. Depletion of Pols ι, κ, θ, ν, or Rev1 resulted in large decreases in the TLS frequency across a single minor groove lesion, again demonstrating that these are the TLS Pols responsible for bypassing minor groove BPDE lesions. Depletion of Pols θ, or ν, resulted in a smaller decrease in TLS activity than depletion of Pols ι, or κ, suggesting that Pols ι, or κ, are the major error prone inserter and extender polymerases respectively, if previous structural studies are taken into account. The large decrease in TLS activity after depletion of Rev1 might be due to both its error-free insertion activity and its ability to acts as a platform for recruitment of other TLS Pols.

BPDE SENSITIVITY AFTER DEPLETION OF TLS POLS

Deregulation of TLS polymerases can be harmful for the survival of a cell affected by the cognate DNA damage of such polymerase. Deficiency of Pol η results in the xeroderma pigmentosum variant, these cells are hypersensitive to UV irradiation, matching the role of Pol η's error-free role in TLS across cis-syn TT dimers (45-49). Similarly, defects of Pol κ have revealed a hypersensitivity towards BPDE (42). However, sensitivity towards minor groove N^2-BPDE-dG adducts has not been extensively studied with the other TLS polymerases. Increased sensitivity would confirm an important role in lesion bypass by any of the TLS polymerases and complement the mutagenesis studies.

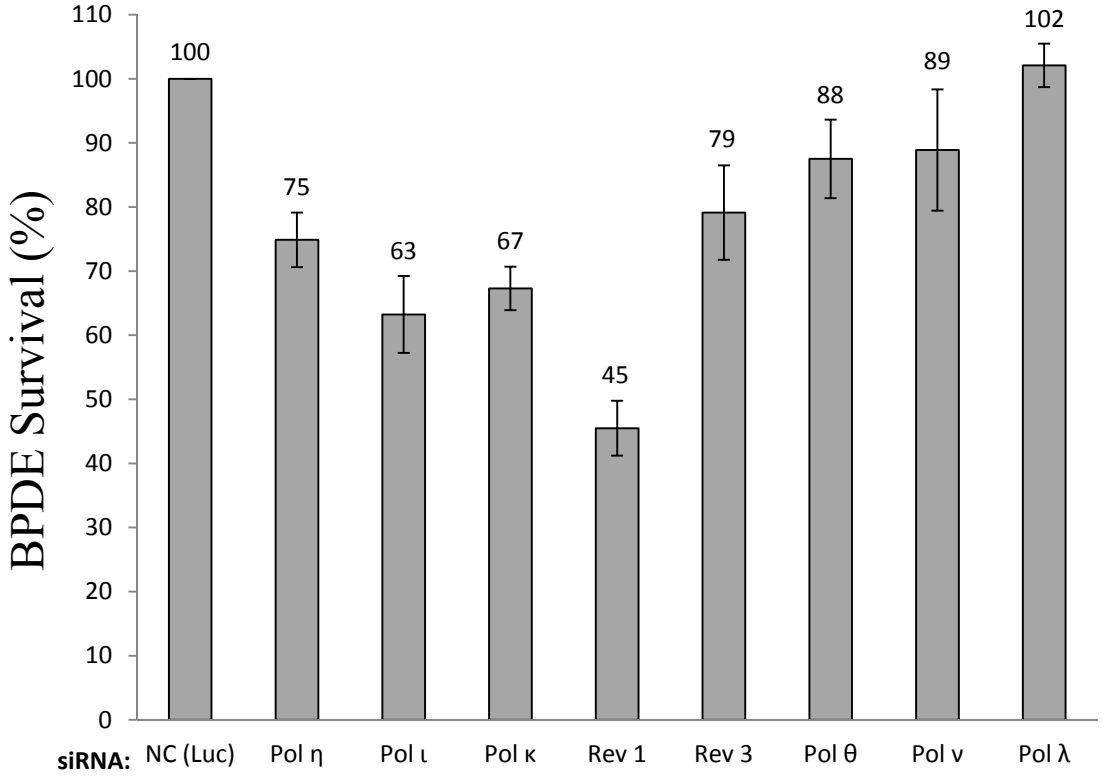


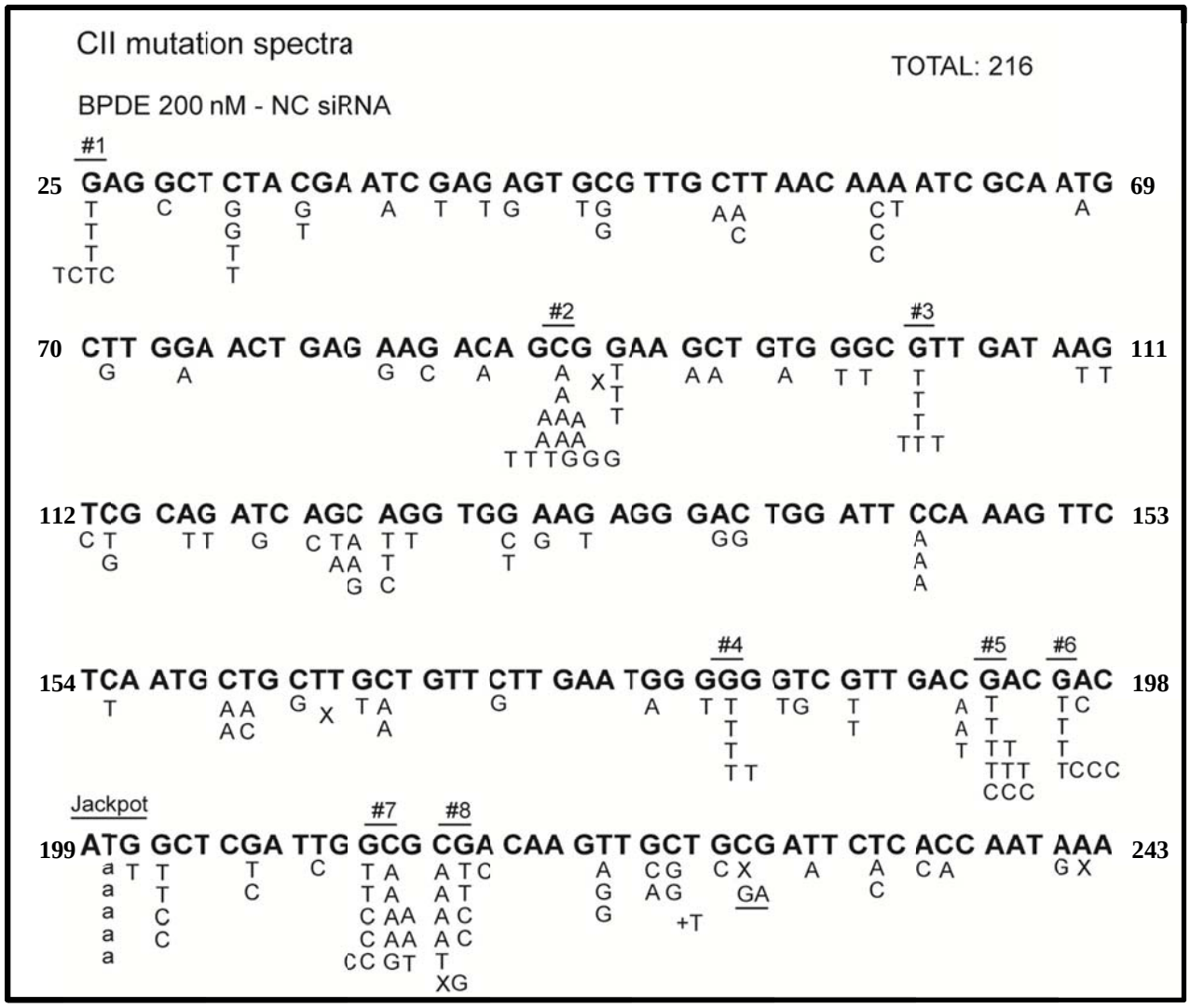
Figure 9. Sensitivity to BPDE after depletion of TLS Pols

Survival of human fibroblasts after siRNA depletion of TLS Pols and exposure to 500 nM BPDE.

BPDE sensitivity assay data for human fibroblasts closely mirrors the results from the *cII* mutagenesis and the TLS plasmid assay (refer to Table 4, Table 6, and Figure 9). The high sensitivity of human cells to BPDE upon Rev1 knockdown points to the central role of Rev1 in replication across BPDE lesions and, coupled with previous structural and biochemical studies of Rev1, would suggest that Rev1's role is to act as the major error-free inserter polymerase across BPDE lesions. Sensitivity of BPDE after depletion of pols θ and ν increases slightly suggesting that these TLS pols play a smaller role in lesion bypass of minor groove adducts. Sensitivity to pols ι and κ , together with the *cII* mutagenesis and the TLS assay data, and previous structural and biochemical studies, further suggest that Pol κ might be the major extender polymerase in these lesions and that the major error-prone inserter polymerase is Pol ι not ν. Sensitivity increases after depletion of Pol η might be due to its role in bypassing N^6-BPDE adducts. Sensitivity depletion of Pol ζ might be due to its role in recombinatorial repair.

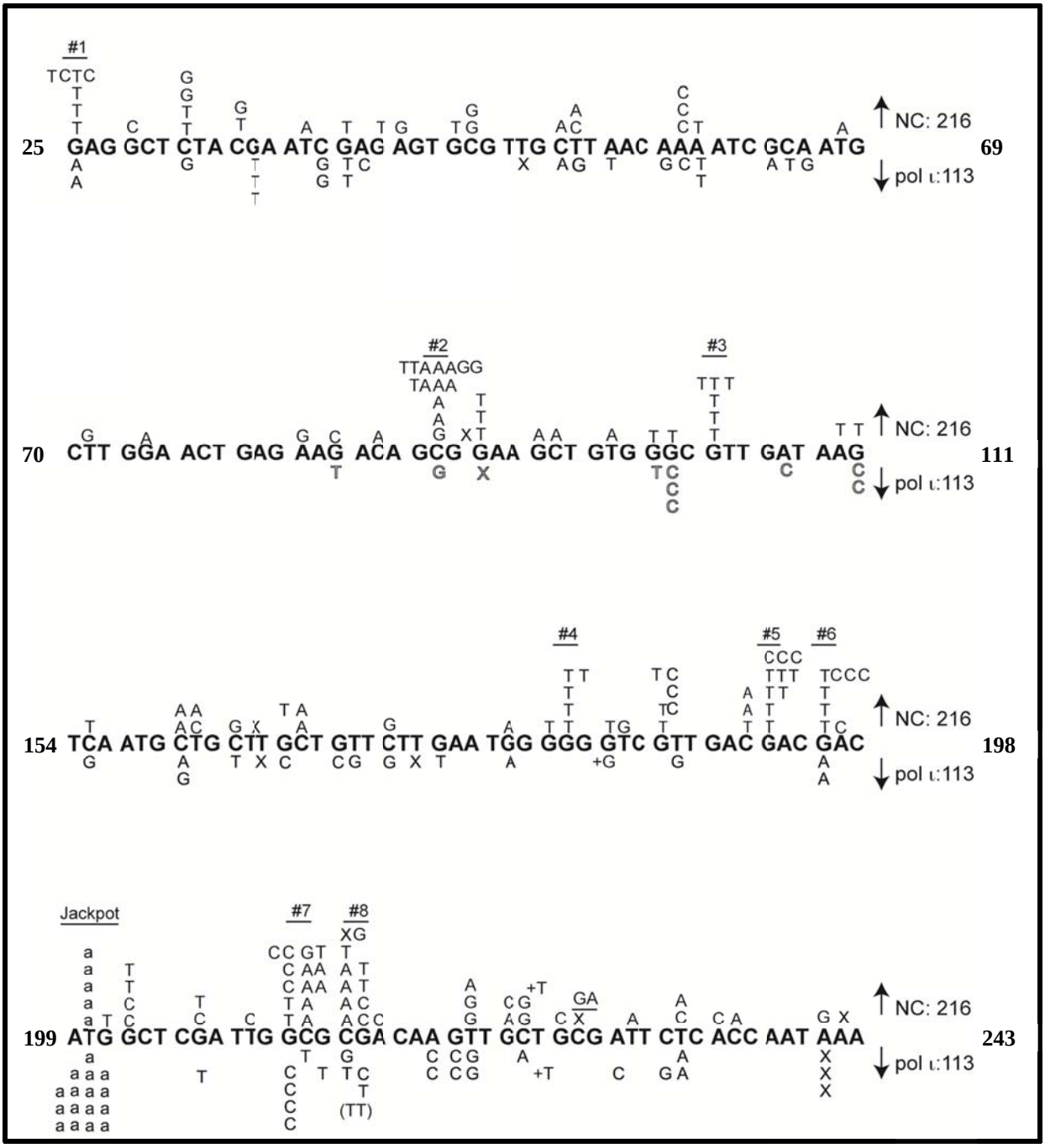
MUTATION SPECTRA AFTER DEPLETION OF TLS POLS IN THE *cII* GENE

Mutations vary depending on the gene and DNA damage but they tend to accumulate at specific hotspots. These mutational hotspots reflect intrinsic properties of the mutagenesis process and can provide insights into the molecular mechanisms of mutagenesis. In order to understand the role that the TLS Pols play in mutagenesis across BPDE lesions the *cII* gene from mutant plaques was amplified by PCR, sequenced, and analyzed for mutational hotspots.



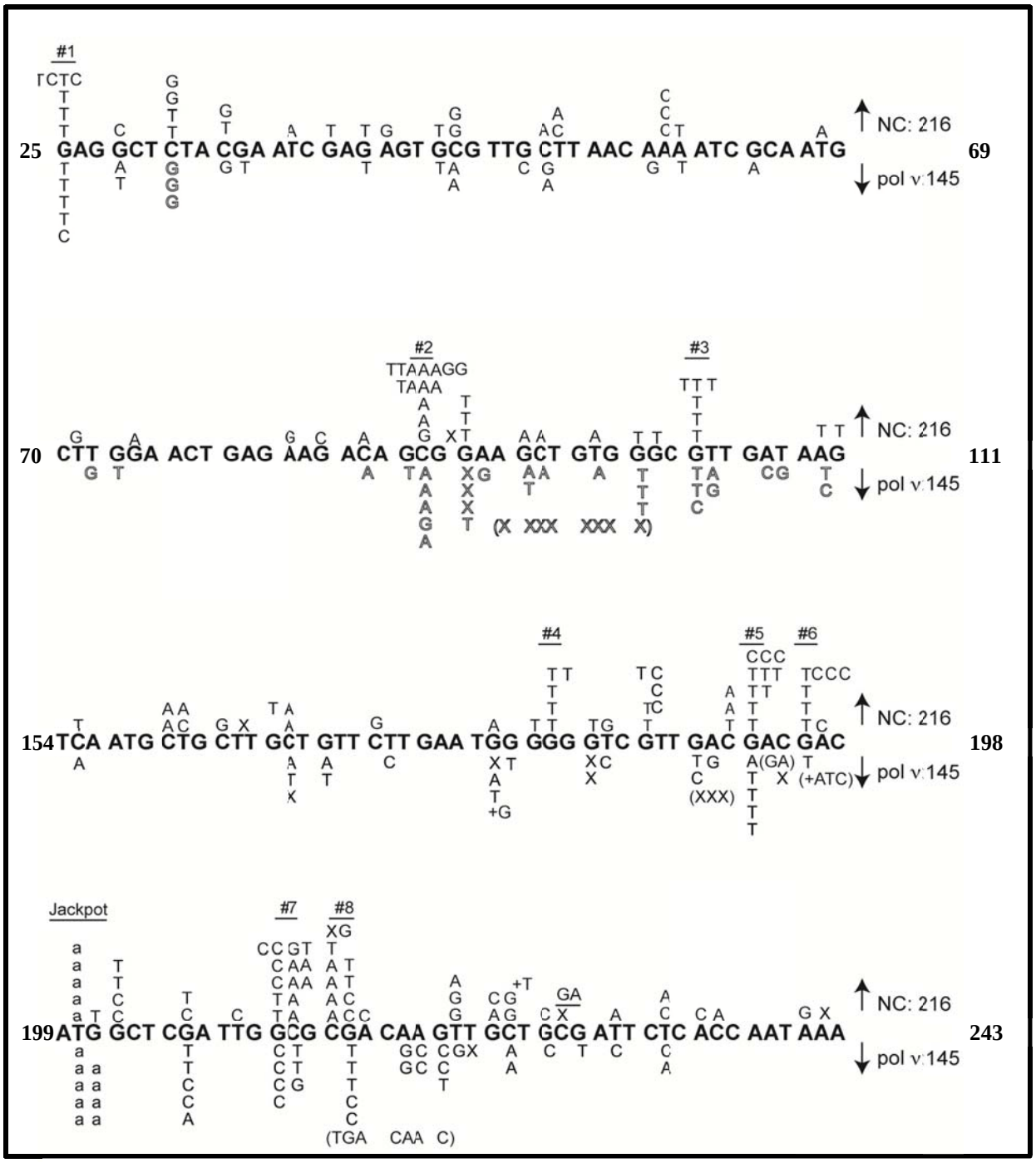
'ol

tations
leotides



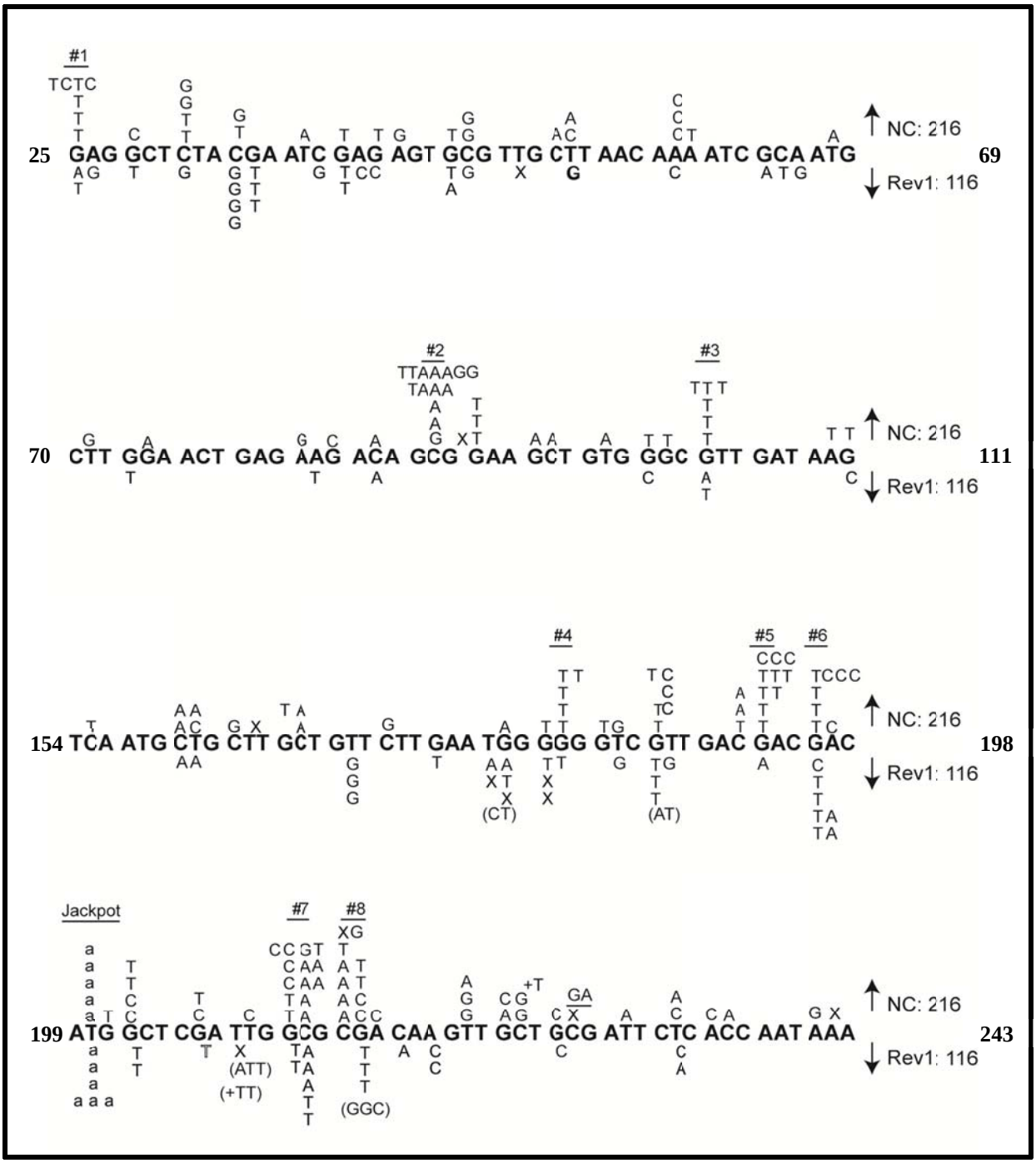
ted cells

cII gene in
cII gene
ntly



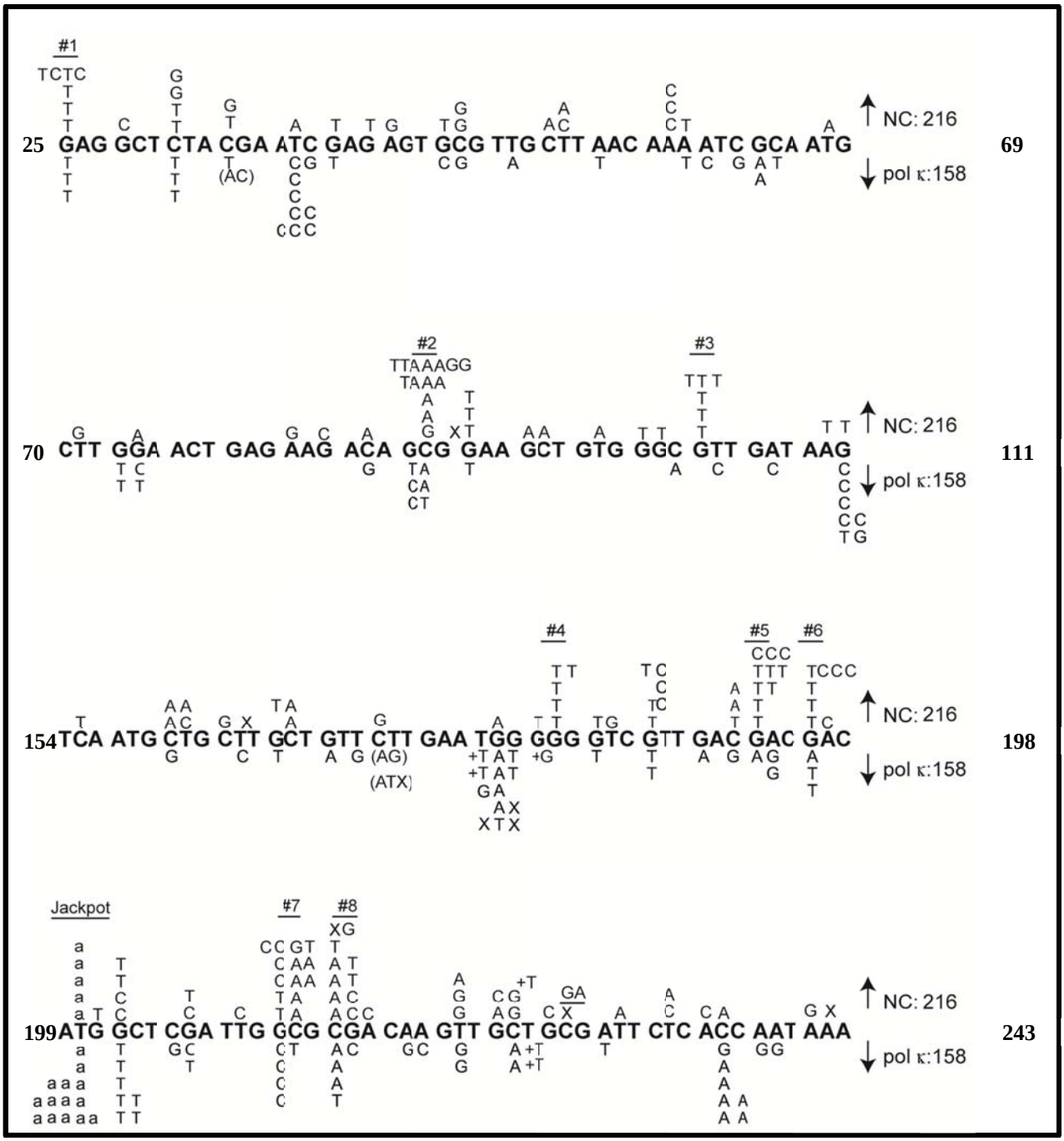
l cells

I gene in
I gene and
curring



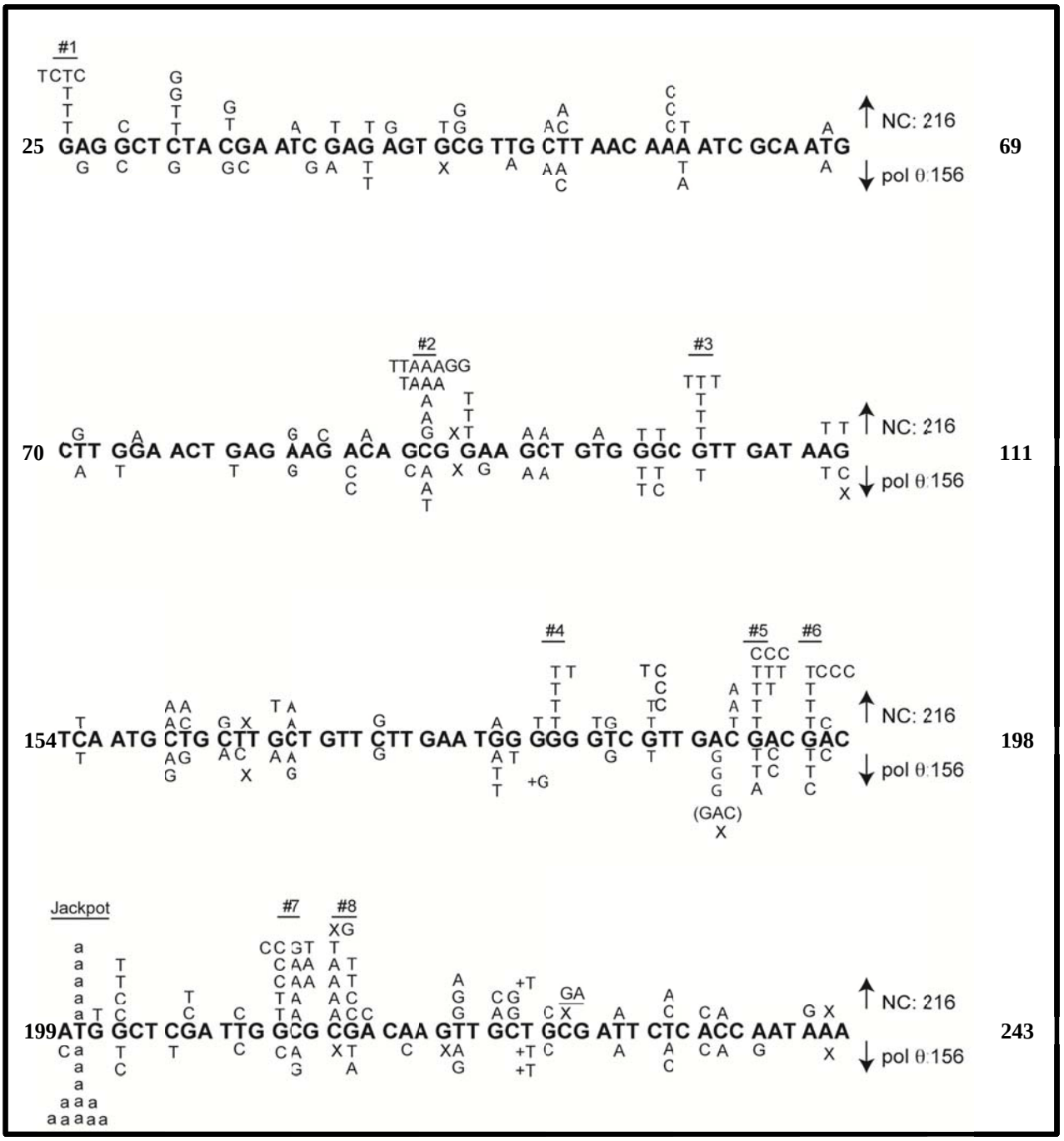
l cells

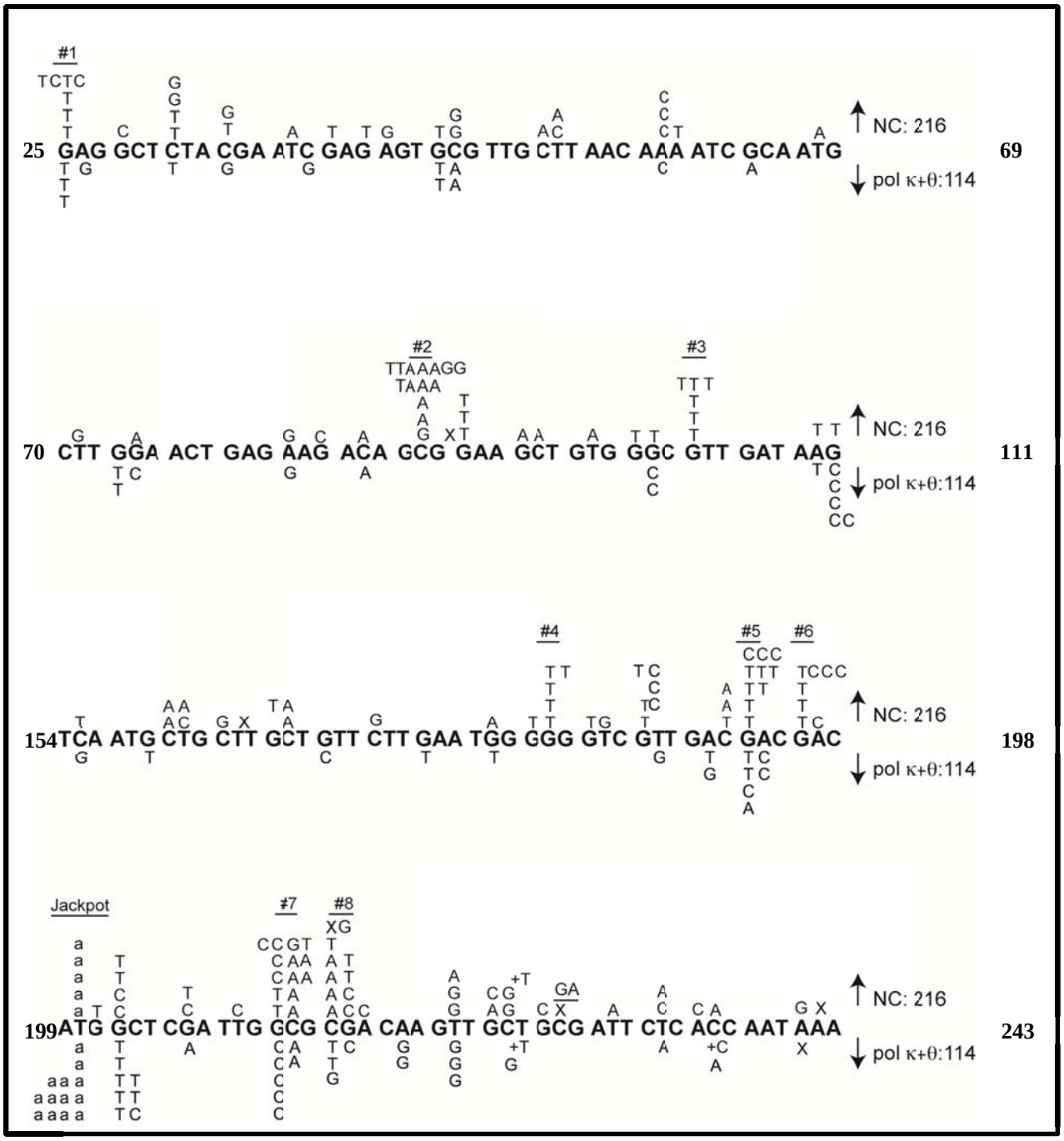
gene in
gene and
urring

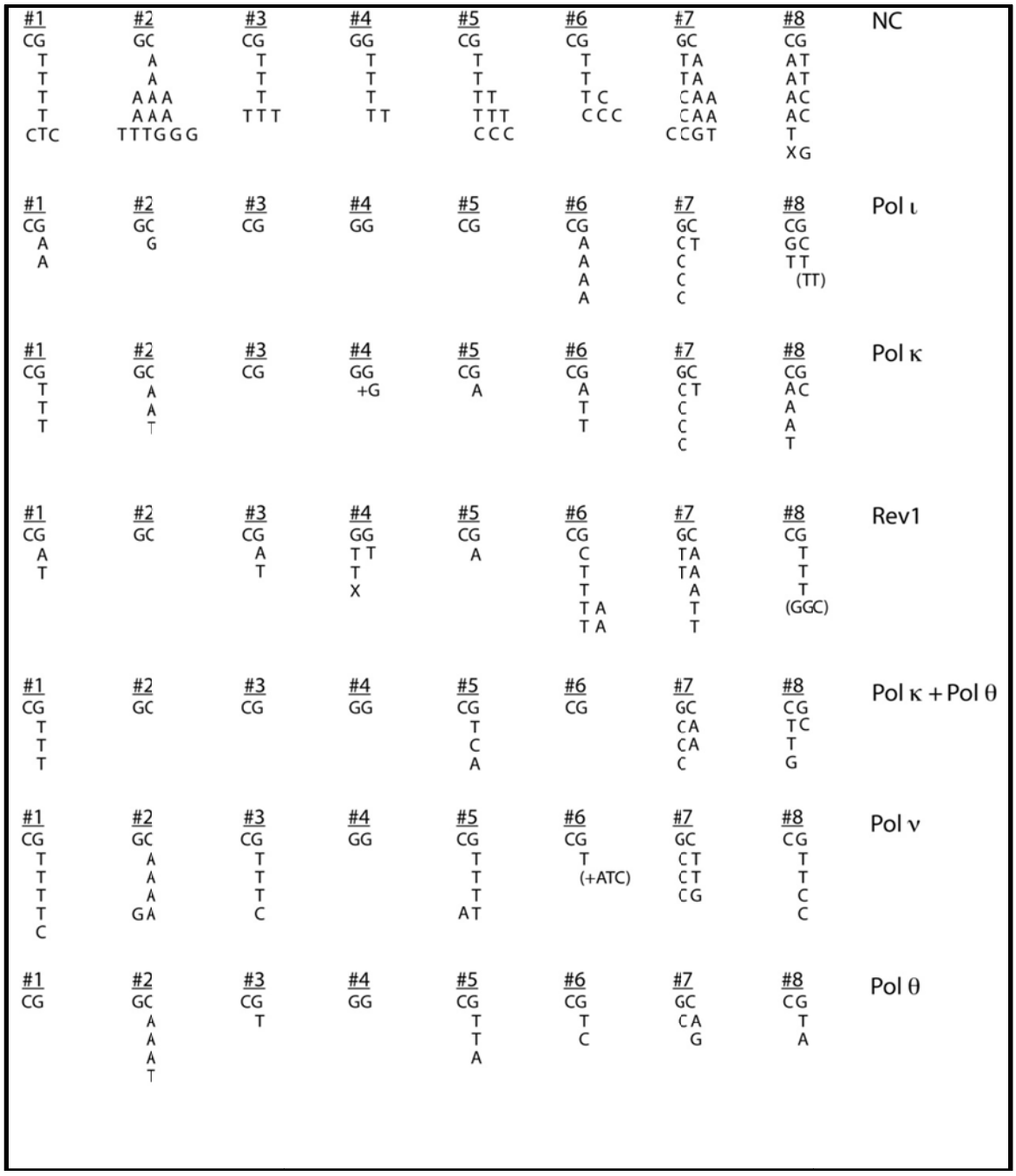


l cells

gene in
gene and
urring


#1
25 GAG GCT CTA CGA ATC GAG AGT GCG TTG CTT AAC AAA ATC GCA ATG 69
NC: 216
pol θ:156
#2
#3
70 CTT GGA ACT GAG AAG ACA GCG GAA GCT GTG GGC GTT GAT AAG 111
NC: 216
pol θ:156
#4
#5
#6
154 TCA ATG CTG CTT GCT GTT CTT GAA TGG GGG GTC GTT GAC GAC GAC 198
NC: 216
pol θ:156
Jackpot
#7
#8
199 ATG GCT CGA TTG GCG CGA CAA GTT GCT GCG ATT CTC ACC AAT AAA 243
NC: 216
pol θ:156


#1
25 GAG GCT CTA CGA ATC GAG AGT GCG TTG CTT AAC AAA ATC GCA ATG 69
NC: 216
pol κ+θ:114
#2
#3
70 CTT GGA ACT GAG AAG ACA GCG GAA GCT GTG GGC GTT GAT AAG 111
NC: 216
pol κ+θ:114
#4
#5
#6
154 TCA ATG CTG CTT GCT GTT CTT GAA TGG GGG GTC GTT GAC GAC GAC 198
NC: 216
pol κ+θ:114
Jackpot
#7
#8
199 ATG GCT CGA TTG GCG CGA CAA GTT GCT GCG ATT CTC ACC AAT AAA 243
NC: 216
pol κ+θ:114


#1 #2 #3 #4 #5 #6 #7 #8 NC
CG GC CG GG CG CG GC CG
T A T T T T TA AT
T A T T TT TA AT
T AAA T TT CAA AC
T AAA TTT CAA AC
CTC TTTGGG CCC CCGT T
XG
#1 #2 #3 #4 #5 #6 #7 #8 Pol ι
CG GC CG GG CG CG GC CG
A G A CT GC
A C C TT
C C (TT)
C
#1 #2 #3 #4 #5 #6 #7 #8 Pol κ
CG GC CG GG CG CG GC CG
T A +G A A CT AC
T A C A
T T C T
C
#1 #2 #3 #4 #5 #6 #7 #8 Rev1
CG GC CG GG CG CG GC CG
A A TT C TA T
T T X T TA T
A T T (GGC)
TA TA T
#1 #2 #3 #4 #5 #6 #7 #8 Pol κ + Pol θ
CG GC CG GG CG CG GC CG
T T CA TC
T C CA T
A C G
#1 #2 #3 #4 #5 #6 #7 #8 Pol ν
CG GC CG GG CG CG GC CG
T A T T T (+ATC) CT T
T A T T CT T
T A T T CG C
AT C
#1 #2 #3 #4 #5 #6 #7 #8 Pol θ
CG GC CG GG CG CG GC CG
A T T T CA T
A C A G A
A T
T

cells

Mutational spectra data from control siRNA experiments in the BBMEF cells reveal that the majority of mutations induced after BPDE treatment are G→T transversions at GC base pairs (Figure 10). Importantly, this correlates with the mutational fingerprint obtained from tissues in studies of tobacco smoke carcinogenesis (14, 23-27). Mutations accumulated at 8 different hot spots throughout the *cII* gene (Figure 10). Depletion of Pol ι results in the disappearance of a large number of hot spots 2, 3, 4, and 5 and decreases in hot spots 1 and 6 with only hot spots 7 and 8 remaining, reflecting the probable role of Pol ι as the main error prone inserter across BPDE lesions (Figure 11 and 17). In contrast, depletion of Pol ν results in the disappearance of hot pots 4 and decreases in hot spot 6 while the remaining hot spots were unaffected, reflective of Pol ν's minor role in insertion across BPDE lesions (Figure 12 and 17). The large differences in hot spot patterns are probably indicative of the different roles of Pols ι and ν in insertion across BPDE lesions.

The mutagenesis experiments suggested a role for Pol κ in conjunction with Pol ι in lesion bypass of BPDE minor groove adducts. The pattern of hot spots after depletion of Pol κ is similar to Pol ι with decreases in all the hot spots except 2, 7, and 8 probably reflecting the importance of Pol κ as the major extender polymerase across BPDE lesions (Figure 14). Interestingly, hot spot 7 was only decreased when Pol θ (Figure 15 and 17) was depleted and the hot spot pattern after Pol θ depletion resulted in decreases of hot spots 7 and 8 which are both maintained after depletion of Pol ι (Figure 11 and 17) and Pol κ (Figure 14 and 17) suggesting that Pol θ is not involved with Pol κ and might have a smaller role with Pol ι. Simultaneous depletion of both Pols κ and θ resulted in large decreases in all the mutational hot spots except 7 (Figure 15 and 17) further adding to the evidence that Pol κ and θ act in different pathways to replicate across BPDE lesions. This large decrease in mutational hot spots would be expected if TLS across BPDE lesions was blocked by depleting the Pols responsible for the extension step of TLS. Depletion of

Rev1 resulted in decreases throughout the *cII* gene and in most of the mutational hot spots with only hot spots 4, 6, and 7 remaining (Figure 13 and 17). This might reflect the probable role of Rev1 as a structural element in TLS opposite BPDE lesion. This pattern is also interesting since Pol ν had a large effect in hot spots 4 and 6 and Pol θ was the only polymerase to affect hot spot 7. This might indicate that if Rev1 is serving as a structural platform for TLS in BPDE minor groove lesions it might be acting only with Pols ι and κ and not with Pols ν and Pol θ.

MUTAGENICITY OF TLS OPPOSITE N^2-BPDE-DG

The blue mutant colonies from the TLS heteroduplex plasmid system assay were utilized for amplification of the *lacZ* gene by PCR and the samples were analyzed by restriction enzyme and sequencing to determine the types of mutations that occur after depletion of the TLS pols in response to BPDE damage.

Table 7. Effects of siRNA knockdowns of TLS Pols on the frequencies of mutations opposite N^2-BPDE-dG

siRNA KO	No of Kan^+ colonies	Nucleotide Inserted				Mutation Frequency
		A	G	C	T	%
XPA NC (Luc)	135	25	1	107	1	18.5

As determined from Table 7, TLS opposite N^2-BPDE-dG is highly mutagenic with mutations occurring ~18.5% of the time in NER deficient $XPA^{-/-}$ human fibroblasts. The majority of the mutations were G→T transversions similar to the *cII* mutations seen in response to BPDE damage.

REQUIREMENT OF TLS POLS FOR DNA REPLICATION FOCI IN BPDE TREATED HUMAN CELLS

The results from previous aims suggest the existence of at least three pathways of bypass of BPDE lesions. These include a Rev1 pathway utilizing either Pols θ or κ for extension, an error-prone pathway utilizing Pol ι and Pol κ or θ for extension and finally a third minor error-prone pathway utilizing Pol ν with θ or κ for extension. It now becomes important to understand how these TLS Pols are regulated and how the cell activates these pathways in response to BPDE treatment. It has been previously reported that the TLS Pols are regulated by recruitment to the lesion sites by the clamp loader PCNA. Rev1 has also been suggested to play a structural role by mediating recruitment of the TLS Pols at the lesion site (35). We find that Rev1 knockdown results in a decrease in mutagenesis (Table 4). This is surprising since Rev1 is limited in its polymerase activity to insertion of a deoxycytidyl which should result in error-free bypass of the N^2-dG BPDE lesion (35). Replication foci formation studies were performed in order to further explore the idea that Rev1 is playing a structural role in promoting lesion bypass by the error-prone pathways.

Table 8. Effect of BPDE on foci formation in normal human fibroblasts carrying GPP-tagged TLS Pols

GFP-Pol	siRNA	% Foci Positive Cells	% Change
GFP-Pol ι	NC (Luc)	31.3	100
	Pol η	27.5	91
	Pol κ	20.8	66
	Pol θ	35	112
	Pol ν	37	118
	Rev1	21	69
GFP-Pol κ	NC (Luc)	23.5	100
	Pol η	19.1	81
	Pol ι	11.8	34
	Pol θ	19.8	94
	Pol ν	21	89
	Rev1	9	38
GFP-Rev1	No Damage	17.1	-
	NC (Luc)	44.5	100
	Pol η	51.9	117
	Pol κ	27.6	62
	Pol ν	46.5	104
	Pol θ	57	128

Normal cells were treated with BPDE 500nm for 30 min at 37 °C

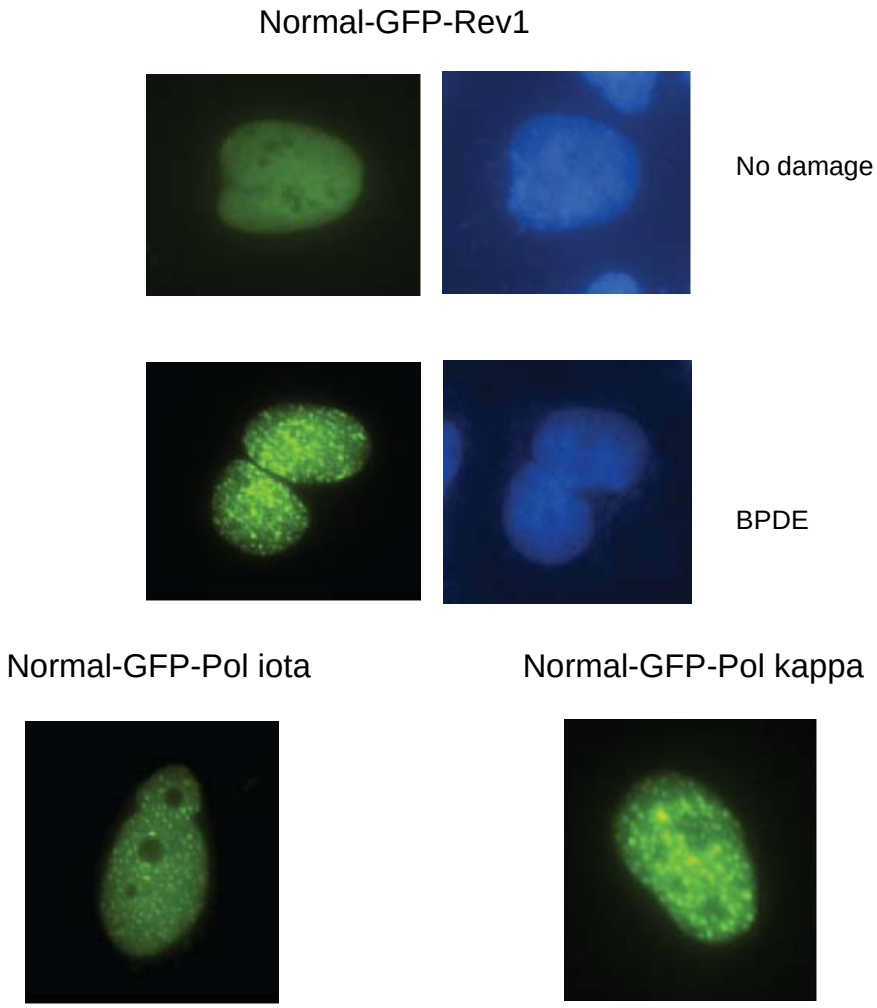


Figure 18. TLS pols DNA replication foci formation after treatment with BPDE

Human fibroblasts were transfected with GFP tagged TLS pols. Twenty-four hours after transfection the cells were treated with 500 nM BPDE, incubated for 6 h then fixed with paraformaldehyde and stained with DAPI.

For this purpose, it was determined whether the knockdown of Rev1 results in a reduction in the accumulation of Pols ι or κ into replication foci in response to BPDE treatment. Exposure of cells to BPDE results in the accumulation of TLS Pols into replication foci at the lesion site (Figure 18). Importantly, Rev1 knockdown resulted in a decrease in replication foci formation for TLS Pols ι and κ (Table 8), suggesting that Rev1 plays a structural role in the recruitment of these Pols to the lesion site. Depletion of Pol ι resulted in a decrease in Pol κ replication foci, while depletion of Pol κ resulted

in a decrease in Pol ι foci, further suggesting that these pols are involved in the same lesion bypass pathway across BPDE lesions. Depletion of Pols η, ν, or θ had no effect on Pols ι, κ, or Rev1 foci formation (Table 8) which is expected since Pol η is not involved in lesion bypass of N^2-BPDE-dG lesions and Pols ν and θ are involved in a different minor mutagenic bypass across BPDE lesions. Interestingly, depletion of Pol κ resulted in a decrease in Rev1 foci after BPDE treatment (Table 8). A simple model of foci accumulation would imply a stepwise assembly where Rev1 recruits Pols ι and κ. The fact that Pol κ affects Rev1 foci formation is rather surprising since it implies that Pol κ can itself modulate the assembly of Rev1 at lesion sites. It will be interesting to see whether the same effect is present after depletion of Pol ι. The implications for this finding are that recruitment of TLS Pols to lesion sites is not a simple stepwise model, rather it is probable that the TLS Pols are being assembled into multi-protein complexes and that the interactions between TLS Pols and other unidentified proteins are essential for successful assembly at DNA lesion sites.

THE ROLE OF POL ζ IN DAMAGE TOLERANCE ACROSS BPDE MINOR GROOVE ADDUCTS

Mutagenesis data from exposure to BPDE in a chromosomal gene and single N^2-BPDE-dG demonstrate that in normal lesion bypass Pol ζ is not utilized by the cell. A small effect on sensitivity occurred after depletion of Pol ζ and exposure to BPDE. Other studies have noted an effect in sensitivity and cell cycle progression in human fibroblasts after reduction of hRev7 expression. However, mutagenesis and the types of mutations after BPDE exposure remained unchanged (125). Studies *in vitro* have been contradictory with some finding no bypass activity (56), error-free extension (126), or error-prone incorporation (127). Studies in yeast have revealed that Pol ζ is required for mutations (128, 129), as well as in human cells in gapped plasmid assays (40, 53, 130). However,

mutagenesis studies in human cells with the HPRT assay have found no effects in mutations after depletion of Rev3 or Rev7 (125, 131). This might be due to the fact that lesion bypass in a gapped plasmid would closely resemble bypass of lesions in yeast, whose TLS occurs post-replicatively in gaps (132, 133) and the role of Pol ζ would be in gap filling rather than TLS coordinated with the replication fork machinery.

Pol ζ introduces complex mutations with multiple changes within short DNA stretches (134-136), which might be due to its role in recombinatorial repair (137-139). Interestingly, removal of the NER (140, 136), base excision repair (BER) (141), or homologous recombination (HR) pathways (136) results in an elevated level of endogenous replication blocking lesions which are channeled into alternate pathways of repair under which Pol ζ, recruited by Rev1, has a high contribution to spontaneous mutagenesis under these conditions. Further evidence for this lies on studies inhibiting template switching mechanisms through Rad 52 or Mms2 depletion, leading to activation of Pol ζ and increases in mutagenesis (142, 143) and the role of Pol ζ in inhibiting HR and stimulating non-homolohous end joining (NHEJ) (144). Furthermore, increases in replisome impairment through undamaged DNA by defects of Pol δ or ε results in a similar mutator phenotype attributed to Pol ζ activity (139). This all suggests that Pol ζ/Rev1 provide an efficient and generalized mutagenic means of replicating at stalled forks when other methods of repair or DNA damage tolerance have been exhausted, possibly through template switching and strand realignment followed by extension of the mismatched primer termini (139, 145). This increase in mutagenesis prevents DSB formation and chromosomal rearrangements and the lack of this generalized Pol ζ pathway results in increases in tumorigenesis (145, 146).

Table 9. Effect of TLS Pols on HR deficient Fibroblast after BPDE induced mutations in the *cII* gene

siRNA KO	Mutation Frequency ($X10^{-5}$)
No BPDE	11.4 ± 2.9
NC (GFP) or (Luc)	38.1 ± 3.3
Rad51	61.2 ± 8.0
Rad52	55.9
Rev1	22.6 ± 2.0
Rev3 (Subunit of Pol ζ)	40.4 ± 1.8
Rad51 +Rev3 (Subunit of Pol ζ)	37.3 ± 1.7
Rad51 +Rev1	24.5 ± 1.8

BBMEF cells were treated with BPDE 200 nm for 30 min at 37 °C

In order to test the role of Pol ζ across BPDE lesions, HR was inhibited by depletion of either Rad51 or Rad52 (Table 9) resulting in an increase in mutagenesis as the error-free recombinatorial repair was blocked. This is unsurprising, as previous studies in human cancer cells have demonstrated similar increases in mutagenicity and cytotoxicity by depletion of HR proteins (147- 150). Interestingly, this increase in mutagenesis was attributed solely to increases in the activity of Pol ζ, as depletion of Rev3, the catalytic subunit of Pol ζ, resulted in mutagenesis decreasing to wt levels. Importantly, this activity of Pol ζ was regulated by the activity of Rev1 since depletion of Rev1 resulted in the loss of this increase in mutagenesis to levels similar to depletion of Rev1 alone (Table 9).

REGULATION OF TLS ACROSS BPDE LESIONS

DNA Damage Responses After BPDE Treatment

We have now identified the TLS Pols responsible for bypassing minor groove adducts. Next, we wanted to identify the methods by which the cell regulates the activities of the

TLS Pols to lesion bypass of minor groove lesions. Individual TLS Pols have different specificities and their activities must be closely regulated to particular DNA lesions. The DNA clamp proliferating cell nuclear antigen (PCNA) plays a key structural role in DNA replication and repair mechanisms (151). Ubiquitination of PCNA is believed to play a role in the polymerase switch from replicative to TLS Pols during lesion bypass (152). The interaction of the TLS Pols with PCNA increases their efficiency of incorporation (153). The Rad6/Rad18 group of proteins have been demonstrated to be able to monoubiquitinate PCNA at K164 allowing the polymerase switch (152, 154). Opposite this it was speculated that the Rad5 group would be able to polyubiquitinate PCNA and activate template switching mechanisms (152). The mammalian homologues of Rad5 are the E3 ubiquitin ligase HLTF and SHPRH. Their roles in TLS are more complicated than initially though. Across UV lesions HLTF enhances PCNA monoubiquitination and recruitment of Pol η to replication foci while inhibiting the activity of SHPRH. When treated with an alkylating agent however, HLTF is degraded and SHPRH interacts with Rad18 to promote Pol κ recruitment to sites of DNA damage (102).

Table 10. Effect of HLTF on BPDE induced mutations in the *cII* gene

siRNA KO	Mutation Frequency ($X10^{-5}$)
No BPDE	11.4 ± 2.9
NC (GFP) or (Luc)	41.7 ± 3.3
HLTF	18.0 ± 2.4

BBMEF cells were treated with BPDE 200 nm for 30 min at 37 °C

In order to explore whether ubiquitination and the Rad5 homolog HLTF was playing a role across BPDE lesions depletion of HLTF was performed in BBMEF cells followed by mutagenesis assays in the *cII* gene. As seen from Table 10, depletion of HLTF resulted in a decrease in mutagenesis suggesting that HLTF is responsible for part of the regulation

of TLS Pols in lesion bypass of minor groove adducts through a possible mechanism involving ubiquitination.

In addition to ubiquitination, evidence has pointed to a role of post translational modifications such as phosphorylation as a method of regulation of the TLS Pols. Ataxia Telangiectasia and Rad3 related (ATR) is known to regulate cellular responses to DNA replication stress and the activity of DNA repair proteins (155, 156). Pol η is phosphorylated after UV damage at S587 and T617 through ATR and Protein Kinase C (PKC) (157). This phosphorylation mediates Pol η's recruitment to replication foci. Additionally, Chk1 a downstream effector of ATR has a PCNA interacting motif and a study revealed that Chk1 can recruit Pol η to sites of UV damage while surprisingly Pol η can itself increase phosphorylation of Chk1's S317 (158). Yeast Rev1 has similarly been associated with recruitment and phosphorylation by the yeast's ATR homolog MEC1 (159) which further promote activity of a Pol ζ/Rev1 complex (207). ATR, Chk1, and Replication Protein A (RPA) have been implicated in PCNA foci formation after BPDE treatment (160).

Table 11. Effect of Checkpoint genes on BPDE induced mutations in the *cII* gene.

siRNA KO	Mutation Frequency ($X10^{-5}$)
No BPDE	11.4 ± 2.9
NC (GFP) or (Luc)	18.8 ± 2.3
ATR	13.6 ± 0.9
Chk1	11.5 ± 1.0

BBMEF cells were treated with BPDE 100 nm for 30 min at 37 °C

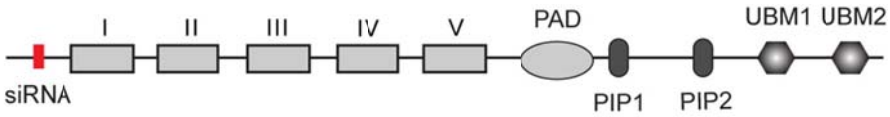
In order to explore whether ATR and phosphorylation were involved in regulation of lesion bypass across BPDE lesions depletion of ATR and its downstream effector Chk1

was performed in BBMEF cells followed by mutagenesis assays in the *cII* gene. Either ATR or Chk1 depletions were extremely toxic to the cell after BPDE treatment suggestive of the central role of ATR in the cellular DNA damage response. Therefore, the BPDE dosage was decreased to 100 nM for these experiments. As seen from Table 11, depletion of either ATR or Chk1 resulted in a decrease in mutagenesis compared to the siRNA control suggesting that ATR is involved in regulating the activities of the TLS Pols possibly through phosphorylation.

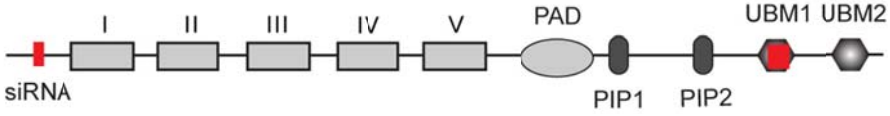
Mutagenesis of Conserved Protein Domains in TLS Pols

The TLS Pols have conserved Ubiquitin Binding Motifs (UBM or UBZ), which might play a role in their regulation across various DNA lesions (161-164). The TLS Pols also contain conserved PCNA Interacting Protein Boxes (PIP) which allows interactions with PCNA (153, 165, 166). Studies with TLS Pols containing deletions of their PIP domains resulted in interference with the TLS ability of the polymerase in both Pol η and UV lesions (167), and Pol κ and Tg lesions (168). In order to explore the roles of ubiquitination, phosphorylation, and protein-protein interactions in the regulation of lesion bypass across BPDE lesions, site directed mutagenesis was performed to create UBM/UBZ substitution mutants, PIP substitution mutants, phosphorylation deficient, or phosphomimetic mutants of TLS Pols ι, κ, or Rev1 (Figures 19-21).

hPol ι



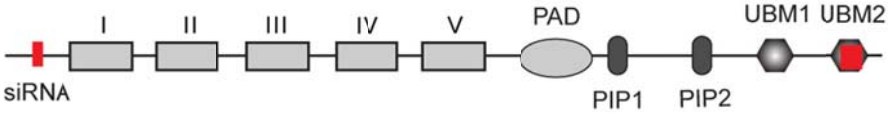
UBM I Mutants



1. UBM I D503A

2. UBZ II LP510,511AA

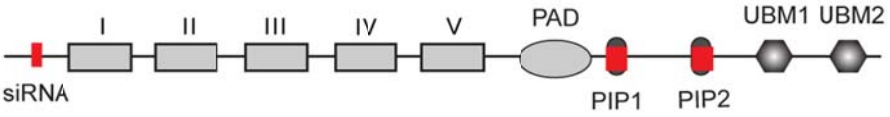
UBM II Mutants



3. UBM I D684A

4. UBM II LP691,692AA

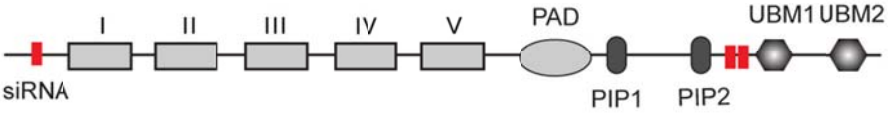
PIP Mutants



5. PIP I YY426,427AA

6. PIP II FF546,547AA

Phospho Mutants



7. S591A

8. S610A

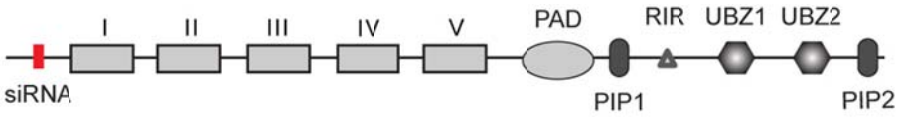
9. S591D

10. S610D

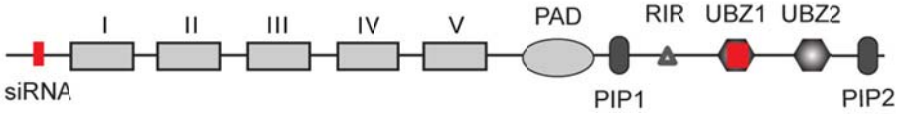
11. S591E

12. S610E

hPol κ

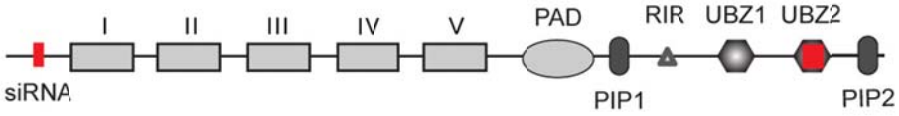


UBZ I Mutants



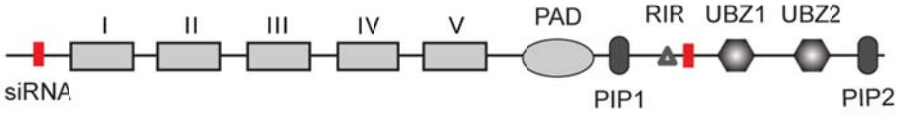
1. UBZ I H642A
2. UBZ II D644A

UBZ II Mutants

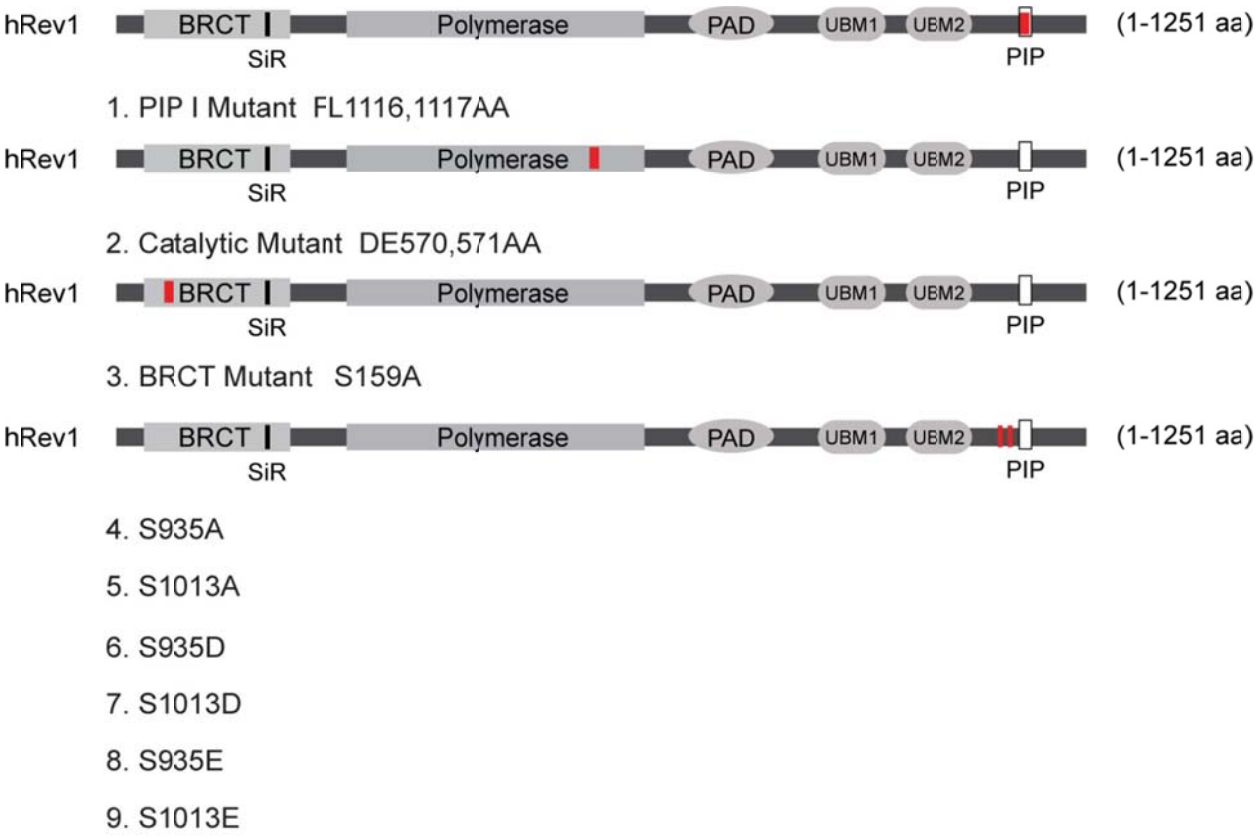


3. UBZ I H797A
4. UBZ II D799A

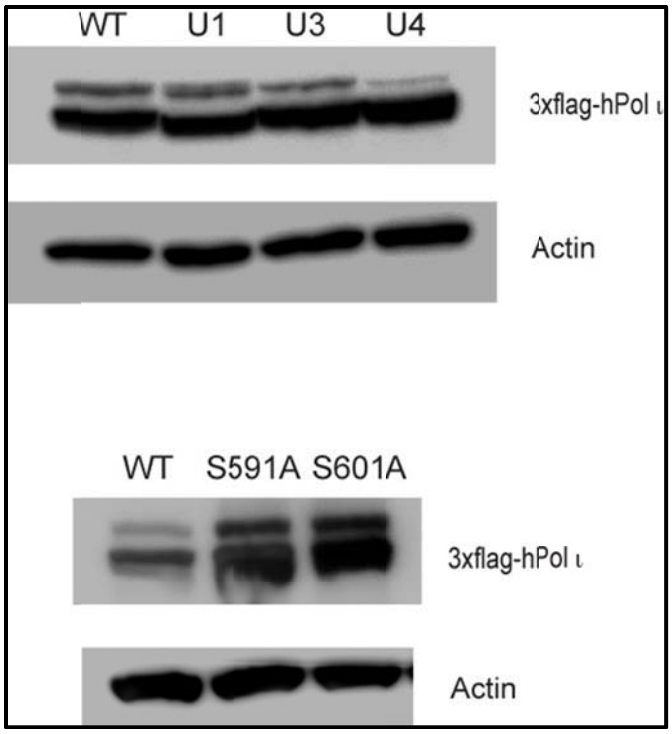
Phospho Mutants



5. S596A
6. S596D
7. S596E



Human fibroblasts were transfected by lipofection with eukaryotic expression vectors containing the TLS Pols mutants. The cells were then selected with Zeocin and positive stable pools were analyzed for positive TLS mutant Pol expression by western blotting with anti-Flag antibody (Figure 22). The stable transfectants will be utilized in the future in mutagenesis and TLS experiments in order to explore the roles of these protein domains in regulation of bypass across BPDE lesions.

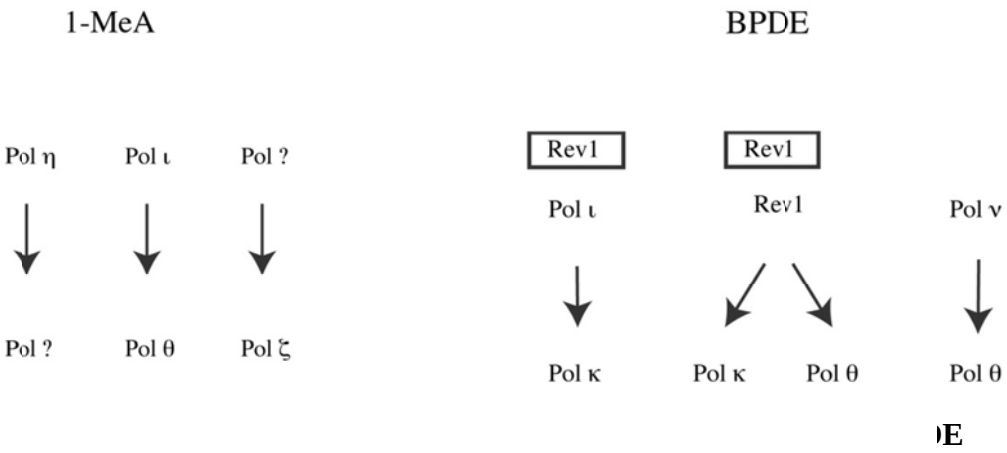

WT
U1
U3
U4
3xflag-hPol ι
Actin
WT
S591A
S601A
3xflag-hPol ι
Actin

mutants

Chapter 5 Discussion

ROLE OF TLS POLS IN BYPASS OF 1-MEA AND N^2-BPDE-DG LESIONS

We have determined the role that TLS Pols play in bypassing 1-MeA and N^2-BPDE-dG lesions. We have identified a role for Pols η, ι, θ, and ζ in the bypass of 1-MeA lesions. Furthermore, based on epistasis analysis after simultaneous depletion of TLS Pols and TLS frequency we have identified three independent pathways of 1-MeA bypass involving: 1) Pol η; 2) Pol ι and θ; and 3) Pol ζ (Figure 23). Depending on the lesion, bypass can be performed by one or two polymerases. In the case of 1-MeA, due to previous structural studies it is probable that Pol ι is functioning as an inserter polymerase and Pol θ would serve as an extender polymerase. Pol η has been known to be able to carry out both the insertion and extension steps across UV lesions and could be performing both steps across 1-MeA lesions. For the Pol ζ pathway, it is probable that Pol ζ, which has been known to play a role in extension of mismatch primer termini across various lesions, is functioning as an extension Pol. Currently, it is unknown whether another TLS Pol is involved in insertion across the 1-MeA lesion through which Pol ζ would then extend from the lesion. Biochemical studies of the TLS Pols bypassing a 1-MeA lesion will clarify the role of the various TLS Pols as inserters or extenders across 1-MeA lesions. Further TLS frequency experiments after depletion with other TLS Pols such as λ, ν, or μ might reveal a function for the bypass with Pol ζ across 1-MeA lesions. Such experiments will be performed in the future.



)E

The mechanisms of bypass for BPDE lesions are more complex than across 1-MeA. The Pols that are involved in this bypass are Pols ι, κ, ν, θ, and Rev1. Epistasis analysis and mutagenesis studies in the *cII* gene have allowed us to identify at least 3 pathways through which the cell bypasses these lesions: 1) Rev1 with the help of Pols κ or θ; 2) Pol ι with the help of Pol κ; and 3) Pol ν with the help of Pol θ (Figure 23). In addition to this, we have identified a structural role for Rev1 in the formation of replication foci for Pols ι and κ (Table 8), this role is further supported by the similar mutational hotspots between Rev1 and Pols ι and κ (Figures 10, 11, 12, 14, and 17). Furthermore, *cII* mutagenesis suggests a role for Pol κ in the same pathway as that of Pol ι (Table 5). Mutational spectra data suggests that Pol ι and κ might be involved in the same pathway, since the locations of hotspots closely resemble each other (Figures 11, 14, and 17). Simultaneous depletion of Pols ι and the other TLS Pols in the TLS assay with the heteroduplex plasmid will be performed in the future to further support this model. However, based on *cII* mutation spectra data and hotspot analysis, the pattern of hotspots after depletion of Pols ι or θ do not overlap suggesting that they might be involved in different pathways (Figures 11, 15, and 17). The effect of depletion of Pol θ in mutagenesis (Table 4) and TLS frequency (Table 6) is also smaller than that of Pol ι which would not be expected if they are acting in the same pathway. The roles of

Pols ν and θ are more limited than Pols ι, κ, and Rev1, mutagenesis, sensitivity, as well as TLS frequency data, demonstrate a larger effect with Pols ι, κ, and Rev1 (Tables 4 and 6; Figure 9) which would be expected if they are acting in the same bypass pathway as each other and in different pathways as Pols ι, κ, and Rev1. Similarly, Pol ν and θ, share similar hotspots and this might indicate that they are functioning in the same pathway (Figures 12, 15 and 17). More importantly, Rev1 does not act as a structural element for Pols ν and θ (Table 8) which would suggest that these Pols are regulated differently than Pols ι and κ and are probably not involved in a pathway with them. Nevertheless, in order to fully understand the roles of these TLS Pols to each other it is necessary in the future to carry out simultaneous depletion of the TLS Pols and test for epistasis by measuring the effects in TLS frequency. Biochemical experiments of lesion bypass with purified TLS Pols will help clarify the role of insertion vs extension across BPDE lesions.

THE ROLE OF TLS IN REPLICATION ACROSS VARIOUS LESIONS

Previous work has identified the role of TLS Pols in replicating across cis-syn TT, (6-4) TT photoproduct, and Tg. Across these lesions TLS provides a substantial contribution ~40% of TLS frequency in XPA fibroblasts (49, 175, 202, 203). Surprisingly, owing to the fact that biochemical studies of TLS Pols have revealed low fidelity and efficiencies, the TLS Pols bypass these lesions mostly in an error-free manner with only ~2% of mutations occurring. Similarly, opposite 1-MeA lesions TLS contributes substantially to the lesion bypass ~65% in XPA fibroblasts (Table 1) and surprisingly all three pathways replicating past the lesion do so in an error-free manner with <2% of mutations occurring. In contrast, lesion bypass across BPDE lesions is far more mutagenic ~20% in XPA fibroblasts (Table 6) and occurs less frequently ~16% (Table 7). The reasons for this are not known but are probably influenced by the structure of the lesion, the identity of the TLS Pols recruited by the cell for the bypass, and the role of as of now unidentified

accessory proteins. Structurally, the lesions previously studied in our lab do not cause large distortions with the exception of the (6-4) photoproduct which bends the DNA helix by ~44^{o}. In contrast, the CPD lesion does not interfere with WC base pairing and has only modest effects on the DNA structure. The Tg lesion does not interfere with WC base pairing but becomes non-planar preventing extension synthesis from the lesion. The lesions now studied have larger effects on the DNA structure and base pairing. The 1-MeA lesion affects the base pair structure of adenine switching the base orientation to *syn* and forcing the base to adopt Hoogsteen base pairing, furthermore the methyl group prevents WC base pairing. The BPDE lesion is a large bulky adduct that prevents normal WC base pairing. The adduct can be accommodated in the minor groove of DNA resulting in little perturbation to the DNA helix structure. In order to be able to replicate across the 1-MeA lesion, a TLS Pol must be able to accommodate the methyl group or be able to replicate without utilizing WC base pairing. Pol η with its large active site would be capable of accommodating the methyl group. Pol ι utilizes Hoogsteen base pairing and would not be blocked by lesions that interfere with WC H-bonding. The BPDE lesion on the other hand is far larger and distorted. Again, Pol ι utilizing Hoogsteen base pairing would be capable of bypassing the lesion but other Pols would be blocked by the large bulky adduct. Rev1 by flipping the bulky adduct from the active site and utilizing its peculiar protein directed catalytic mechanism would not be inhibited by this lesion. The bulkiness of the adduct might interfere with correct or fast insertion across the lesion which might influence the fidelity of the reaction or force the cell to utilize a more error-prone TLS Pol. Finally, from replication foci studies and previous studies of Rev1 it is known that Rev1 can affect the placement of TLS Pols to lesion sites possibly due to the formation of multiprotein assemblies (201). These assemblies could contribute to the regulation and fidelity of the bypass. The identification of the identities of these proteins as well as their role in the regulation of TLS will be an important area of research that will need to be studied if TLS is to be understood.

SELECTION OF TLS POLS TO LESION SITES

An important consideration for TLS is how does the cell select for the TLS Pols to be recruited to each lesion site? Biochemical studies with Pols ν and η have revealed the ability to replicate across Tg lesions (204, 205). Pol η is in fact highly efficient for replicating across this lesion *in vitro* but will do so with a higher error rate (205). Yet *in vivo* studies of TLS have not indicated a role for these TLS Pols across the Tg lesion (175). This implies that the selection of TLS Pols is not based on their catalytic efficiencies alone and the fact that, across UV, Tg, and 1-MeA lesions mutagenesis is low, implies that the cell adapted the TLS machinery so that error-free TLS is utilized over more efficient but mutagenic TLS.

Previous studies of TLS frequency across various lesions have revealed small statistically insignificant differences between TLS across the leading or lagging strand of DNA (49, 175, 202, 203). This is significant because it suggests an important method of regulation. A lesion on the leading strand will present a strong block for fork progression while a lesion on the lagging strand would not be a significant block for fork progression but would block Okazaki fragment completion. It could be anticipated that TLS would be more active on the leading strand while other methods such as template switching could be utilized for the lagging strand, in this way, replication would be rapidly completed across a lesion. The fact that TLS activity is very similar suggests that TLS in both strands is regulated by the same genetic controls and that the stalling of the replication fork is closely coordinated with the stalled replisome irrespective of on which strand the lesion is located. Therefore, TLS across mammalian cells is closely coordinated and possibly regulated with the replication machinery. The fact that PCNA has been shown to be able to regulate TLS supports this idea (152). This link of TLS with replication makes it imperative that in order to understand the cell's lesion bypass system TLS must be

studied in a situation where the replisome is present and not post-replicatively in artificial gaps.

Furthermore, previous work with the TLS Pol Rev1 has implied that it is an indispensable component of TLS mediated by the Y family Pols, the requirement of Rev1 for foci formation in Pols η, ι, and κ across UV lesions (202) and Pols ι and κ across BPDE lesions (Table 8) and the ability of the TLS Pols to modulate Rev1 foci formation implies the functional and physical interactions with other proteins and the formation of multiprotein assemblies at DNA lesion sites. The identity of these proteins is not known but they could regulate the activities of TLS Pols. This is probable since the basic structural features of the TLS Pols do not offer a sufficient explanation of their selection or fidelity to various DNA lesions. Modulation by protein-protein interactions, and association with other proteins as well as the replication ensemble might allow for regulation of the efficiency and fidelity of bypass across lesions. Mutagenesis studies with HLTF (Table 10) and ATR (Table 11) suggests this is the case and that at least across BPDE, TLS might be regulated by ubiquitination in the case of HLTF and phosphorylation in the case of ATR/Chk1. Future studies of TLS Pols conserved domains, which we have generated, might help decipher the methods of regulation as well as the components regulating the TLS machinery. These studies will help elucidate the genetic control by which the cell integrates, signals, and activate the repair pathways to coordinate TLS. Importantly, these studies might helped link TLS to other repair pathways and might provide clues to the regulation of replication and DNA repair tolerance mechanisms.

THE ROLE OF POL ζ IN BPDE LESION BYPASS

Mutagenesis (Table 4) and TLS frequency data (Table 6) have demonstrated that across BPDE lesions Pol ζ does not play a role in replicative bypass. Previous studies with gapped plasmids have demonstrated a wide role for Pol ζ across a variety of DNA lesions (130), including BPDE lesions. The current studies as well as studies with the HPRT assay system in human fibroblasts (135) do not support a role for Pol ζ in lesion bypass of BPDE. Gapped plasmid TLS occurs post-replicatively in gaps (132, 133) and is a good model system for explaining gap filling or bypass that would occur on simpler organisms such as yeast. However, the regulation of TLS in humans is more complex, as mentioned before, previous studies of Rev1 and UV (202), as well as the current DNA replication foci studies (Table 8) suggest the possibility of multi-protein assemblies with the replisome. TLS assays of the leading and lagging strand demonstrate small differences in TLS frequencies, this and the fact that many of the replication machinery of the cell, such as PCNA, is closely associated with TLS in human cells suggests that in humans TLS is regulated in concert with the stalled replisome and quite differently than in gap filling. Such model will not be sufficient to study TLS in human cells and lack the genetic controls that the cell utilizes for TLS.

Pol ζ introduces complex mutations with multiple changes within short DNA stretches. Inhibition of HR in cancer cells has been demonstrated to increase mutagenicity and cytotoxicity. Similarly, inhibition of other repair pathways such as NER or BER result in an increase in mutagenesis, which like depletion of HR, could be attributed to increases in the levels of replication blocking lesions and channeling these lesions into alternate lesion bypass pathways. Across BPDE lesions TLS does not provide the only means to bypass the lesion making recombination and template switching mechanisms critical methods for the cell to avoid replication blocking lesions. In a situation where the cell is

faced with high levels of BPDE lesions and a large part of its repertoire for dealing with these lesions is inhibited, the cell would have to rely on TLS for the entirety of its bypass. Unlike other DNA lesions, the frequency of TLS across BPDE lesions is lower than across UV, Tg, or 1-MeA lesions. This might be attributed to the bulky nature of BPDE and the difficulty for the cell to correctly bypass the lesion. When faced with high levels of replication blocking lesions the cell might utilize a generalized TLS mechanism and avoid the BPDE lesion at the cost of loss on the ability to regulate TLS and increasing mutagenesis but preventing the formation of DSBs and protecting the stability of the replication fork. The current data (Table 9) on recombination deficient TLS supports this. Importantly, this generalized method of TLS has been demonstrated before with UV lesions (136), with replisome impairment (139), or in cancer cells (147 -150). This method of TLS might be similar to the TLS observed in gapped plasmids (130) and might not be as stringently regulated by the cell. It would be interesting to explore whether this generalized method of TLS is utilized predominantly by cancer cells and whether cancer cells derive a benefit in their growth or survival from it. However, it has to be noted that although this method of TLS exists, in a situation where a normal human cell encounters a BPDE lesion the cell will not utilize Pol ζ for bypassing the lesion and will instead utilize one of the 3 pathways listed above for bypassing the lesion (Figure 23).

FUTURE DIRECTIONS

The data presented here demonstrates the differences of how a cell bypasses different DNA replication blocking lesions. More important than understanding which polymerases are recruited to the different types of DNA lesions is the emerging picture of TLS as a highly regulated mechanism of DNA damage tolerance that has evolved to promote the fidelity of replication. Understanding the higher mechanisms of regulation of TLS and the identities of the multiprotein complex that are responsible for recruiting the

TLS Pols as well as their regulation will be necessary in the future, if TLS is to be understood. These knowledge might help increase the understanding that lesion bypass plays in mutagenesis and carcinogenesis and provide novel targets for therapeutics.

Appendix A: Rights and Permissions

Figure 1.

License Number	3821510534771
License date	Mar 03, 2016
Licensed content publisher	Nature Publishing Group
Licensed content publication	Nature Chemical Biology
Licensed content title	DNA damage tolerance: when it's OK to make mistakes
Licensed content author	Debbie J Chang, Karlene A Cimprich
Licensed content date	Jan 15, 2009
Type of Use	reuse in a dissertation / thesis
Volume number	5
Issue number	2
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
High-res required	no
Figures	Figure 1. Overview of DNA damage tolerance pathways
Author of this NPG article	no
Your reference number	None
Title of your thesis / dissertation	Role of TLS Polymerases in Replication of DNA Lesions in Human Cells
Expected completion date	May 2016
Estimated size (number of pages)	115
Total	0.00 USD

Figure 2.

License Number	3821511145799
License date	Mar 03, 2016
Licensed content publisher	Nature Publishing Group
Licensed content publication	Nature Reviews Molecular Cell Biology
Licensed content title	Repairing DNA-methylation damage
Licensed content author	Barbara Sedgwick
Licensed content date	Feb 1, 2004
Type of Use	reuse in a dissertation / thesis
Volume number	5
Issue number	2
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
High-res required	no
Figures	Sites of methylation on the bases and sugar–phosphate backbone of DNA
Author of this NPG article	no

Your reference number	None
Title of your thesis / dissertation	Role of TLS Polymerases in Replication of DNA Lesions in Human Cells
Expected completion date	May 2016
Estimated size (number of pages)	115
Total	0.00 USD

Figure 3.

License Number	3821520392995
License date	Mar 03, 2016
Licensed content publisher	Elsevier
Licensed content publication	Biochimica et Biophysica Acta
Licensed content title	The electronic structure of the purine-pyrimidine pairs of DNA
Licensed content author	Bernard Pullman,Alberte Pullman
Licensed content date	December 1959
Licensed content volume number	36
Licensed content issue number	2
Number of pages	8
Type of Use	reuse in a thesis/dissertation
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Original figure numbers	Figure 1. The purine-pyrimidine pairs of DNA
Title of your thesis/dissertation	Role of TLS Polymerases in Replication of DNA Lesions in Human Cells
Expected completion date	May 2016
Estimated size (number of pages)	115
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 USD
VAT/Local Sales Tax	0.00 USD / 0.00 GBP
Total	0.00 USD

Figure 4.

License Number	3821511402609
License date	Mar 03, 2016
Licensed content publisher	Nature Publishing Group
Licensed content publication	Nature Reviews Cancer
Licensed content title	Chemical-induced DNA damage and human cancer risk
Licensed content author	Miriam C. Poirier
Licensed content date	Aug 1, 2004
Type of Use	reuse in a dissertation / thesis
Volume number	4
Issue number	8
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations

Number of figures/tables/illustrations	1
High-res required	no
Figures	Minor groove lesion
Author of this NPG article	no
Your reference number	None
Title of your thesis / dissertation	Role of TLS Polymerases in Replication of DNA Lesions in Human Cells
Expected completion date	May 2016
Estimated size (number of pages)	115
Total	0.00 USD

Figure 5.

License Number	3821520012621
License date	Mar 03, 2016
Licensed content publisher	Nature Publishing Group
Licensed content publication	Nature Reviews Cancer
Licensed content title	Nature and nurture – lessons from chemical carcinogenesis
Licensed content author	Andreas Luch
Licensed content date	Jan 20, 2005
Type of Use	reuse in a dissertation / thesis
Volume number	5
Issue number	2
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
High-res required	no
Figures	Metabolism and activation of BaP
Author of this NPG article	no
Your reference number	None
Title of your thesis / dissertation	Role of TLS Polymerases in Replication of DNA Lesions in Human Cells
Expected completion date	May 2016
Estimated size (number of pages)	115
Total	0.00 USD

Figure 6.

License Number	3824960656487
License date	Mar 09, 2016
Licensed content publisher	Elsevier
Licensed content publication	Mutation Research/Reviews in Mutation Research
Licensed content title	Detailed review of transgenic rodent mutation assays
Licensed content author	Iain B. Lambert,Timothy M. Singer,Sherri E. Boucher,George R. Douglas
Licensed content date	September 2005
Licensed content volume number	590
Licensed content issue number	1-3
Number of pages	280
Type of Use	reuse in a thesis/dissertation

Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Original figure numbers	Fig 1-2
Title of your thesis/dissertation	Role of TLS Polymerases in Replication of DNA Lesions in Human Cells
Expected completion date	May 2016
Estimated size (number of pages)	115
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 USD
VAT/Local Sales Tax	0.00 USD / 0.00 GBP
Total	0.00 USD

Figure 7.

License Number	3824960656487
License date	Mar 09, 2016
Licensed content publisher	Elsevier
Licensed content publication	Mutation Research/Reviews in Mutation Research
Licensed content title	Detailed review of transgenic rodent mutation assays
Licensed content author	Iain B. Lambert,Timothy M. Singer,Sherri E. Boucher,George R. Douglas
Licensed content date	September 2005
Licensed content volume number	590
Licensed content issue number	1-3
Number of pages	280
Type of Use	reuse in a thesis/dissertation
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Original figure numbers	Fig 1-2
Title of your thesis/dissertation	Role of TLS Polymerases in Replication of DNA Lesions in Human Cells
Expected completion date	May 2016
Estimated size (number of pages)	115
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 USD
VAT/Local Sales Tax	0.00 USD / 0.00 GBP
Total	0.00 USD

Bibliography

1. Pagès V, Fuchs RP. (2002). How DNA lesions are turned into mutations within cells? Oncogene. 21(58):8957-66

2. Hoeijmakers JH. (2001). Genome maintenance mechanisms for preventing cancer. Nature. 411(6835):366-74.

3. Setlow RB. (1978). Repair deficient human disorders and cancer. Nature. 271(5647):713-7.

4. Pullman B, Pullman A. (1959). The electronic structure of the purine-pyrimidine pairs of DNA. Biochim Biophys Acta.36:343-50.

5. Phillips DH. (1983). Fifty years of benzo(a)pyrene. Nature. 303(5917):468-72.

6. Marnett LJ. (2000). Oxyradicals and DNA damage. Carcinogenesis. 21(3):361-70.

7. Marnett LJ. Plastaras JP. (2001). Endogenous DNA damage and mutation. Trends Genet. 17(4):214-21.

8. Marnett LJ. (2002). Oxy radicals, lipid peroxidation and DNA damage. Toxicology. 181-182:219-22.

9. Thomas K. Theodor S. (1993). Reaction of glucose with guanosine. Tetrahedron Letters. 34 (46): 7389 – 7390.

10. Seidel W, Pischetsrieder M. (1998). Reaction of guanosine with glucose under oxidative conditions. Bioorg Med Chem Lett. 8(15):2017-22.

11. Yuan B, You C, Andersen N, Jiang Y, Moriya M, O'Connor TR, Wang Y. (2011). The roles of DNA polymerases κ and ι in the error-free bypass of N2-carboxyalkyl-2'-deoxyguanosine lesions in mammalian cells. J Biol Chem. 286(20):17503-11.

12. Belous AR, Hachey DL, Dawling S, Roodi N, Parl FF. (2007). Cytochrome P450 1B1-mediated estrogen metabolism results in estrogen-deoxyribonucleoside adduct formation. Cancer Res. 67(2):812-7.

13. Cavalieri E, Frenkel K, Liehr JG, Rogan E, Roy D. (2000). Estrogens as endogenous genotoxic agents--DNA adducts and mutations. J Natl Cancer Inst Monogr. (27):75-93.

14. Pfeifer GP, Denissenko MF, Olivier M, Tretyakova N, Hecht SS, Hainaut P. (2002) Tobacco smoke carcinogens, DNA damage and p53 mutations in smoking-associated cancers. Oncogene. 21(48):7435-51.

15. Chang DJ, Cimprich KA. (2009). DNA damage tolerance: when it's OK to make mistakes. Nat Chem Biol. 5(2):82-90.

16. Johnson RE, Washington MT, Prakash S, Prakash L. (1999). Bridging the gap: a family of novel DNA polymerases that replicate faulty DNA. Proc Natl Acad Sci U S A. 96(22):12224-6.

17. Baynton K, Fuchs RP. (2000). Lesions in DNA: hurdles for polymerases. Trends Biochem Sci. 25(2):74-9.

18. Hanahan D, Weinberg RA. (2011). Hallmarks of cancer: the next generation. Cell. 144(5):646-74.

19. Luch A. (2005). Nature and nurture - lessons from chemical carcinogenesis. Nat Rev Cancer.5(2):113-25.

20. Jha P. (2009). Avoidable global cancer deaths and total deaths from smoking. Nat Rev Cancer. 9(9):655-64.

21. Wynder EL, Hoffmann D. (1968). Experimental tobacco carcinogenesis. Science. (3856):862-71.

22. DeMarini DM, Landi S, Tian D, Hanley NM, Li X, Hu F, Roop BC, Mass MJ, Keohavong P, Gao W, Olivier M, Hainaut P, Mumford JL. (2001). Lung tumor KRAS and TP53 mutations in nonsmokers reflect exposure to PAH-rich coal combustion emissions. Cancer Res. 61(18):6679-81.

23. Denissenko MF, Pao A, Tang M, Pfeifer GP. (1996). Preferential formation of benzo[a]pyrene adducts at lung cancer mutational hotspots in P53. Science. 274(5286):430-2.

24. DeMarini DM, Landi S, Tian D, Hanley NM, Li X, Hu F, Roop BC, Mass MJ, Keohavong P, Gao W, Olivier M, Hainaut P, Mumford JL. (2001). Lung tumor KRAS and TP53 mutations in nonsmokers reflect exposure to PAH-rich coal combustion emissions. Cancer Res. 61(18):6679-81.

25. Hainaut P, Pfeifer GP. (2001). Patterns of p53 G-->T transversions in lung cancers reflect the primary mutagenic signature of DNA-damage by tobacco smoke. Carcinogenesis. 22(3):367-74.

26. Smith LE, Denissenko MF, Bennett WP, Li H, Amin S, Tang M, Pfeifer GP. (2000). Targeting of lung cancer mutational hotspots by polycyclic aromatic hydrocarbons. J Natl Cancer Inst. 92(10):803-11.

27. Feng Z, Hu W, Chen JX, Pao A, Li H, Rom W, Hung MC, Tang MS. (2002). Preferential DNA damage and poor repair determine ras gene mutational hotspot in human cancer. J Natl Cancer Inst. 94(20):1527-36.

28. Choi JY, Angel KC, Guengerich FP. (2006). Translesion synthesis across bulky N2-alkyl guanine DNA adducts by human DNA polymerase kappa. J Biol Chem. 281(30):21062-72

29. Minko IG, Harbut MB, Kozekov ID, Kozekova A, Jakobs PM, Olson SB, Moses RE, Harris TM, Rizzo CJ, Lloyd RS. (2008). Role for DNA polymerase kappa in the processing of N2-N2-guanine interstrand cross-links. J Biol Chem. 283(25):17075-82

30. Suzuki N, Ohashi E, Hayashi K, Ohmori H, Grollman AP, Shibutani S. (2001). Translesional synthesis past acetylaminofluorene-derived DNA adducts catalyzed by human DNA polymerase kappa and Escherichia coli DNA polymerase IV. Biochemistry. 40(50):15176-83.

31. Yuan B, Cao H, Jiang Y, Hong H, Wang Y. (2008). Efficient and accurate bypass of N2-(1-carboxyethyl)-2'-deoxyguanosine by DinB DNA polymerase in vitro and in vivo. Proc Natl Acad Sci U S A. 105(25):8679-84.

32. Yuan B, You C, Andersen N, Jiang Y, Moriya M, O'Connor TR, Wang Y. (2011). The roles of DNA polymerases κ and ι in the error-free bypass of N2-carboxyalkyl-2'-deoxyguanosine lesions in mammalian cells. J Biol Chem. 286(20):17503-11

33. Jhappan C, Noonan FP, Merlino G. (2003). Ultraviolet radiation and cutaneous malignant melanoma. Oncogene. 22(20):3099-112.

34. Kensler TW, Qian GS, Chen JG, Groopman JD. (2003). Translational strategies for cancer prevention in liver. Nat Rev Cancer. 3(5):321-9.

35. Prakash S, Johnson RE, Prakash L. (2005). Eukaryotic translesion synthesis DNA polymerases: specificity of structure and function. Annu Rev Biochem. 74:317-53.

36. Suzuki N, Ohashi E, Hayashi K, Ohmori H, Grollman AP, Shibutani S. (2001). Translesional synthesis past acetylaminofluorene-derived DNA adducts catalyzed by human DNA polymerase kappa and Escherichia coli DNA polymerase IV. Biochemistry. 40(50):15176-83.

37. Ohashi E, Ogi T, Kusumoto R, Iwai S, Masutani C, Hanaoka F, Ohmori H. (2000). Error-prone bypass of certain DNA lesions by the human DNA polymerase kappa. Genes Dev. 14(13):1589-94.

38. Zhang Y, Yuan F, Wu X, Wang M, Rechkoblit O, Taylor JS, Geacintov NE, Wang Z. (2000). Error-free and error-prone lesion bypass by human DNA polymerase kappa in vitro. Nucleic Acids Res. 28(21):4138-46.

39. Shen X, Sayer JM, Kroth H, Ponten I, O'Donnell M, Woodgate R, Jerina DM, Goodman MF. (2002). Efficiency and accuracy of SOS-induced DNA polymerases replicating benzo[a]pyrene-7,8-diol 9,10-epoxide A and G adducts. J Biol Chem. 277(7):5265-74

40. Avkin S, Goldsmith M, Velasco-Miguel S, Geacintov N, Friedberg EC, Livneh Z. (2004). Quantitative analysis of translesion DNA synthesis across a benzo[a]pyrene-guanine adduct in mammalian cells: the role of DNA polymerase kappa. J Biol Chem279(51):53298-305.

41. Pence MG, Blans P, Zink CN, Fishbein JC, Perrino FW. (2011). Bypass of N^2-ethylguanine by human DNA polymerase κ. DNA Repair (Amst). 10(1):56-64.

42. Ogi T, Shinkai Y, Tanaka K, Ohmori H. (2002). Polkappa protects mammalian cells against the lethal and mutagenic effects of benzo[a]pyrene. Proc Natl Acad Sci U S A. 99(24):15548-53.

43. Woodgate, R. (1999). "A plethora of lesion-replicating DNA polymerases." Genes Dev 13(17): 2191-2195.

44. Little JW, Mount DW. (1982). The SOS regulatory system of Escherichia coli. Cell. 29(1):11-22.

45. Johnson, R. E., S. Prakash, et al. (1999). Efficient Bypass of a Thymine-Thymine Dimer by Yeast DNA Polymerase, Pol eta. Science 283(5404): 1001-1004.

46. Washington MT, Johnson RE, Prakash L, Prakash S. (2001). Accuracy of lesion bypass by yeast and human DNA polymerase eta. Proc Natl Acad Sci U S A. 98(15):8355-60.

47. Masutani C, Kusumoto R, Yamada A, Dohmae N, Yokoi M, Yuasa M, Araki M, Iwai S, Takio K, Hanaoka F. (1999). The XPV (xeroderma pigmentosum variant) gene encodes human DNA polymerase eta. Nature. 399(6737):700-4.

48. Johnson RE, Kondratick CM, Prakash S, Prakash L. (1999). hRAD30 mutations in the variant form of xeroderma pigmentosum. Science. 285(5425):263-5.

49. Yoon, J.-H., L. Prakash, et al. (2009). Highly error-free role of DNA polymerase eta in the replicative bypass of UV-induced pyrimidine dimers in mouse and human cells. Proc Natl Acad Sci U S A. 106(43): 18219-18224.

50. Nair, D. T., R. E. Johnson, et al. (2008). Protein-template-directed synthesis across an acrolein-derived DNA adduct by yeast Rev1 DNA polymerase. Structure 16(2): 239-245.

51. Lone, S., S. A. Townson, et al. (2007). Human DNA polymerase kappa encircles DNA: implications for mismatch extension and lesion bypass. Mol Cell 25(4): 601-614.

52. Carlson, K. D., R. E. Johnson, et al. (2006). Human DNA polymerase kappa forms nonproductive complexes with matched primer termini but not with mismatched primer termini. Proc Natl Acad Sci U S A. 103(43):15776-81.

53. Hashimoto K, Cho Y, Yang IY, Akagi JI, Ohashi E, Tateishi S, de Wind N, Hanaoka F, Ohmori H, Moriya M. (2012). The vital role of pol ζ and REV1 in mutagenic, but not correct, DNA synthesis across benzo[a]pyrene-dG and the recruitment of pol ζ by REV1 to a replication-stalled site. J Biol Chem. 287(12):9613-22.

54. Mukhopadhyay S, Clark DR, Watson NB, Zacharias W, McGregor WG. (2004). REV1 accumulates in DNA damage-induced nuclear foci in human cells and is implicated in mutagenesis by benzo[a]pyrenediolepoxide. Nucleic Acids Res32(19):5820-6.

55. Zhao B, Wang J, Geacintov NE, Wang Z. (2006). Poleta, Polzeta and Rev1 together are required for G to T transversion mutations induced by the (+)- and (-)-trans-anti-BPDE-N2-dG DNA adducts in yeast cells. Nucleic Acids Res. 34(2):417-25.

56. Rechkoblit O, Zhang Y, Guo D, Wang Z, Amin S, Krzeminsky J, Louneva N, Geacintov NE. (2002). trans-Lesion synthesis past bulky benzo[a]pyrene diol epoxide N2-dG and N6-dA lesions catalyzed by DNA bypass polymerases. J Biol Chem.277(34):30488-94.

57. Branzei D, Foiani M. (2007). Template switching: from replication fork repair to genome rearrangements. Cell. 131(7):1228-30.

58. Bartkova J, Horejsí Z, Koed K, Krämer A, Tort F, Zieger K, Guldberg P, Sehested M, Nesland JM, Lukas C, Ørntoft T, Lukas J, Bartek J. (2005). DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. Nature. 434(7035):864-70.

59. Parris CN, Seidman MM. (1992). A signature element distinguishes sibling and independent mutations in a shuttle vector plasmid. Gene. 117(1):1-5.

60. Rodin SN, Rodin AS. (2000). Human lung cancer and p53: the interplay between mutagenesis and selection. Proc Natl Acad Sci U S A. 97(22):12244-9.

61. Hainaut P, Pfeifer GP. (2001). Patterns of p53 G-->T transversions in lung cancers reflect the primary mutagenic signature of DNA-damage by tobacco smoke. Carcinogenesis. 22(3):367-74.

62. Stratagene. (2009).”Figure 2”. λ select-cII Mutation Detection System for Big Blue Rodents. Instruction manual. 3.

63. Poirier MC. (2004). Chemical-induced DNA damage and human cancer risk. Nat Rev Cancer.4(8):630-7.

64. Richardson C, Jasin M. (2000). Frequent chromosomal translocations induced by DNA double-strand breaks. Nature. 405(6787):697-700.

65. Negrini S, Gorgoulis VG, Halazonetis TD. (2010) Genomic instability an evolving hallmark of cancer. Nat Rev Mol Cell Biol.11(3):220-8.

66. Pagès V, Fuchs RP. (2002). How DNA lesions are turned into mutations within cells? Oncogene. 21(58):8957-66.

67. Semizarov D, Frost L, Sarthy A, Kroeger P, Halbert DN, Fesik SW. (2003). Specificity of short interfering RNA determined through gene expression signatures. Proc Natl Acad Sci U S A.100(11):6347-52

68. Persengiev SP, Zhu X, Green MR. (2004). Nonspecific, concentration-dependent stimulation and repression of mammalian gene expression by small interfering RNAs (siRNAs). RNA.10(1):12-8.

69. Geacintov NE, Shahbaz M, Ibanez V, Moussaoui K, Harvey RG. (1988). Base-sequence dependence of noncovalent complex formation and reactivity of benzo[a]pyrene diol epoxide with polynucleotides. Biochemistry.27(22):8380-7.

70. Lindahl T and Wood RD. Quality control by DNA repair. Science. 1999 Dec 3;286(5446):1897-905.

71. Friedberg, EC; Walker, GC; Siede, W; Schultz, RA (2006). DNA repair and mutagenesis (2nd ed.).

72. Prakash S, Prakash L. Translesion DNA synthesis in eukaryotes: a one- or two-polymerase affair. Genes Dev. 2002 Aug 1;16(15):1872-83.

73. Loeb LA, Monnat RJ Jr. DNA polymerases and human disease. Nat Rev Genet. 2008 Aug;9(8):594-604.

74. Goodman MF, Woodgate R. Translesion DNA polymerases. Cold Spring Harb Perspect Biol. 2013 Oct 1;5(10):a010363.

75. Sale JE, Lehmann AR, Woodgate R. Y-family DNA polymerases and their role in tolerance of cellular DNA damage. Nat Rev Mol Cell Biol. 2012 Feb 23;13(3):141-52.

76. Sedgwick 2004. Repairing DNA-methylation damage. Nat Rev Mol Cell Biol. 2004 Feb;5(2):148-57.

77. Fu D, Calvo JA, Samson LD. Balancing repair and tolerance of DNA damage caused by alkylating agents. Nat Rev Cancer. 2012 Jan 12;12(2):104-20.

78. Bodell WJ, Singer B. Influence of hydrogen bonding in DNA and polynucleotides on reaction of nitrogens and oxygens toward ethylnitrosourea. Biochemistry. 1979 Jun 26;18(13):2860-3.

79. Lawley PD, Brookes P. The action of alkylating agents on deoxyribonucleic acid in relation to biological effects of the alkylating agents. Exp Cell Res. 1963;24:SUPPL9:512-20.

80. Yang H, Zhan Y, Fenn D, Chi LM, Lam SL. Effect of 1-methyladenine on double-helical DNA structures. FEBS Lett. 2008 May 14;582(11):1629-33

81. Delaney JC, Essigmann JM. Mutagenesis, genotoxicity, and repair of 1-methyladenine, 3-alkylcytosines, 1-methylguanine, and 3-methylthymine in alkB Escherichia coli. Proc Natl Acad Sci U S A. 2004 Sep 28;101(39):14051-6.

82. Ma W, Panduri V, Sterling JF, Van Houten B, Gordenin DA, Resnick MA. The transition of closely opposed lesions to double-strand breaks during long-patch base excision repair is prevented by the coordinated action of DNA polymerase delta and Rad27/Fen1. Mol Cell Biol. 2009 Mar;29(5):1212-21. doi: 10.1128/MCB.01499-08

83. Sogo JM, Lopes M, Foiani M. Fork reversal and ssDNA accumulation at stalled replication forks owing to checkpoint defects. Science. 2002 Jul 26;297(5581):599-602.

84. Trewick SC, Henshaw TF, Hausinger RP, Lindahl T, Sedgwick B. Oxidative demethylation by Escherichia coli AlkB directly reverts DNA base damage. Nature. 2002 Sep 12;419(6903):174-8.

85. Falnes PØ, Johansen RF, Seeberg E. AlkB-mediated oxidative demethylation reverses DNA damage in Escherichia coli. Nature. 2002 Sep 12;419(6903):178-82.

86. Beranek DT1, Weis CC, Swenson DH. A comprehensive quantitative analysis of methylated and ethylated DNA using high pressure liquid chromatography. Carcinogenesis. 1980 Jul;1(7):595-606.

87. Gomes JD, Chang CJ. Reverse-phase high-performance liquid chromatography of chemically modified DNA. Anal Biochem. 1983 Mar;129(2):387-91.

88. Margison GP, Margison JM, Montesano R. Methylated purines in the deoxyribonucleic acid of various Syrian-golden-hamster tissues after administration of a hepatocarcinogenic dose of dimethylnitrosamine. Biochem J. 1976 Sep 1;157(3):627-34.

89. Faustman-Watts EM, Goodman JI. DNA-purine methylation in hepatic chromatin following exposure to dimethylnitrosamine or methylnitrosourea. Biochem Pharmacol. 1984 Feb 15;33(4):585-90.

90. Bodell WJ, Singer B. Influence of hydrogen bonding in DNA and polynucleotides on reaction of nitrogens and oxygens toward ethylnitrosourea. Biochemistry. 1979 Jun 26;18(13):2860-3.

91. Rydberg B, Lindahl T. Nonenzymatic methylation of DNA by the intracellular methyl group donor S-adenosyl-L-methionine is a potentially mutagenic reaction. EMBO J. 1982;1(2):211-6.

92. Näslund M, Segerbäck D, Kolman A. S-adenosylmethionine, an endogenous alkylating agent. Mutat Res. 1983 Mar;119(3):229-32.

93. Stern LL, Mason JB, Selhub J, Choi SW. Genomic DNA hypomethylation, a characteristic of most cancers, is present in peripheral leukocytes of individuals who are homozygous for the C677T polymorphism in the methylenetetrahydrofolate reductase gene. Cancer Epidemiol Biomarkers Prev. 2000 Aug;9(8):849-53.

94. Zhao C, Tyndyk M, Eide I, Hemminki K. Endogenous and background DNA adducts by methylating and 2-hydroxyethylating agents. Mutat Res. 1999 Mar 8;424(1-2):117-25.

95. Lawley PD. Methylation of DNA by N-methyl-N-nitrosourethane and N-methyl-N-nitroso-N'-nitroguanidine. Nature. 1968 May 11;218(5141):580-1.

96. Hamilton JT1, McRoberts WC, Keppler F, Kalin RM, Harper DB. Chloride methylation by plant pectin: an efficient environmentally significant process. Science. 2003 Jul 11;301(5630):206-9.

97. Bertsch HP. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Supplement 7. International Agency for Research in Cancer. 1987.

98. Dinglay S, Trewick SC, Lindahl T, Sedgwick B. Defective processing of methylated single-stranded DNA by E. coli AlkB mutants. Genes Dev. 2000 Aug 15;14(16):2097-105.

99. Sedgwick B, Bates PA, Paik J, Jacobs SC, Lindahl T. Repair of alkylated DNA: recent advances. DNA Repair (Amst). 2007 Apr 1;6(4):429-42.

100. Kataoka H, Yamamoto Y, Sekiguchi M. A new gene (alkB) of Escherichia coli that controls sensitivity to methyl methane sulfonate. J Bacteriol. 1983 Mar;153(3):1301-7.

101. Ringvoll J, Nordstrand LM, Vågbø CB, Talstad V, Reite K, Aas PA, Lauritzen KH, Liabakk NB, Bjørk A, Doughty RW, Falnes PØ, Krokan HE, Klungland A. Repair deficient mice reveal mABH2 as the primary oxidative demethylase for repairing 1meA and 3meC lesions in DNA. EMBO J. 2006 May 17;25(10):2189-98.

102. Lin JR, Zeman MK, Chen JY, Yee MC, Cimprich KA. SHPRH and HLTF act in a damage-specific manner to coordinate different forms of postreplication repair and prevent mutagenesis. Mol Cell. 2011 Apr 22;42(2):237-49.

103. Takenaka K, Ogi T, Okada T, Sonoda E, Guo C, Friedberg EC, Takeda S. Involvement of vertebrate Polkappa in translesion DNA synthesis across DNA monoalkylation damage. J Biol Chem. 2006 Jan 27;281(4):2000-4.

104. Huang D, Piening BD, Paulovich AG. The preference for error-free or error-prone postreplication repair in Saccharomyces cerevisiae exposed to low-dose methyl methanesulfonate is cell cycle dependent. Mol Cell Biol. 2013 Apr;33(8):1515-27

105. Eeken JC, Romeijn RJ, de Jong AW, Pastink A, Lohman PH. Isolation and genetic characterisation of the Drosophila homologue of (SCE)REV3, encoding the catalytic subunit of DNA polymerase zeta. Mutat Res. 2001 Apr 4;485(3):237-53.

106. Sonoda E, Okada T, Zhao GY, Tateishi S, Araki K, Yamaizumi M, Yagi T, Verkaik NS, van Gent DC, Takata M, Takeda S. Multiple roles of Rev3, the catalytic subunit of polzeta in maintaining genome stability in vertebrates. EMBO J. 2003 Jun 16;22(12):3188-97.

107. Conde F, San-Segundo PA. Role of Dot1 in the response to alkylating DNA damage in Saccharomyces cerevisiae: regulation of DNA damage tolerance by the error-prone polymerases Polzeta/Rev1. Genetics. 2008 Jul;179(3):1197-210

108. Conde F, Ontoso D, Acosta I, Gallego-Sánchez A, Bueno A, San-Segundo PA. Regulation of tolerance to DNA alkylating damage by Dot1 and Rad53 in Saccharomyces cerevisiae. DNA Repair (Amst). 2010 Oct 5;9(10):1038-49.

109. Cosman M, Fiala R, Hingerty BE, Amin S, Geacintov NE, Broyde S, Patel DJ. Solution conformation of the (+)-cis-anti-[BP]dG adduct opposite a deletion site in a DNA duplex: intercalation of the covalently attached benzo[a]pyrene into the helix with base displacement of the modified deoxyguanosine into the minor groove. Biochemistry. 1994 Sep 27;33(38):11518-27.

110. Feng B, Gorin A, Hingerty BE, Geacintov NE, Broyde S, Patel DJ. Structural alignment of the (+)-trans-anti-benzo[a]pyrene-dG adduct positioned opposite dC at a DNA template-primer junction. Biochemistry. 1997 Nov 11;36(45):13769-79.

111. Reeves DA, Mu H, Kropachev K, Cai Y, Ding S, Kolbanovskiy A, Kolbanovskiy M, Chen Y, Krzeminski J, Amin S, Patel DJ, Broyde S, Geacintov NE. Resistance of bulky DNA lesions to nucleotide excision repair can result from extensive aromatic lesion-base stacking interactions. Nucleic Acids Res. 2011 Nov 1;39(20):8752-64.

112. Gillet LC, Schärer OD. Molecular mechanisms of mammalian global genome nucleotide excision repair. Chem Rev. 2006 Feb;106(2):253-76.

113. Sugasawa K, Shimizu Y, Iwai S, Hanaoka F. A molecular mechanism for DNA damage recognition by the xeroderma pigmentosum group C protein complex. DNA Repair (Amst). 2002 Jan 22;1(1):95-107.

114. Khan QA, Dipple A. Diverse chemical carcinogens fail to induce G(1) arrest in MCF-7 cells. Carcinogenesis. 2000 Aug;21(8):1611-8.

115. Wang A, Gu J, Judson-Kremer K, Powell KL, Mistry H, Simhambhatla P, Aldaz CM, Gaddis S, MacLeod MC. Response of human mammary epithelial cells to DNA damage induced by BPDE: involvement of novel regulatory pathways. Carcinogenesis. 2003 Feb;24(2):225-34

116. Paules RS, Cordeiro-Stone M, Mass MJ, Poirier MC, Yuspa SH, Kaufman DG. Benzo[alpha]pyrene diol epoxide I binds to DNA at replication forks. Proc Natl Acad Sci U S A. 1988 Apr;85(7):2176-80.

117. Yamagiwa K, Ichikawa K. Über die künstliche Erzeugung von Papillom. Verh Jap Path Ges 1915; 5: 142–8.

118. Bachmann WE, Kennaway EL, and Kennaway NM. The Rapid Production of Tumours by Two New Hydrocarbons. Yale J Biol Med. 1938 Dec; 11(2): 97–102.

119. Cook JW, Hewett CL and Hieger I. The isolation of a cancer-producing hydrocarbon from coal tar. Parts I, II, and III. J. Chem. Soc., 1933, 395-405.

120. Cook JW, Haslewood GAD, Hewett CL, Hieger I, Kennaway EL, and Mayneord WV. Chemical Compounds as Carcinogenic Agents. Am J Cancer February 1937 29; 219.

121. Weinstein IB, Jeffrey AM, Jennette KW, Blobstein SH, Harvey RG, Harris C, Autrup H, Kasai H, Nakanishi K. Benzo(a)pyrene diol epoxides as intermediates in nucleic acid binding in vitro and in vivo. Science. 1976 Aug 13;193(4253):592-5.

122. Meehan T, Straub K, Calvin M. Benzo[alpha]pyrene diol epoxide covalently binds to deoxyguanosine and deoxyadenosine in DNA. Nature. 1977 Oct 20;269(5630):725-7.

123. Mumford JL, He XZ, Chapman RS, Cao SR, Harris DB, Li XM, Xian YL, Jiang WZ, Xu CW, Chuang JC. Lung cancer and indoor air pollution in Xuan Wei, China. Science. 1987 Jan 9;235(4785):217-20.

124. Hoffmann D, Hoffmann I, El-Bayoumy K. The less harmful cigarette: a controversial issue. a tribute to Ernst L. Wynder. Chem Res Toxicol. 2001 Jul;14(7):767-90.

125. Neal JA, Fletcher KL, McCormick JJ, Maher VM. The role of hRev7, the accessory subunit of hPolζ, in translesion synthesis past DNA damage induced by benzo[a]pyrene diol epoxide (BPDE). BMC Cell Biol. 2010 Dec 10;11:97.

126. Simhadri S, Kramata P, Zajc B, Sayer JM, Jerina DM, Hinkle DC, Wei CS. Benzo[a]pyrene diol epoxide-deoxyguanosine adducts are accurately bypassed by yeast DNA polymerase zeta in vitro. Mutat Res. 2002 Oct 31;508(1-2):137-45.

127. Xie Z, Braithwaite E, Guo D, Zhao B, Geacintov NE, Wang Z. Mutagenesis of benzo[a]pyrene diol epoxide in yeast: requirement for DNA polymerase zeta and involvement of DNA polymerase eta. Biochemistry. 2003 Sep 30;42(38):11253-62.

128. Zhao B, Wang J, Geacintov NE, Wang Z. Poleta, Polzeta and Rev1 together are required for G to T transversion mutations induced by the (+)- and (-)-trans-anti-BPDE-N2-dG DNA adducts in yeast cells. Nucleic Acids Res. 2006 Jan 13;34(2):417-25.

129. Broomfield S, Hryciw T, Xiao W. DNA postreplication repair and mutagenesis in Saccharomyces cerevisiae. Mutat Res. 2001 Aug 9;486(3):167-84.

130. Shachar S, Ziv O, Avkin S, Adar S, Wittschieben J, Reissner T, Chaney S, Friedberg EC, Wang Z, Carell T, Geacintov N, Livneh Z. Two-polymerase mechanisms dictate error-free and error-prone translesion DNA synthesis in mammals. EMBO J. 2009 Feb 18;28(4):383-93

131. Li Z, Zhang H, McManus TP, McCormick JJ, Lawrence CW, Maher VM. hREV3 is essential for error-prone translesion synthesis past UV or benzo[a]pyrene diol epoxide-induced DNA lesions in human fibroblasts. Mutat Res. 2002 Dec 29;510(1-2):71-80.

132. Daigaku Y, Davies AA, Ulrich HD.Ubiquitin-dependent DNA damage bypass is separable from genome replication. Nature. 2010 Jun 17;465(7300):951-5

133. Karras GI, Jentsch S. The RAD6 DNA damage tolerance pathway operates uncoupled from the replication fork and is functional beyond S phase. Cell. 2010 Apr 16;141(2):255-67.

134. Zhang H, Chatterjee A, Singh KK. Saccharomyces cerevisiae polymerase zeta functions in mitochondria. Genetics. 2006 Apr;172(4):2683-8.

135. Northam MR, Robinson HA, Kochenova OV, Shcherbakova PV. Participation of DNA polymerase zeta in replication of undamaged DNA in Saccharomyces cerevisiae. Genetics. 2010 Jan;184(1):27-42.

136. Harfe BD, Jinks-Robertson S. DNA polymerase zeta introduces multiple mutations when bypassing spontaneous DNA damage in Saccharomyces cerevisiae. Mol Cell. 2000 Dec;6(6):1491-9.

137. Holbeck SL, Strathern JN. A role for REV3 in mutagenesis during double-strand break repair in Saccharomyces cerevisiae. Genetics. 1997 Nov;147(3):1017-24.

138. Sharma S, Shah NA, Joiner AM, Roberts KH, and Canman CE. DNA Polymerase ζ Is a Major Determinant of Resistance to Platinum-Based Chemotherapeutic Agents. Mol Pharmacol. 2012 Jun; 81(6): 778–787.

139. Northam MR1, Moore EA, Mertz TM, Binz SK, Stith CM, Stepchenkova EI, Wendt KL, Burgers PM, Shcherbakova PV. DNA polymerases ζ and Rev1 mediate error-prone bypass of non-B DNA structures. Nucleic Acids Res. 2014 Jan;42(1):290-306.

140. Swanson RL, Morey NJ, Doetsch PW, Jinks-Robertson S. Overlapping specificities of base excision repair, nucleotide excision repair, recombination, and translesion synthesis pathways for DNA base damage in Saccharomyces cerevisiae. Mol Cell Biol. 1999 Apr;19(4):2929-35.

141. Xiao W, Chow BL. Synergism between yeast nucleotide and base excision repair pathways in the protection against DNA methylation damage. Curr Genet. 1998 Feb;33(2):92-9.

142. Ulrich HD. Timing and spacing of ubiquitin-dependent DNA damage bypass. FEBS Lett. 2011 Sep 16;585(18):2861-7.

143. Broomfield S, Chow BL, Xiao W. MMS2, encoding a ubiquitin-conjugating-enzyme-like protein, is a member of the yeast error-free postreplication repair pathway. Proc Natl Acad Sci U S A. 1998 May 12;95(10):5678–5683

144. Xu G, Chapman JR, Brandsma I, Yuan J, Mistrik M, Bouwman P, et al. REV7 counteracts DNA double-strand break resection and affects PARP inhibition. Nature 2015 Mar 23.

145. Makarova AV, Burgers PM. Eukaryotic DNA polymerase ζ. DNA Repair (Amst). 2015 May;29:47-55.

146. Brondello JM, Pillaire MJ, Rodriguez C, Gourraud PA, Selves J, Cazaux C, Piette J. Novel evidences for a tumor suppressor role of Rev3, the catalytic subunit of Pol zeta. Oncogene. 2008 Oct 16;27(47):6093-101

147. Akerman GS, Rosenzweig BA, Domon OE, McGarrity LJ, Blankenship LR, Tsai CA, Culp SJ, MacGregor JT, Sistare FD, Chen JJ, Morris SM. Gene expression profiles and genetic damage in benzo(a)pyrene diol epoxide-exposed TK6 cells. Mutat Res. 2004 May 18;549(1-2):43-64.

148. Chuang SM, Wang LH, Hong JH, Lin YW. Induction of Rad51 protein levels by p38 MAPK decreases cytotoxicity and mutagenicity in benzo[a]pyrene-exposed human lung cancer cells. Toxicol Appl Pharmacol. 2008 Aug 1;230(3):290-7.

149. Luo W1, Fan W, Xie H, Jing L, Ricicki E, Vouros P, Zhao LP, Zarbl H. Phenotypic anchoring of global gene expression profiles induced by N-hydroxy-4-acetylaminobiphenyl and benzo[a]pyrene diol epoxide reveals correlations between expression profiles and mechanism of toxicity. Chem. Res. Toxicol., 18 (2005), 619–629.

150. Nwagbara O1, Darling-Reed SF, Tucker A, Harris C, Abazinge M, Thomas RD, Gragg RD. Induction of cell death, DNA strand breaks, and cell cycle arrest in DU145 human prostate carcinoma cell line by Benzo[a]pyrene and Benzo[a]pyrene-7,8-diol-9,10-epoxide. Int. J. Environ. Res. Public Health, 4 (2007), 10–14.

151. Moldovan GL, Pfander B, Jentsch S. PCNA, the maestro of the replication fork. Cell. 2007 May 18;129(4):665-79.

152. Hoege C, Pfander B, Moldovan GL, Pyrowolakis G, Jentsch S. RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. Nature. 2002 Sep 12;419(6903):135-41.

153. Haracska L, Acharya N, Unk I, Johnson RE, Hurwitz J, Prakash L, Prakash S. A single domain in human DNA polymerase iota mediates interaction with PCNA: implications for translesion DNA synthesis. Mol Cell Biol. 2005 Feb;25(3):1183-90.

154. Freudenthal BD, Gakhar L, Ramaswamy S, Washington MT. Structure of monoubiquitinated PCNA and implications for translesion synthesis and DNA polymerase exchange. Nat Struct Mol Biol. 2010 Apr;17(4):479-84.

155. Cortez D, Guntuku S, Qin J, Elledge SJ. ATR and ATRIP: partners in checkpoint signaling. Science. 2001 Nov 23;294(5547):1713-6.

156. Guo X, Deng Y, Lin Y, Cosme-Blanco W, Chan S, He H, Yuan G, Brown EJ, Chang S. Dysfunctional telomeres activate an ATM-ATR-dependent DNA damage response to suppress tumorigenesis. EMBO J. 2007 Nov 14;26(22):4709-19.

157. Chen YW, Cleaver JE, Hatahet Z, Honkanen RE, Chang JY, Yen Y, Chou KM. Human DNA polymerase eta activity and translocation is regulated by phosphorylation. Proc Natl Acad Sci U S A. 2008 Oct 28;105(43):16578-83.

158. Göhler T, Sabbioneda S, Green CM, Lehmann AR. ATR-mediated phosphorylation of DNA polymerase η is needed for efficient recovery from UV damage. J Cell Biol. 2011 Jan 24;192(2):219-27.

159. Sabbioneda S, Bortolomai I, Giannattasio M, Plevani P, Muzi-Falconi M. Yeast Rev1 is cell cycle regulated, phosphorylated in response to DNA damage and its binding to chromosomes is dependent upon MEC1. DNA Repair (Amst). 2007 Jan 4;6(1):121-7.

160. Bi X, Barkley LR, Slater DM, Tateishi S, Yamaizumi M, Ohmori H, Vaziri C. Rad18 regulates DNA polymerase kappa and is required for recovery from S-phase checkpoint-mediated arrest. Mol Cell Biol. 2006 May;26(9):3527-40.

161. Bienko M, Green CM, Crosetto N, Rudolf F, Zapart G, Coull B, Kannouche P, Wider G, Peter M, Lehmann AR, Hofmann K, Dikic I. Ubiquitin-binding domains in Y-family polymerases regulate translesion synthesis. Science. 2005 Dec 16;310(5755):1821-4.

162. Plosky BS, Vidal AE, Fernández de Henestrosa AR, McLenigan MP, McDonald JP, Mead S, Woodgate R. Controlling the subcellular localization of DNA polymerases iota and eta via interactions with ubiquitin. EMBO J. 2006 Jun 21;25(12):2847-55.

163. Guo C, Tang TS, Bienko M, Dikic I, Friedberg EC. Requirements for the interaction of mouse Polkappa with ubiquitin and its biological significance. J Biol Chem. 2008 Feb 22;283(8):4658-64.

164. Zhuang Z, Johnson RE, Haracska L, Prakash L, Prakash S, Benkovic SJ. Regulation of polymerase exchange between Poleta and Poldelta by monoubiquitination of PCNA and the movement of DNA polymerase holoenzyme. Proc Natl Acad Sci U S A. 2008 Apr 8;105(14):5361-6.

165. Vidal AE, Kannouche P, Podust VN, Yang W, Lehmann AR, Woodgate R. Proliferating cell nuclear antigen-dependent coordination of the biological functions of human DNA polymerase iota. J Biol Chem. 2004 Nov 12;279(46):48360-8

166. Warbrick, E. (1998), PCNA binding through a conserved motif. Bioessays, 20: 195–199.

167. Acharya N, Yoon JH, Hurwitz J, Prakash L, Prakash S. DNA polymerase eta lacking the ubiquitin-binding domain promotes replicative lesion bypass in humans cells. Proc Natl Acad Sci U S A. 2010 Jun 8;107(23):10401-5

168. Yoon JH, Acharya N, Park J, Basu D, Prakash S, Prakash L. Identification of two functional PCNA-binding domains in human DNA polymerase κ. Genes Cells. 2014 Jul;19(7):594-601.

169. Zhang Y, Yuan F, Wu X, Rechkoblit O, Taylor JS, Geacintov NE, Wang Z. Error-prone lesion bypass by human DNA polymerase eta. Nucleic Acids Res. 2000 Dec 1;28(23):4717-24.

170. Langie SA, Knaapen AM, Ramaekers CH, Theys J, Brun J, Godschalk RW, van Schooten FJ, Lambin P, Gray DA, Wouters BG, Chiu RK. Formation of lysine 63-linked poly-ubiquitin chains protects human lung cells against benzo[a]pyrene-diol-epoxide-induced mutagenicity. DNA Repair (Amst). 2007 Jun 1;6(6):852-62.

171. Klarer AC, Stallons LJ, Burke TJ, Skaggs RL, McGregor WG. DNA polymerase eta participates in the mutagenic bypass of adducts induced by benzo[a]pyrene diol epoxide in mammalian cells. PLoS One. 2012;7(6):e39596.

172. Bergoglio V, Bavoux C, Verbiest V, Hoffmann JS, Cazaux C. Localisation of human DNA polymerase kappa to replication foci. J Cell Sci. 2002 Dec 1;115(Pt 23):4413-8.

173. Washington MT, Minko IG, Johnson RE, Haracska L, Harris TM, Lloyd RS, Prakash S, Prakash L. Efficient and error-free replication past a minor-groove N2-guanine adduct by the sequential action of yeast Rev1 and DNA polymerase zeta. Mol Cell Biol. 2004 Aug;24(16):6900-6.

174. Seki M, Masutani C, Yang LW, Schuffert A, Iwai S, Bahar I, Wood RD. High-efficiency bypass of DNA damage by human DNA polymerase Q. EMBO J. 2004 Nov 10;23(22):4484-94.

175. Yoon JH, Roy Choudhury J, Park J, Prakash S, Prakash L. A role for DNA polymerase θ in promoting replication through oxidative DNA lesion, thymine glycol, in human cells. J Biol Chem. 2014 May 9;289(19):13177-85

176. Conde J, Yoon JH, Roy Choudhury J, Prakash L, Prakash S. Genetic Control of Replication through N1-methyladenine in Human Cells. J Biol Chem. 2015 Dec 11;290(50):29794-29800.

177. Chan SH, Yu AM, McVey M. Dual roles for DNA polymerase theta in alternative end-joining repair of double-strand breaks in Drosophila. PLoS Genet. 2010 Jul 1;6(7):e1001005.

178. Yousefzadeh MJ, Wyatt DW, Takata K, Mu Y, Hensley SC, Tomida J, Bylund GO, Doublié S, Johansson E, Ramsden DA, McBride KM, Wood RD. Mechanism of suppression of chromosomal instability by DNA polymerase POLQ. PLoS Genet. 2014 Oct 2;10(10):e1004654.

179. Fernandez-Vidal A, Guitton-Sert L, Cadoret JC, Drac M, Schwob E, Baldacci G, Cazaux C, Hoffmann JS. A role for DNA polymerase θ in the timing of DNA replication. Nat Commun. 2014 Jul 3;5:4285.

180. Zahn KE, Averill AM, Aller P, Wood RD, Doublié S. Human DNA polymerase θ grasps the primer terminus to mediate DNA repair. Nat Struct Mol Biol. 2015 Apr;22(4):304-11

181. Frank EG, Sayer JM, Kroth H, Ohashi E, Ohmori H, Jerina DM, Woodgate R. Translesion replication of benzo[a]pyrene and benzo[c]phenanthrene diol epoxide adducts of deoxyadenosine and deoxyguanosine by human DNA polymerase iota. Nucleic Acids Res. 2002 Dec 1;30(23):5284-92.

182. Washington MT, Minko IG, Johnson RE, Wolfle WT, Harris TM, Lloyd RS, Prakash S, Prakash L. Efficient and error-free replication past a minor-groove DNA adduct by the sequential action of human DNA polymerases iota and kappa. Mol Cell Biol. 2004 Jul;24(13):5687-93.

183. Wolfle WT, Johnson RE, Minko IG, Lloyd RS, Prakash S, Prakash L. Human DNA polymerase iota promotes replication through a ring-closed minor-groove adduct that adopts a syn conformation in DNA. Mol Cell Biol. 2005 Oct;25(19):8748-54.

184. Johnson RE, Haracska L, Prakash L, Prakash S. Role of hoogsteen edge hydrogen bonding at template purines in nucleotide incorporation by human DNA polymerase iota. Mol Cell Biol. 2006 Sep;26(17):6435-41.

185. Takata K, Shimizu T, Iwai S, Wood RD. Human DNA polymerase N (POLN) is a low fidelity enzyme capable of error-free bypass of 5S-thymine glycol. J Biol Chem. 2006 Aug 18;281(33):23445-55.

186. Yamanaka K, Minko IG, Takata K, Kolbanovskiy A, Kozekov ID, Wood RD, Rizzo CJ, Lloyd RS. Novel enzymatic function of DNA polymerase nu in translesion DNA synthesis past major groove DNA-peptide and DNA-DNA cross-links. Chem Res Toxicol. 2010 Mar 15;23(3):689-95.

187. Lee YS, Gao Y, Yang W. How a homolog of high-fidelity replicases conducts mutagenic DNA synthesis. Nat Struct Mol Biol. 2015 Apr;22(4):298-303.

188. Nelson JR, Lawrence CW, Hinkle DC. Deoxycytidyl transferase activity of yeast REV1 protein. Nature. 1996 Aug 22;382(6593):729-31.

189. Haracska L, Prakash S, Prakash L. Yeast Rev1 protein is a G template-specific DNA polymerase. J Biol Chem. 2002 May 3;277(18):15546-51.

190. Swan MK, Johnson RE, Prakash L, Prakash S, Aggarwal AK. Structure of the human Rev1-DNA-dNTP ternary complex. J Mol Biol. 2009 Jul 24;390(4):699-709.

191. Choi JY, Guengerich FP. Kinetic analysis of translesion synthesis opposite bulky N2- and O6-alkylguanine DNA adducts by human DNA polymerase REV1.J Biol Chem. 2008 Aug 29;283(35):23645-55.

192. Gibbs PE, Kilbey BJ, Banerjee SK, Lawrence CW. The frequency and accuracy of replication past a thymine-thymine cyclobutane dimer are very different in Saccharomyces cerevisiae and Escherichia coli. J Bacteriol. 1993 May;175(9):2607-12.

193. Haracska L, Unk I, Johnson RE, Johansson E, Burgers PM, Prakash S, Prakash L. Roles of yeast DNA polymerases delta and zeta and of Rev1 in the bypass of abasic sites. Genes Dev. 2001 Apr 15;15(8):945-54.

194. Avkin S, Adar S, Blander G, Livneh Z. Quantitative measurement of translesion replication in human cells: evidence for bypass of abasic sites by a replicative DNA polymerase. Proc Natl Acad Sci U S A. 2002 Mar 19;99(6):3764-9.

195. Guo C, Fischhaber PL, Luk-Paszyc MJ, Masuda Y, Zhou J, Kamiya K, Kisker C, Friedberg EC. Mouse Rev1 protein interacts with multiple DNA polymerases involved in translesion DNA synthesis. EMBO J. 2003 Dec 15;22(24):6621-30.

196. Tissier A, Kannouche P, Reck MP, Lehmann AR, Fuchs RP, Cordonnier A. Co-localization in replication foci and interaction of human Y-family members, DNA polymerase pol eta and REVl protein. DNA Repair (Amst). 2004 Nov 2;3(11):1503-14.

197. Ohashi E, Murakumo Y, Kanjo N, Akagi J, Masutani C, Hanaoka F, Ohmori H. Interaction of hREV1 with three human Y-family DNA polymerases. Genes Cells. 2004 Jun;9(6):523-31.

198. van Gent DC, Hoeijmakers JH, Kanaar R. Chromosomal stability and the DNA double-stranded break connection. Nat Rev Genet. 2001 Mar;2(3):196-206.

199. Witkin EM. Ultraviolet mutagenesis and inducible DNA repair in Escherichia coli. Bacteriol Rev. 1976 Dec; 40(4): 869–907.

200. Rupp WD, Howard-Flanders P. Discontinuities in the DNA synthesized in an excision-defective strain of Escherichia coli following ultraviolet irradiation. J Mol Biol. 1968 Jan 28;31(2):291-304.

201. Lehmann AR. Postreplication repair of DNA in ultraviolet-irradiated mammalian cells. J Mol Biol. 1972 May 28;66(3):319-37.

202. Yoon JH, Park J, Conde J, Wakamiya M, Prakash L, Prakash S. Rev1 promotes replication through UV lesions in conjunction with DNA polymerases η, ι, and κ but not DNA polymerase ζ. Genes Dev. 2015 Dec 15;29(24):2588-602.

203. Yoon JH, Prakash L, Prakash S. Error-free replicative bypass of (6-4) photoproducts by DNA polymerase zeta in mouse and human cells. Genes Dev. 2010 Jan 15;24(2):123-8.

204. Takata K., Shimizu T., Iwai S., Wood R. D. (2006) Human DNA polymerase N (POLN) is a low fidelity enzyme capable of error-free bypass of 5S-thymine glycol. J. Biol. Chem. 281, 23445–23455

205. Kusumoto R., Masutani C., Iwai S., Hanaoka F. (2002) Translesion synthesis by human DNA polymerase η across thymine glycol lesions. Biochemistry 41, 6090–6099]

206. Lambert IB1, Singer TM, Boucher SE, Douglas GR. Detailed review of transgenic rodent mutation assays. Mutat Res. 2005 Sep;590(1-3):1-280.

207. Hirano Y, Sugimoto K. ATR homolog Mec1 controls association of DNA polymerase zeta-Rev1 complex with regions near a double-strand break. Curr Biol. 2006 Mar 21;16(6):586-90.

Vita

Juan Antonio Conde born on October 31st, 1985, is the son of Dr. Jose A Conde and Hilda G Fabela and has an older sister and a younger brother. He graduated Summa Cum Laude in 2007 from Texas A&M University Kingsville with a double major degree in Chemistry and Biomedical Science. He remained in Kingsville to work researching mechanisms of apoptosis and nerve regeneration under the supervision of Drs. Maribel Gonzalez-Garcia and Rafael Perez-Ballestero, earning his MS from Texas A&M University Kingsville in 2011 and publishing work on apoptosis and nerve regeneration during this period. He furthered his teaching skills through tutoring in science to undergraduates and becoming a teaching assistant in embryology, bacteriology, cellular physiology, and biochemistry. He came to Galveston in 2010 and joined the Prakash laboratory where he studied TLS mechanisms on BPDE, UV, and 1-MeA and will graduate with a PhD in 2016.

Education

B.S. Chemistry; Biomedical Sciences, May 2007, Texas A&M University Kingsville, Kingsville TX

M.S. Chemistry, May 2011, Texas A&M University Kingsville, Kingsville TX

Publications

Conde J, Yoon JH, Roy Choudhury J, Prakash L, Prakash S. Genetic Control of Replication through N1-methyladenine in Human Cells. J Biol Chem. 2015 Dec 11;290(50):29794-29800.

Yoon JH, Park J, **Conde J**, Wakamiya M, Prakash L, Prakash S. Rev1 promotes replication through UV lesions in conjunction with DNA polymerases η, ι, and κ but not DNA polymerase ζ. Genes Dev. 2015 Dec 15;29(24):2588-602.

Conde JA, Claunch CJ, Romo HE, Benito-Martín A, Ballestero RP, González-García M. Identification of a motif in BMRP required for interaction with Bcl-2 by site-directed mutagenesis studies. J Cell Biochem. 2012 Nov;113(11):3498-508.

Malladi S, Parsa KV, Bhupathi D, Rodríguez-González MA, **Conde JA**, Anumula P, Romo HE, Claunch CJ, Ballestero RP, González-García M. Deletion mutational analysis of BMRP, a proapoptotic protein that binds to Bcl-2. Mol Cell Biochem. 2011 May;351(1-2):217-32.

Pathi SS, Jose S, Govindaraju S, **Conde JA**, Romo HE, Chamakura KR, Claunch CJ, Benito-Martín A, Challa-Malladi M, González-García M, Ballestero RP. zRICH, a protein induced during optic nerve regeneration in zebrafish, promotes neuritogenesis and interacts with tubulin. Brain Res. 2012 Sep 20;1474:29-39.

Summary of Dissertation

Cells are constantly exposed to numerous genotoxic lesions that inhibit replication. The inability of cells to correctly repair these lesions results in mutations or chromosomal aberrations that threaten the integrity of the genome. DNA damage tolerance mechanisms, including translesion synthesis (TLS), alleviate this block at the expense of increasing mutagenesis.

Minor groove DNA lesions result from lipid peroxidation or exposure to environmental pollutants. Prevalent among these lesions are those produced by tobacco products, particularly benzo(a)pyrene-diolepoxide (BPDE), a polycyclic aromatic hydrocarbon strongly associated with carcinogenesis, particularly lung cancer. Alkylating lesions result from exposures to endogenous methylating agents and naturally occurring methyl halides. They might interfere with base pairing and are cytotoxic. The long-term goal is to understand the mechanisms by which replication through such ubiquitous lesions occurs in human cells.

Tumorigenesis is a multistep process associated with accumulation of mutations. Understanding of the biochemical basis of lesion bypass and the role of TLS polymerases will result in insights on how human cells handle exposure to environmental carcinogens and how the TLS processes contribute to cancer avoidance or to cancer risk.

Permanent address: 2292 Eidson Road, Eagle Pass TX 78852

This dissertation was typed by Juan A Conde.