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Regulation of DNA Damage Processing by Covalent Modification of Thymine DNA Glycosylase

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REGULATION OF DNA DAMAGE PROCESSING BY COVALENT MODIFICATION OF THYMINE DNA GLYCOSYLASE

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Abstract

Thymine DNA glycosylase (TDG) is an essential DNA repair enzyme mediating excision of uracil and thymine mispaired with guanine within CpG contexts. Unrepaired, these lesions result in G:C to A:T transitions which are major contributors to genome instability. Interestingly, TDG interacts functionally with transcriptional regulators and participates in directed cytosine demethylation at promoters. TDG is subject to multiple post-translational modifications (PTM) and we undertook an analysis of how these regulate TDG function.

Initially, we examined TDG regulation by small ubiquitin-like modifier (SUMO) and identified a novel SUMO binding motif (SBM1, residues 144-148). We hypothesized that SBM1, along with SBM2 (319-322), would facilitate non-covalent SUMO interactions upon conjugation of SUMO (sumoylation) to lysine 341, altering TDG conformation and function. Biochemical and cell based analyses supported our hypothesis, showing SUMO interactions allosterically regulate TDG protein-protein and substrate interactions, altering TDG subnuclear localization and enzymatic function. Furthermore, sumoylation drastically reduced acetylation of TDG occurring at lysines 70, 94, 95, and 98.

Secondly, we examined TDG regulation by phosphorylation and demonstrated that serines 96 and 99 are phosphorylated by protein kinase C α *in vivo*. Biochemical analysis of covalently modified recombinant TDG showed that acetylation and phosphorylation of TDG are mutually exclusive and both are suppressed by TDG-DNA interactions. Furthermore, acetylated TDG did not interact stably with DNA or efficiently excise thymine from G:T mispairs, while phosphorylated TDG was indistinguishable from unmodified protein.

Lastly, we examined TDG regulation in aging cells. Immunostaining showed TDG redistributed from nucleus to cytoplasm in aged cells. Interestingly, treatment with histone deacetylase inhibitors resulted in similar redistribution and immunoblotting indicated that an increase in TDG modification consistent with sumoylation or monoubiquitination had occurred. Similar results were obtained by exposing cells to oxidative stress. Analysis of a sumoylation-minus mutant of TDG identified sumoylation as an important regulator of TDG localization. Interestingly, we found extensive colocalization of TDG with sites of active transcription which was reduced by phorbol ester treatments which surprisingly promoted entry into heterochromatic regions from which TDG is generally excluded.

Together, these findings suggest that TDG function may be regulated by PTM, consequently affecting genome stability and expression.

Keywords: thymine DNA glycosylase (TDG), DNA repair, base excision repair (BER), 5-methylcytosine, spontaneous hydrolytic deamination, epigenetic modification, carcinogenesis, acetylation, phosphorylation, sumoylation.

Co-Authorship

Chapter 2: SUMO-1-dependent allosteric regulation of thymine DNA glycosylase alters subnuclear localization and CBP/p300 recruitment

Ryan Mohan produced figures 2.1C, 2.2C, 2.3C, 2.4C, 2.4D, 2.5D, 2.7B, 2.7C, 2.7D, 2.7E, S.2.1, S.2.2, S.2.3 (with JG), S.2.4, S.2.6, and "data not shown": TDG(1-121) is not a modular DNA-binding domain; TDG does not appreciably interact with baculovirus-expressed FLAG-PML.

Jason Gagliardi prepared recombinant mock-sumoylated and sumoylated TDG used in this study. J. Gagliardi also performed quick-change PCR reactions to create expression vectors for TDG SUMO-binding motif (SBM) point mutants. M. Tini produced FLAG-PML. Otherwise, proteins used in these studies were produced by R. D. Mohan.

Chapter 3: Opposing regulatory roles of phosphorylation and acetylation in DNA mispair processing by thymine DNA glycosylase

Yuhua Dong performed quick-change PCR reactions to create expression vectors for TDG phosphorylation mutants TDG(S96-99A) and TDG(S96-99D), as well as acetylation mutant TDG(K94-95-98R).

Chapter 4: Subnuclear localization of TDG is regulated by posttranslational modifications.

The pCMX-based mammalian expression vector for YFP-SUMO was constructed by Marc Tini.

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List of Abbreviations, Symbols, and Nomenclature

A	adenine
ABCD assay	avidin-biotin coupled DNA binding assay
AcCoA	acetyl coenzyme A
ADAR	adenosine deaminases acting on RNA
ADP ribose	adenosine diphosphate ribose
AD1 & AD2	activation domains 1 and 2
Alu	short DNA sequence originally characterized by the action of the Alu restriction endonuclease and comprising the most abundant mobile elements in the human genome
AP	apurinic/apyrimidinic
APE	apurinic/apyrimidinic endonuclease
AP1	sequence-specific transcription factor, also known as jun oncogene, AP1, Jun, C-JUN
ATM	ataxia telangiectasia mutated
ATP	adenosine triphosphate
ATR	ataxia telangiectasia and RAD3 related
BER	base excision repair
bp	base pair(s)

BSA	bovine serum albumin
C	cytosine
°C	degrees Celsius
CARM1	coactivator associated methyltransferase 1
CBP	cyclic-AMP response element binding protein (CREB) binding protein
cDNA	complementary DNA: single-stranded DNA that is complementary to messenger RNA or DNA that has been synthesized from messenger RNA by reverse transcription
CFP	cyan fluorescent protein
ChIP	chromatin immunoprecipitation
CH3	cysteine/histidine-rich domain 3
C-Jun	sequence-specific transcription factor, also known asjun oncogene, AP1, AP-1, JUN
cm	centimeters
CMV	cytomegalovirus
COS7	SV40-transformed, T antigen expressing, African Green Monkey kidney fibroblast cell line

CpG	cytosine next to guanine separated by a phosphate within the linear sequence of DNA. CpG represents cytosine-phosphate-guanine
CRIP1	cysteine-rich protein 1, a LIM/double zinc finger protein
CREB	cyclic-AMP response element binding protein
CSA/ERCC8	excision repair cross-complementing rodent repair deficiency, complementation group 8
CSB/ERCC6	excision repair cross-complementing rodent repair deficiency, complementation group 6
CSB/ERCC8	excision repair cross-complementing rodent repair deficiency, complementation group 8
CtIP	C-terminal Binding Protein (CtBP) interacting protein
CY3	cyanine 3
Da	Dalton: unit of mass used to express atomic and molecular mass equivalent to $1.660\,538\,782(83) \times 10^{-24}$ g
DAPI	4',6-diamidino-2-phenylindole
Daxx	death associated protein 6
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid

DNA-PKcs	DNA protein kinase catalytic subunit
DNA polβ/ε	DNA polymerase β/ε
Dnmt3a	DNA methyltransferase 3a
DSB	DNA double strand breaks
dTMP	deoxythymidine monophosphate
DTT	dithiothreitol
dTTP	thymidine triphosphate
dUMP	deoxyuridine monophosphate
dUTP	deoxyuridine triphosphate
EDTA	2,2',2",2'"-(ethane-1,2-diyldinitrilo)tetraacetic acid
EGF	epidermal growth factor
EGTA	glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid
EMSA	electrophoretic mobility shift assay
eMUG	*E. coli* Mismatch-specific uracil DNA-glycosylase
ER	estrogen receptor
ERCC/XPF	excision-repair, complementing defective, in Chinese hamster cells/ DNA repair endonuclease XPF.

ERCC5/XPG	excision repair cross-complementing rodent repair deficiency, complementation group 5/ DNA repair protein complementing XP-G cells.
ERCC8	excision repair cross-complementing rodent repair deficiency, complementation group 8
ERα	estrogen receptor α
FdUMP	fluorodeoxyuridine monophosphate
FdUTP	fluorodeoxyuridine triphosphate
FEN1	flap endonuclease 1
FITC	fluorescein isothiocyanate
FLAG	protein peptide tag consisting of amino acids DYKDDDDK (1012 Da)
FUMP	fluorouracil monophosphate
FUTP	fluorouridine triphosphate
FOXO4	forkhead box protein O4
G	guanine
GAL4	a sequence specific yeast transcription activator protein
GFP	green fluorescent protein
GG	global genome

GMP	guanine monophosphate
Gö6976	PKCα chemical inhibitor
GST	glutathione-S-transferase
SAE1	SUMO1 activating enzyme subunit 1
SAE2	SUMO activating enzyme 2
THF	tetrahydrofuran nucleotide – an abasic site analog
G1	gap 1 phase of the cell cycle
G2	gap 2 phase of the cell cycle
HAT	histone acetyltransferase
HDAC	histone deacetylase
HDACi	histone deacetylase inhibitor
HDAC1,6	histone deacetylase 1,6
HEK 293T	hypotriploid human epithelial cell line originating from embryonic kidney and transformed with adenovirus 5 DNA. These cells express the vitronectin receptor and contain an Adenovirus 5 DNA sequence from nts 1 to 4344 integrated into chromosome 19 (19q13.2)
HeLa	human epithelial adenocarcinoma cell line originating from cervix of 31 year-old adult female. The HeLa cell line also

	contains human papilloma virus 18 sequences and expresses p53 at low levels.
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIS	polyhistidine, 6xHIS, or hexahistidine protein tag
HNPCC	hereditary nonpolyposis colon cancer
hOGG1	human 8-oxoguanine glycosylase
HP	high passage
HPLC	high-pressure liquid chromatography
HR	homologous recombination
HSF1,2	heat shock transcription factor 1, 2
h	hour
H1	histone 1
H2A	histone 2A
H2AX	phosphorylate histone variant
H2B	histone 2B
H_2O_2	hydrogen peroxide
H3	histone 3
H4	histone 4

IgG	immunoglobulin G
IMR-90	normal diploid Human fetal lung fibroblast cell line capable of 58 doublings before onset of senescence
IN080	ATP-dependent chromatin remodeling complex
kDa	kilodalton
Ku70/80	in eukaryotes Ku is a heterodimer of two polypeptides, Ku70 and Ku80 (also known as XRCC6 and XRCC5 respectively)
LP	low passage
LRD	lysine-rich domain
M	marker or meter where appropriate
M2	anti-FLAG monoclonal antibody
MBD	methyl binding domain
MBD4	methyl binding domain protein 4
MCF-7	Human breast adenocarcinoma cell line which is estrogen receptor (ER) and cytokeratin positive; desmin, endothelin, GFAP, neurofilament, and vimentin negative
mc	main chain
mCi	millicurie

MG	methyl-guanine
MGMT	O6-methyl-guanine methyltransferase
MG132	amino acid peptide inhibitor of the proteasome, N-(benzyloxycarbonyl)leucinylleucinylleucinal Z-Leu-Leu-Leu-al
Min	minute
Mi-2	subunit of the NuRD complex possessing a SNF2-type ATP-dependent nucleosome remodeling activity
MLH1	mutL homolog 1
MLH3	mutL homolog 3
MMR	DNA mismatch repair
MRE11	repair factor
MRN	Mrell/Rad50/NbsI
mRNA	messenger RNA
MSH2,3,6	mutS homolog 2, 3, 6
MutSα	consisting of MSH2-MSH6
MutSβ	consisting of MSH2-MSH3
Myb	Myb transcription factor
NaCl	sodium chloride

NaOH	sodium hydroxide
nCaRE s	responsive elements
NER	nucleotide excision repair
NF-Kappa β	nuclear factor of kappa light polypeptide gene enhancer in B-cells
NHEJ	non-homologous end joining
NIH 3T3	fibroblast cell line established from an NIH Swiss *Mus musculus* embryo
NLS	nuclear localization signal
NMR	Nuclear magnetic resonance
NS	non specific
NSB1	repair factor
NUP358	nucleoporin of 358 kDa
NuRD	nucleosome remodeling and deacetylase complex
OGG1	8-oxoguanine glycosylase
P19	*Mus musculus* epithelial cells, derived from a teratocarcinoma induced in a C3H/He mouse, which differentiate into neural and glial like cells upon treatment with 500 nM retinoic acid, or into cardiac and skeletal muscle-like cells upon treatment with 1% DMSO

PAGE	polyacrylamide gel electrophoresis
PARP	Poly (ADP-ribose) polymerase
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline + 0.05% tween-20
pCAF	p300/CBP-associated factor
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
pH	unit of measurement of acidity or basicity
PIC	preinitiation complex
PKC	protein kinase C
PMA	phorbol-12-myristate-13-acetate
PML	promyelocytic leukaemia protein
PMS1	postmeiotic segregation increased 1
PMS2	postmeiotic segregation increased 2
PNK	polynucleotide kinase
Polβ	DNA polymerase β

POD(s)	PML oncogenic domain
Po1 II	RNA polymerase II
PPARα	nuclear receptor peroxisome proliferator-activated receptor alpha
p-pol II	phosphorylated RNA polymerase II
PRMT1	protein arginine N-methyltransferase
pS2/TFF1	trefoil factor 1 is also known as pS2 as well as BCEI, HPS2, HP1.A, pNR-2, D21S21
pTDG	phosphorylated TDG
PTM	post-translational modification(s)
PVDF	polyvinylidene fluoride
P19	embryonic carcinoma stem cells
qPCR	quantitative polymerase chain reaction
Rad50	repair factor
RAD51	(XRCC2,XRCC3,RAD51B,RAD51C,RAD51D)
RanBP2	RAN binding protein 2
RanGAP1	Ran GTPase activating protein 1
RARα	retinoic acid receptor alpha

RASSF1A	Ras association (RalGDS/AF-6) domain family member 1
Rb	retinoblastoma protein
RNA	ribonucleic acid
RNA	polymerase
RPA	replication protein A
RUNX3	hypermethylation of tumor suppressor genes
RXR	retinoid x receptor
S100P	S100 calcium binding protein P
SAE1	small ubiquitin-like modifier (SUMO)-1 activating enzyme subunit 1
SAE2	small ubiquitin-like modifier (SUMO) activating enzyme 2
SAM	S-adenosyl methionine
SBM1,2	SUMO binding motifs 1 and 2
SDS	sodium dodecyl sulfate
SET	SET nuclear oncogene, also known as 2PP2A, IGAAD, TAF-I, I2PP2A, IPP2A2, PHAPII, TAF-IBETA
SF9	*Spodoptera frugiperda* epithelial cells derived from ovary which can be used to replicate baculovirus expression vectors and prepare the encoded recombinant proteins

Sp3	Sp3 transcription factor
Sp100	Speckled, 100-KD, a component of the PODs
SRC1,2,3	steroid receptor coactivators 1,2 and 3
SUMO	small ubiquitin-like modifier
SV40	simian virus 40
SWI/SNF	SWItch/Sucrose NonFermentable
T	thymine
TAF1 β	protein
TBS	tris-buffered saline
TBS-T	tris-buffered saline + 0.05% tween-20
TCR	transcription coupled repair
TDG	thymine DNA glycosylase where mTDG and hTDG refer to mouse and human TDG respectively
TFIIA,B,D,F,H	transcription factors
trans-acting	generally refers to processes acting from a different molecules, as opposed to acting on the same molecule (cis-acting)
Tris-HCl	2-Amino-2-hydroxymethyl-propane-1,3-diol, corrected to desired acidity or basicity (pH) with HCl

TSA	trichostatin A
TTF1	thyroid transcription factor 1
U	uracil
Uba2	ubiquitin-like modifier activating enzyme 2
Ubc9	ubiquitin-conjugating enzyme 9 - E2 conjugating enzyme for small ubiquitin-like modifier
UDG	uracil DNA glycosylase
v/v	volume to volume – used to indicate when solutions are to be mixed by relative volume
Wisp2	WNT1 inducible signaling pathway protein 2
WNT5A	wingless-type MMTV integration site family, member 5A
XPA	xeroderma pigmentosum, complementation group A
XPB	xeroderma pigmentosum group B
XPC-hHR23B	xeroderma pigmentosum, complementation group C-RAD23 homolog B complex
XPD	xeroderma pigmentation group D
XRCC1,2,3,4	X-ray repair complementing defective repair in Chinese hamster cells
YFP	yellow fluorescent protein

μCi	microcurie, 1 μCi is equivalent to 3.7×10^4 disintegrations per second, or 2.22×10^6 disintegrations per minute of a radioisotope
μg, ng, g	microgram, nanogram, gram
μL, mL	microlitre, millilitre
μM, mM, nM	micromolar, millimolar, nanomolar
μm, mm, nm	micrometer, millimeter, nanometer
V, mA	volts, milliamperes
5–meC	5-methylcytosine
5-FU	5–fluorouracil
5-HU	5-hydroxymethyluracil
2D-PAGE	two dimensional polyacrylamide gel electrophoresis
4αPMA	4α-Phorbol 12-myristate 13-acetate

Chapter 1: Introduction

1.1 Overview

Maintenance of genomic stability is crucial to ensure the fidelity of information contained within nucleotide sequences comprising genes and corresponding regulatory regions. The integrity of the genome is constantly undermined by DNA damage that occurs via distinct mechanisms, which potentially generate harmful mutations. This is problematic as precise regulation of gene expression is critical for normal development and homeostasis. Gene expression is dynamic process, tightly regulated and highly responsive to the cellular milieu. Occurring at every stage of the process, regulation of gene expression may be facilitated by proteins, RNA, and regulatory regions of the gene. Which of these is the predominant regulator of gene expression has been explored in studies performed on a mouse model of Down syndrome in which mice bear a copy of human chromosome 21. Despite being regulated by mouse proteins/RNAs in the mouse cellular milieu, the human chromosome 21 recruited transcription factors, directed modification of chromatin structure, and expressed genes as they would be in human cells rather than they were on the mouse equivalent chromosome. This strongly suggested that the predominant regulator of gene expression is the primary sequence of the gene itself rather than the local gene regulatory proteins, RNA, and cellular context that expression occurs within (337). This finding highlights the importance of ensuring stability of the primary genome sequence.

There are four major sources of DNA damage. First, DNA is an inherently unstable molecule and decays over time, predominantly through spontaneous hydrolysis creating abasic sites and deamination products (202). Secondly, metabolism produces highly reactive byproducts which create diverse types of DNA damage (67, 286). Thirdly, mutations may also arise due to deletion or misincorporation of DNA bases during

replication or DNA repair. Lastly, exogenous agents such as industrial chemical vinyl chloride may also damage DNA (11, 31, 329). It is estimated that there are as many as 10^5 spontaneous nucleotide damage events per day in each cell (202). A large body of evidence indicates that DNA damage causes mutations which are associated with genetic diseases, aging, and carcinogenesis (20, 43, 250). To ensure stable maintenance and inheritance of genetic material, several DNA repair pathways have evolved to repair DNA lesions. The pathway tasked with correcting the products of oxidative stress is the base excision repair (BER) pathway. BER is initiated by DNA glycosylase specific for the damaged base, and the context in which it is found. The substrate specificity of most glycosylases is overlapping, creating redundancy between the glycosylases which results in subtle effects in cell culture and animal models when an individual glycosylase is deficient. BER enzyme Thymine DNA glycosylase (TDG) however, is an important mediator of genome stability that is unique amongst glycosylases as mice deficient in TDG do not develop past mid-gestation (M. Tini, unpublished), indicating that TDG, unlike any other glycosylase studied to date, has a non-redundant role in cell function [M. Tini, unpublished, (44, 60, 83, 128)].

TDG is subject to multiple post-translational modifications (PTMs). PTMs such as phosphorylation and acetylation have long been recognized as crucial modulators of cell signaling and operate through diverse mechanisms to regulate protein function. PTM can act on substrate proteins to alter electrostatic charge, affecting conformation or enzymatic function. This may lead to changes in affinities for protein and non-protein partners, resulting in altered association with protein complexes. PTM have also been shown to be important regulators of subcellular localization. The focus of this thesis is an examination of how multiple PTM may act in concert to regulate protein function to affect genome stability and gene expression. Specifically, we focus on how PTM act to regulate TDG

function through modulation of subcellular localization, enzymatic function, and intra/intermolecular interactions.

Mechanisms of DNA damage

Watson and Crick were the first to correctly elucidate the structure of DNA as a double helix comprising two anti-parallel polynucleotide strands made continuous by linkage of the phosphate backbone and the two strands are held together by hydrogen bonding between the DNA bases adenine (A), thymine (T), cytosine (C), and guanine (G) such that pairing is always between A and T (A:T) or G and C (G:C) (333) (Figure 1.1.A). This simple arrangement represents a powerful mechanism for storage and propagation of biological information. Agents which damage DNA do so via diverse chemical reactions resulting in alterations to the DNA molecular structure preventing its normal metabolism and replication fidelity. Additional sources of damage may arise due to inherent instability of the DNA molecule itself, resulting in spontaneous damage such as hydrolytic deamination of cytosine and 5-meC (Figure 1.1.B). Because of its centrality to biological function, DNA damage may potentially affect all biological processes directly or indirectly.

Figure 1.1. DNA structure. A. The structure of unmodified DNA showing correct pairing between adenine (A) and thymine (T); cytosine (C) and guanine (G). B. DNA damage via hydrolytic deamination of cytosine and 5-methyl-cytosine (5-meC) produces uracil (U) and thymine lesions respectively.

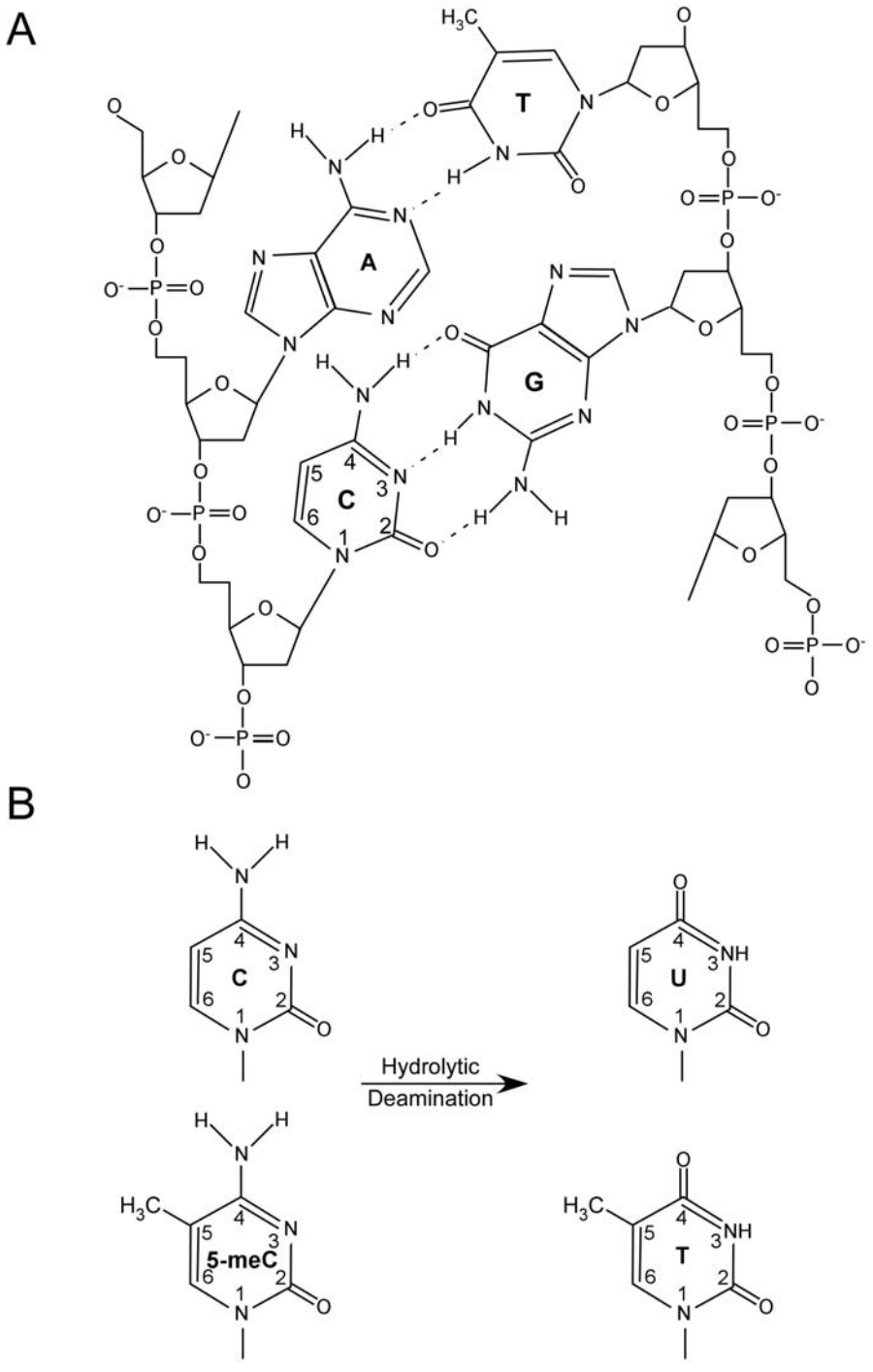

Figure 1.1

Hundreds of distinct DNA damage products have been documented making a comprehensive discussion of each beyond the scope of this thesis. However, DNA damaging agents may be categorized in four ways. First, environmental toxins, ranging from sunlight and ionizing radiation to food and industrial chemicals, have been shown to interact with DNA and produce genotoxic lesions which may be carcinogenic (106, 111, 194, 339). Some of these DNA adducts have been proposed to useful biomarkers for exposure to environmental toxins as well as risk of carcinogenesis (295, 322). Secondly, cellular metabolism, including oxidative respiration and lipid peroxidation, produces reactive oxygen species such as superoxide anions, hydroxyl radicals, and hydrogen peroxide. These are powerful mediators of DNA damage and account for over 100 different types of DNA damage (41, 150). Thirdly, DNA is prone to spontaneous degradation over time arising from base hydrolysis producing abasic sites, or from hydrolytic deamination of bases. For example, deamination of cytosine, 5-methylcytosine, guanine, and adenine produces uracil, thymine, xanthine, and hypoxanthine, respectively (41, 234, 263). Mutations may also arise due to deletion or misincorporation of DNA bases during replication or DNA repair potentially altering coding or gene regulatory sequences. These may alter regulation of gene expression or mRNA processing and may result in the production of aberrant gene products (63, 347) [see Table 1.1, adapted from Dalhus et al., 2009 (63)].

Physical breakage of the DNA double-helix, or DNA double strand breaks (DSBs), may be created by ionizing radiation, x-rays, industrial chemicals, reactive oxygen species, excessive base excision repair (see below), replication of single strand DNA breaks, uncapped telomeres, stalled/collapsed replication forks, as well as through natural process related to immunological function.

Consequences of DNA damage

The cell has multiple strategies for responding to DNA damage. Initial responses to damage which may interfere with cellular processes may include initiation of transient cell cycle arrest (16, 107, 110, 293, 321). Specific points for cell cycle arrest are cell cycle stages G1, S, G2, and M and may include inhibition of transcription, DNA replication, and chromosome segregation. During this time, cellular DNA repair mechanisms attempt to correct the damage (357). If exposure to DNA damage is too severe to allow re-entry into the cell cycle, pre-programmed cell death may occur, or alternatively, the cell may enter a type of permanent cell cycle arrest termed senescence (1, 95, 190, 196). When DNA damage persists, it may result in alterations to normal DNA sequences which can be incorporated into the genome as mutations. Mutations in different contexts have been shown to contribute to aging and diseases including cancer in humans (121, 122, 142, 207). It is important to note that DNA damage may also be a source of biological diversity and its occurrence over time, leads to greater allelic diversity and robustness of species. Without some level of genome instability there would be no genetic diversity and the resulting genetic monoculture becomes highly susceptible to catastrophic events such as outbreaks of disease (76).

Table 1.1 Representative sources of DNA base damage along with common lesions and the corresponding major repair pathways in humans.

Damaging agent	Prototypical lesions	Major repair pathway	Representative repair enzymes
Alkylating agents	06-mG	DR	Akyltransferases, MGMT
	1-mA	DR	Oxidoreductases, ABH2
	3-mA, 3-mG, 7-mA, 7-mG	BER	Glycosylases, AAG
Hydrolysis	Abasic sites	BER	Endonucleases, APE
	Deamination forming uracil/thymine	BER	Glycosylases, UNG, TDG
ROS	8-oxoG, faPyA/G, TG, 5-ohC, DHU, DHT, U	BER	Glycosylases, OGG1, NTH1, TDG, MBD4
Replication errors	Base mismatches	MMR	Mismatch proteins, MutSα, MutSβ
	Insertion/deletion loops	MMR	Mismatch proteins, MutSα, MutSβ
	DSB	HR/NHEJ	DSB repair proteins, DNA-PK_{CS}
UV radiation	Bulky adducts	NER	NER proteins, XPA, XPF, XPG
	CPDs, 6-4 PDs	NER	Photolyases: CPD and (6-4) photolyases
	DSB	HR/NHEJ	DSB repair proteins, DNA-PK_{CS}
Industrial chemicals	$3,N^4$-ethenocytosine	BER	TDG
Chemotherapeutics	5-FU	BER	TDG, MBD4

ROS, reactive oxygen species; DSB, DNA double strand break; 5-FU, 5-fluorouracil; DR, direct reversal; BER, base excision repair; MMR, mismatch repair; NER, nucleotide excision repair; HR/NHEJ, homologous recombination/non-homologous end joining; MGMT, O6-methylguanine methyltransferase; ABH2, alkylation repair homolog 2; AAG, alkyladenine DNA glycosylase; APE, apurinic apyriminic endonuclease; UNG, uracil N glycosylase, TDG, thymine DNA glycosylase; OGG1, 8-oxoguanine DNA glycosylase; NTH1, mammalian homologs of *Escherichia coli* endonuclease III (Nth); MBD4, methyl-CpG-binding domain protein 4; MutSα, the MSH2-MSH6 dimer; MutSβ, the MSH2-MSH3 dimer; DNA-PK_{CS}, DNA protein kinase catalytic subunit; XPA, XPF, XPG, xeroderma pigmentosum group A, F, G; CPD photolyase, cyclobutane pyrimidine dimer photolyase;

DNA repair pathways

DNA repair occurs through a number of multi-protein pathways: homologous recombination (HR), non-homologous end joining (NHEJ), DNA mismatch repair (MMR), nucleotide excision repair (NER) (including transcription coupled repair), and base excision repair (BER). An alternative mechanism is available which utilizes a single protein to enact repair. The basic mechanisms underlying these pathways are briefly discussed below with emphasis on base excision repair, which is the focus of this thesis.

Direct DNA damage reversal

Alkylating agents contained in tobacco smoke and grilled food (69, 71) and endogenous enzymes (163, 202, 284) induce formation of a variety of methyl-DNA lesions, of which O^6-methyl-guanine are the most carcinogenic (52, 72, 163, 164, 210, 251, 273, 349). DNA-alkyltransferases are capable of reversing this damage through removal of the alkyl group in a one step reaction. For example, O^6-methylguanine methyltransferase (MGMT) removes aberrant methyl groups from O^6-methyl-guanine and O^6-methyl-thymine base lesions which are then transferred to an internal cysteine residue in a one step reaction which results in inactivation of MGMT (204). Following inactivation, MGMT is ubiquitinated and degraded (300, 345). Some products of alkylation damage are also repaired by the nucleotide excision repair pathway (described below) which competes with alkyltransferases to repair alkylated bases (285). In the case of O^6-methyl-guanine, the lesion is not efficiently repaired by the nucleotide excision repair system because the excised methyl-base is easily mistaken for a normal base and may be replaced into the position from which it was just excised, causing futile rounds of repair which continue until replication allows pairing with cytosine or thymine (62, 80, 165). The irreversible strategy used by MGMT ensures forward progression of the repair step although

degradation of MGMT in the final step of repair limits the robustness of MGMT-mediated repair to the number of molecules of MGMT available. Once these are degraded, new MGMT proteins must be expressed. The alkylating/methylating chemotherapeutic temozolomide is used to capitalize on this limitation as treatment with pharmacological doses of temozolomide produces large amounts of MGMT substrate DNA damage. Repair of these lesions exhaust MGMT-mediated repair by depleting the cellular compliment of MGMT. The following rounds of futile mismatch repair then contribute to DNA fragmentation and cytotoxicity (62, 80, 139, 165, 252, 309). Larger adducts, such as ethylated guanine, do not fit into the substrate pocket of MGMT and are mainly repaired by nucleotide excision repair (see below) (34, 312).

Homologous recombination and non-homologous end joining

DNA double strand breaks (DSBs) may be created by ionizing radiation, x-rays, industrial chemicals, reactive oxygen species, excessive base excision repair (see below), replication of single strand DNA breaks, uncapped telomeres, stalled/collapsed replication forks, as well as through class switch recombination – a natural process related to immunological function (183, 318). DSBs are repaired by either homologous recombination (HR) or non-homologous end joining (NHEJ) (19).

Homologous recombination is initiated by alterations in chromatin (chromatin is discussed below) structure at DSB sites which activate Mre11/Rad50/Nbs1 (MRN) complex. The MRN complex concurrently binds the ends of the DSB and activates ataxia telangiectasia mutated (ATM) kinase, directing it toward the site of breakage. There, ATM, ataxia telangiectasia and Rad3 related (ATR), and DNA protein kinase catalytic subunit (DNA-PK_{CS}) phosphorylate histone variant H2AX, marking the DSB. At the DSB ends the MRN complex mediates 5'-3' strand trimming, leaving 3' overhangs suitable for DNA recombination. The 3' overhang is populated by replication protein A

(RPA) which recruits RAD51-related proteins XRCC2, XRCC3, RAD51B, RAD51C, and RAD51D, forming a filamentous structure. The sister chromatid is positioned for recombination by cohesions and the filamentous RAD51 proteins direct recombination to matching sequences.

Non-homologous end joining is initiated by Ku70/80 heterodimers which bind and align the broken DNA ends (46, 331). Ku70/80 then recruits DNA-PK_{CS} to the DSB site and activates its kinase function (348). Together, DNA-PK_{CS} and Artemis proteins promote processing of the DNA ends to produce ends compatible for ligation (218). Lastly, XRCC4 ligates the aligned compatible ends, returning continuity of the strand (113).

HR is mostly error free due to the presence of an undamaged template whereas NHEJ often results in a small gain or loss of nucleotides during end processing to create ligatable DNA end fragments (219, 220)

The pathway chosen for repair of DSBs is highly dependent on which stage of the cell cycle the break was detected. HR is preferred during S and G2 stages of the cell cycle when there is a second, undamaged copy of the DNA sequence available due to the presence of a sister chromatid. Otherwise, during G0, G1, and early S-phase NHEJ is preferred (86). Interestingly, the type of break also contributes to the decision of which pathway is chosen for repair. For example, repair of DSBs caused by ionizing radiation in G1 are not be repaired by NHEJ. Instead repair is delayed until S or G2 so that HR may take place after DNA replication (12). The decision to use HR or NHEJ is influenced by cell cycle dependent post-translational modification of proteins which may direct progression of repair. For example, CtIP/Sae2 is a substrate for phosphorylation by cyclin dependent kinases active during S and G2 and favors initiation of HR over NHEJ (152, 287).

DNA mismatch repair

DNA mismatch repair (MMR) primarily addresses mismatches created during DNA replication and missed by replication-associated proofreading mechanisms (186). These damages may take the form of single base mismatches and single base loops, or insertion and deletion loops (92, 232). Dysfunction in MMR may lead to microsatellite instability and dramatic increases in mutation rates leading to carcinogenesis, particularly hereditary nonpolyposis colon cancer (HNPCC) in which 60% of cases examined show germline mutations in MMR proteins mutL homolog 1(MLH1) and mutS homolog 2 (MSH2) (94, 157, 315).

MMR is initiated by MutSα (consisting of MSH2-MSH6) and MutSβ (consisting of MSH2-MSH3) which contact DNA and, along with MutLα (consisting of MLH1 and postmeiotic segregation increased 2 (PMS2)) (51), form sliding clamps that scan along DNA until they recognize mispaired or DNA bases or loops. MutSα preferentially recognizes single base mismatches or single base loops, whereas MutSβ recognizes insertions and deletion loops of 2-8 bases (99, 223, 282). Once a lesion is identified MSH3 or MSH6 along with proliferating cell nuclear antigen (PCNA) (58) direct repair toward the damaged strand and a long stretch of the damaged strand is degraded by a 3'-5' exonuclease then resynthesized correctly by DNA polymerase δ and PCNA (208).

Nucleotide excision repair and transcription coupled repair

Nucleotide excision repair (NER) allows processing of a diverse set of DNA-helix distorting lesions. When used to correct DNA damage during transcription, the process is termed transcription-coupled repair (TCR) while all non-transcription-associated NER is referred to as global genome (GG) NER (242). NER is initiated upon detection of a disrupted base-pair causing distortion of the DNA helix by the xeroderma pigmentosum,

complementation group C-RAD23 homolog B (XPC-hHR23B) complex (303). Non-helix-distorting lesions generally are not addressed by NER but by base excision repair (see below) (308). Initiation of TCR occurs when a lesion is sufficient to stop RNA polymerase from proceeding at which point it is temporarily stopped by excision repair cross-complementing rodent repair deficiency, complementation group 6 (CSB/ERCC6) and excision repair cross-complementing rodent repair deficiency, complementation group 8 (CSA/ERCC8) proteins to allow NER to proceed. After stabilizing the paused polymerase, CSA and CSB recruit proteins which contribute to NER (85). From this point, NER and TCR follow the same pathway. Using the helicase activity of its xeroderma pigmentosum group B complementing (XPB) and xeroderma pigmentosum D (XPD) subunits, the TFIIH transcription factor unwinds approximately 30 base pairs surrounding the lesion, at which point xeroderma pigmentosum complementation group A (XPA) verifies that repair is needed (38). If this does not occur, then the NER process is halted and reversed (303). If DNA damage is still detected, replication protein A (RPA) then binds the undamaged strand, stabilizing the open DNA helix. Excision repair cross-complementing rodent repair deficiency, complementation group 5 (ERCC5/XPG) and excision repair cross-complementing rodent repair deficiency, complementation group 1 (ERCC1/XPF) endonucleases then cleave the damaged strand, excising 24-32 bases containing the damaged base. The normal DNA replication machinery then completes repair by filling in the gap using the undamaged strand as a template.

Base excision repair

The process of correcting more subtle, non-helix-distorting mismatches occurs predominantly through the base excision repair (BER) pathway (Figure 1.1) (reviewed by Hoeijmakers, 2001) (143) and is initiated by lesion-specific DNA glycosylases. The substrate specificities of the glycosylases are partially overlapping so that most lesions

may be corrected by more than one glycosylase. This redundancy, along with the ability of TCR (see above) to repair some lesions addressed by BER, is credited with the non-lethal phenotypes seen in mouse models of glycosylase deficiency (204, 336), with the notable exception of TDG (see below) (M. Tini, unpublished). The glycosylases may be divided into two types - monofunctional and bifunctional. Monofunctional glycosylases such as TDG (see below) lack lyase activity which is provided by DNA polymerase β. Lyase activity refers to the ability to catalytically break specific chemical bonds (in this case, referring to the DNA phosphate backbone) by means other than hydrolysis and oxidation, often forming or breaking a double bond in the process. Bifunctional glycosylases such as hOGG1 possess lyase activity and after base excision, cleave the phosphate backbone 3' of the abasic site. Abasic sites may also occur spontaneously and are unstable (98), producing cytotoxic DNA single stranded breaks upon degradation (203). Abasic sites also hinder DNA- and RNA-polymerases, potentially impeding replication or transcription or promoting error-prone bypass synthesis (335). DNA single strand breaks are also prone to forming double-strand breaks which are also cytotoxic. Additional risk of DNA double-strand break production is created from abasic sites arising closely together on opposite DNA strands. Whether produced by glycosylases or arising spontaneously, abasic sites are mostly processed by apurinic/apyrimidinic (AP) endonuclease 1 (APE) which prepares the 3' end of the phosphate backbone so that it becomes substrate for DNA polymerase. In mammalian cells, APE is the major AP endonuclease, processing 95% of the AP-site incision activity present (49, 68). Additionally, APE is essential for cellular proliferation and embryonic development in mice, and mice heterozygous for APE exhibit phenotypes associated with oxidative stress (90, 235, 343). The BER pathway also addresses damage produced by x-rays which can contribute to DNA damage directly by causing single strand breaks, or by reaction with water to create free radicals which then create a variety of lesions including single strand breaks (27, 116, 135, 332). In certain scenarios where single strand breaks are produced

as a by-product of radiation, repair is initiated by PARP which is activated by the presence of the breaks and along with polynucleotide kinase (PNK) and X-ray repair complementing defective repair in Chinese hamster cells 1(XRCC1) prepares the DNA ends for new DNA synthesis (3, 147). The resulting DNA lesions are predominantly addressed by the short-patch BER pathway and to a lesser extent by the long-patch BER pathway, a decision which may partially depend on the availability of ATP at the time of repair (268). The short-patch pathway is completed by DNA polβ which removes the remaining phosphate backbone and inserts a new nucleotide, followed by DNA ligase 3 which returns continuity to the strand, completing repair. XRCC1, which has no enzymatic activity, interacts with many BER core proteins also participates in these final steps and is thought to play a role as a scaffold protein in BER (147). Long-patch repair is completed by PCNA and DNA polβ/ε which synthesize a patch of new bases (2-10 bases) over the lesion after which FEN1 cleaves the displaced strand and DNA ligase 1 reseals the phosphate backbone, returning continuity to the strand. In cases where the BER lesion blocks transcription, the lesion is addressed by TCR and the NER pathway (see above).

Figure 1.2. The base excision repair (BER) pathway. Upon recognition of an aberrant base, the glycosylase binds and excises the mispaired base, generating a cytotoxic abasic site (step Ⅰ) (206). Abasic sites may also be generated through spontaneous hydrolysis. The abasic site is then processed by apurinic/apyrimidinic (AP) endonuclease 1 (APE) which prepares the 3' end of the phosphate backbone so that it may be processed by DNA polymerase (step Ⅱ). BER may also be initiated by DNA single strand breaks (SSBs) which activate Poly(ADP-ribose) polymerase (PARP). PARP, X-ray repair complementing defective repair in Chinese hamster cells 1(XRCC1), and polynucleotide kinase (PNK) bind the SSB and prepare the DNA ends for new DNA synthesis (step Ⅲ). The resulting single nucleotide gap may be processed by the short patch (predominant) or long-patch (minor) pathways. In the short-patch pathway DNA polymerase β in complex with XRCC1 cleaves the phosphate backbone 5' of the abasic site, removing the remaining piece of the base-free phosphate backbone (step Ⅳ), and then replaces the missing base (step Ⅴ). Finally, DNA ligase 3 in complex with XRCC1 returns continuity to the strand (step Ⅵ) (184). In the long-patch pathway the single nucleotide gap is processed by DNA pol δ/ε and PCNA which synthesize a short (2-10 bases) stretch of new DNA around the gap (step Ⅶ) after which FEN1 endonuclease cleaves the DNA flap displaced by synthesis (step Ⅷ) and DNA ligase 1 reseals the phosphate backbone, returning continuity to the strand and completing repair (step Ⅸ). Adapted from Hoeijmakers, 2001(143).

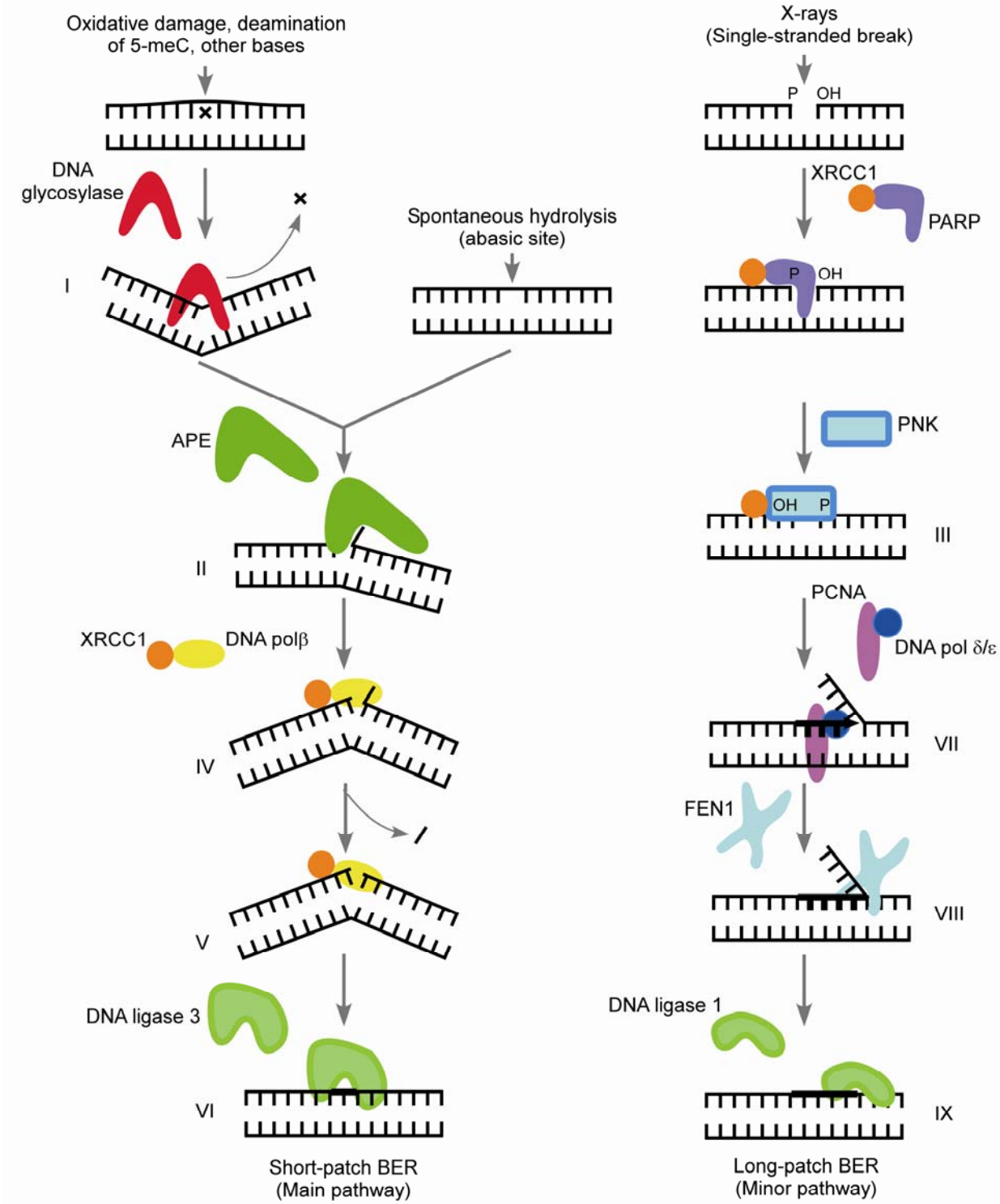
Oxidative damage, deamination
of 5-meC, other bases
DNA
glycosylase
I
Spontaneous hydrolysis
(abasic site)
APE
II
XRCC1
DNA polβ
IV
V
DNA ligase 3
VI
Short-patch BER
(Main pathway)
X-rays
(Single-stranded break)
P
OH
XRCC1
PARP
P
OH
PNK
OH
P
III
PCNA
DNA pol δ/ε
VII
FEN1
VIII
DNA ligase 1
IX
Long-patch BER
(Minor pathway)

Figure 1.2

Posttranslational regulation of the mammalian DNA base excision repair pathway

The BER pathways as described above, and many other DNA repair pathways, are affected by a series of protein complexes which assemble sequentially on the site of the DNA lesion being repaired (140). These complexes may include scaffolding proteins such as XRCC1 (281), which has been implicated in coordinating the entire BER pathway (42, 66, 224, 228, 325), or chromatin modifiers such as the CREB binding protein and its related family member p300 (CBP/p300), which have been identified as potentially acting at sites of DNA damage to acetylate histone tails and promote access for DNA repair proteins to chromatinized DNA (316). As each step of the pathway is completed, the resulting product is passed from the processing enzymes on to either the next protein complex or within the same complex, until the lesion has been repaired (338).

The repair proteins are subject to PTM which may act to modulate enzymatic activity, alter protein-protein interactions (316), and affect stability of the modified DNA repair proteins (118, 127). In this way, the DNA repair response may be tailored to specific cellular conditions such as exposure to DNA damaging oxidative stress (150) or to normal process such as cell cycle progression (118, 127) and transcription (22). Table 1.2 [adapted from Almeida et al., 2007 (3) and Fan et al., 2005 (78)] describes some examples of BER proteins which are subject to PTM and where known, indicates the effect these modifications may have on protein function. Although this thesis focuses on BER, regulation of DNA repair by PTM occurs in many of the other pathways outlined above. For example, NHEJ proteins Ku70 and Ku80 are subject to sumoylation and ubiquitination respectively (18) and in response to DNA DSB ATM dimers autophosphorylate, releasing monomers which propagate the γH2AX mark, assisting in DNA repair and cell cycle checkpoint activation (193).

Table 1.2. Known post-translational modifications (PTM) of base excision repair (BER) proteins

Function	Enzyme	Modification	Consequence on function	Reference
Lesion recognition & strand scission	TDG	Acetylation	Disrupts interaction with APE1, reduces phosphorylation, DNA binding	1
		Phosphorylation	Reduces acetylation by CBP/p300	2
		Sumoylation	Promotes G:C base excision activity while reducing DNA binding	3
	NEIL2	Acetylation	Reduces base excision and AP lyase activities	4
	UNG2	Phosphorylation	Cell cycle-specific: regulates protein turnover, activity and association with RPA	5
		Ubiquination	Leads to proteolytic degradation	6
	AAG	Acetylation	ND	7
	OGG1	Nitrosylation	Inhibits OGG1 glycosylase and/or AP lyase activity (ies)	8
		Phosphorylation	Chromatin associated OGG1 is phosphorylated with no detectable effect on enzymatic activity	9
	MYH	Phosphorylation	Promotes glycosylase activity	10
	MPG	Acetylation	ND	11
	APE	Acetylation	No impact on the endonuclease activity, regulates REDOX functions	12
		Oxidation	Reduces AP site incision activity	13
		Phosphorylation	Reduces AP site incision activity (inconsistent reports), regulates REDOX functions	14
		Ubiquination	Increases cytoplasmic localization and protein degradation	15
	MGMT	Phosphorylation	Reduces activity	16
	Msh2/6 (Mutsα)	Phosphorylation	Required for mismatch-binding activity and nuclear translocation	17
BER scaffold	XRCC1	Phosphorylation	Enhances PNK-binding activity	18
	PARP	Acetylation	Increases catalytic activity	19
		Phosphorylation	Increases catalytic activity	20
		Sumoylation	No impact on enzymatic activity detected	21
		Ubiquination	Inhibition of ubiquitination through cleavage of PARP stabilizes and activates PARP	22
Gap tailoring	FEN1	Acetylation	Reduces substrate binding and nuclease activities	23
		Phosphorylation	Reduces nuclease activities and disrupts interaction with PCNA	24
	WRN	Acetylation	Increases catalytic efficiency	25
		Phosphorylation	Decreases catalytic efficiency	26
		Sumoylation	No effect shown	27
DNA synthesis & ligation	DNA pol β	Acetylation	Reduces dRP lyase activity	28
		Methylation	Increases catalytic efficiency	29
		Phosphorylation	Increases catalytic efficiency	30
	DNA ligase 1	Phosphorylation	No effect shown	31
	DNA ligase 3	Phosphorylation	No effect shown	32
	PCNA	Acetylation	Increases catalytic efficiency	33
		Phosphorylation	Increases catalytic efficiency	34
		Sumoylation	No effect shown	35
		Ubiquination	Inconsistent reports of increased catalytic efficiency	36

TDG, thymine DNA glycosylase; NEIL2, nei like 2; UNG2, uracil-DNA glycosylase; AAG, alkyladenine DNA glycosylase; OGG1, 8-oxoguanine DNA glycosylase; MYH, adenine DNA glycosylase; MPG, N-methylpurine-DNA glycosylase; APE, apurinic/apyrimidinic endonuclease 1; MGMT, O-6-methylguanine-DNA methyltransferase; Msh2/6 (Mutsα), the Msh2-Msh6dimer; XRCC1, X-ray repair complementing defective repair in Chinese hamster cells 1; PARP, Poly-(ADP-ribose) polymerase; FEN1, flap structure-specific endonuclease 1; WRN, Werner syndrome, RecQ helicase-like; DNA pol β, polymerase beta; PCNA, proliferating cell nuclear antigen. References - 1: (243, 316), 2: (243), 3: (128, 244), 4: (21), 5: (212), 6: (83), 7: (199), 8: (156), 9: (64), 10: (117, 265), 11: (199), 12: (22, 23), 13: (169), 14: (88, 216), 15: (40), 16: (299), 17: (55), 18: (215), 19: (131), 20: (166), 21: (105), 22: (227), 23: (87, 130), 24: (138), 25: (28), 26: (52, 163, 164, 273, 349), 27: (167, 341), 28: (129), 29: (75), 30: (181, 216), 31: (24), 32: (70), 33: (168), 34: (274), 35: (124, 141), 36: (149, 296).

Transcription occurs within a chromatin context

The mammalian genome consists of approximately 2.7-3 billion bases (mouse and human respectively) and approximately 22,000 genes (56, 192). Eukaryotic genomes are packaged with specialized protein (histones) into a higher order structure (chromatin) that protects the genome and permits regulated access to genetic information. Regulation of transcription is critical for viability and misregulation of the transcriptional program lead to disease and carcinogenesis (1, 2, 8, 14, 29, 32, 33, 37, 43, 45, 47, 53, 59, 74, 79, 85, 96, 97, 101, 102, 256, 259, 270, 283). Agents such as α-amanitin amatoxin which may be found in the *Amanita* genus of mushrooms such as the Death cap mushroom (*Amanita phalloides*) inhibit the critical transcriptional mediator RNA polymerase II, and are lethal (39).

Chromatin is composed of a core histone octamer consisting of 2 units each of histones H2A, H2B, H3, and H4. Around this core particle 146 bp of duplex DNA is tightly wrapped (214). This unit is repeated every 200 bp in eukaryotic genomes and appears as approximately 11 nm "beads on a string" when imaged by electron microscopy (euchromatin) (233). Further compaction into higher order structures may be achieved through inclusion of the linker histone H1 (heterochromatin) (241) as well as inter-nucleosomal interactions and through chromatin binding proteins. Euchromatin is generally permissive for transcription while heterochromatin is generally repressive (340).

Interestingly, the core histone particles possess outward facing tails which are substrates for PTM. These include acetylation, phosphorylation, methylation, sumoylation, and ubiquitination (182). Histone tails may also be irreversibly modified by proteolytic cleavage (73). These modifications can act to alter histone-DNA interactions (182). For example, acetylation of histone tails has been associated with reducing histone-DNA

interactions and promoting a euchromatic state which increases access to the constituent DNA sequences (115, 226, 294, 323, 324). Histone tails also serve as docking sites for chromatin modifiers, and reversible PTM of specific residues in histone tails can regulate binding of these factors. In turn, these proteins direct further alterations in local chromatin structure cascading to regulate genomic organization. The complex regulation of chromatin structure achieved through histone tail PTM and histone binding proteins, can serve to regulate histone-DNA interactions and DNA metabolism (84, 189, 288, 302).

Four families of ATP-dependent chromatin remodeling complexes have been identified that function to improve access of trans-acting factors to chromosomal DNA. The families are named SWI/SNF, INO80, ISWI, and Mi-2/CHD (10). The mechanism of action for these complexes is still under investigation, but may include actions as drastic as complete removal of local histone octamers to more subtle manipulations involving creation of small loops of DNA lifted from the surface of the core octamer (57, 119, 145, 209) and in either case, may be associated with epigenetic marks that favor continuity of promoter states (255).

A useful intellectual framework for conceptualizing the relationship between histone post-translational modification, chromatin organization, and DNA metabolism has been described by the histone code hypothesis (189, 289, 302, 319). This hypothesis proposes that histone PTM may be written and read by the cell, resulting in a dynamic exchange of information from chromatin to the cell and vice versa. This information is transmitted through numerous chromatin modifiers as described above and collectively these PTM, their mediators and effectors of chromatin remodeling are thought to facilitate a mechanism for exquisite and dynamic regulation of genome structure, stability, replication, and metabolism. Accordingly, misregulation of chromatin state through aberrant PTM of histone tails or misdirection of ATP-dependent chromatin remodeling

complexes is a common feature of malignancies and gene expression studies have shown that this can potentially affect expression of thousands of genes (53, 120, 176, 280).

Transcription is directed by the coordinated action of three classes of transcriptional regulators which act to coordinate transcription temporally, spatially, and quantitatively. These include the basal transcription apparatus, sequence specific transcription factors, and the transcriptional coactivators/repressors (61). When the chromatin state is permissive for transcription factor binding, sequence specific transcription factors such as nuclear receptors may recognize and bind to their cognate gene regulatory regions, this promotes assembly of a preinitiation complex (PIC) which consists of the multimeric RNA pol II (PolII) enzyme and the basal or general transcription factors TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH bound to promoter DNA (278, 313). For high levels of induced transcriptional activation or repression, the sequence specific transcription factors also recruit transcriptional coactivators and corepressors, which are often multi-protein complexes possessing enzymatic activities facilitating covalent modification of chromatin and transcription factors (247). These multiple protein complexes assemble sequentially, some acting as bridging factors which facilite binding of subsequent factors. For example, the transcriptional coactivator steroid receptor coactivators-1, -2, and -3 (SRC1, 2, 3) interact with steroid receptors in a hormone dependent manner and dramatically increases steroid receptor-dependent transcription (50, 262, 326, 327). The SRC coactivators possess several interaction domains, including triple LXXLL motifs necessary for association with steriod receptors (65, 134, 327), and two activation domains, AD1 and AD2, which mediate interactions with the transcriptional coactivators CBP/p300 (CBP/p300 are discussed below), and the coactivator associated methyltransferase 1 (CARM1)/protein arginine N-methyltransferase (PRMT1), respectively. Interestingly, CBP/p300 are histone acetyltransferases; CARM1 and PRMT1 are histone methyltransferases (4, 35, 151, 178, 217, 266, 304, 350); and

SRC1and SRC3 contain putative histone acetyltransferase (HAT) domains (298). These multiple interaction surfaces allow formation of multi-protein complexes and the accompanying enzymatic activities facilitate local chromatin remodeling, transcription factor recruitment, and assembly of RNA polymerase II, resulting in dynamic regulation of transcription in a gene specific manner (279, 346, 354).

Interestingly, increasing evidence indicates a role for DNA repair pathways in chromatin remodeling and transcriptional regulation. For example, demethylation of dimethylated histone H3, lysine 9 during estrogen-induced gene activation was shown to cause local oxidative DNA damage resulting in recruitment of OGG1 as well as DNA topoisomerase II β which contributed to chromatin remodeling essential for induction of estrogen-dependent transcription (267). Additionally, evidence indicates that during signal-dependent transcriptional activation by nuclear receptors as well as other DNA-binding transcription factors, DNA topoisomerase II β generates DNA double-stranded breaks in gene promoter regions, initiating a signal which activates PARP-1 function and subsequent histone H1-HMGB exchange proximal to the break (160). These observations link the DNA double-strand break repair, chromatin remodeling, and transcriptional machinery in a signal cascade generally facilitating induced gene expression. It is interesting to note that this implies that a broad induction of numerous, potentially cytotoxic, DNA lesions occurs during transcriptional activation. DNA repair signaling pathways may also participate in cross-talk with transcriptional pathways in order to facilitate coordination between cellular processes. For example, after binding to mutagenic O(6)-alkylguanine direct DNA damage reversal protein MGMT undergoes a conformational change which exposes an internal estrogen receptor α (ERα) binding site. Binding between MGMT and ERα prevents ERα interaction with its transcriptional coactivator SRC1 and represses estrogen regulated transcription and cell growth, slowing DNA metabolism when its integrity has been compromised (310). BER protein APE also

participates widely in transcriptional activation through its redox activity, acting to regulate the redox state of transcription factors such as Fos-Jun heterodimers, Jun-Jun homodimers, Hela cell AP-1 proteins, NF-kappa B, Myb, and ATF/CREB family members, stimulating their DNA binding activity and promoting transactivation (342). Conversely, APE has also been shown to negatively regulate some negative Ca^{2+} responsive elements (nCaREs) (260). Interestingly, the DNA repair and redox functions of APE are biochemically independent, indicating that the APE DNA damage response does not have to be active for transcriptional regulation to occur (342). Additional mechanisms linking DNA repair and transcription exist; another is discussed below, although a complete discussion of this topic is beyond the scope of this thesis.

Cytosine methylation regulates gene expression and contributes to genome instability

Cytosine may be methylated at the 5' site (5-meC) and approximately 3-4% of cytosines in mammalian genomes are methylated resulting in 1% of all DNA bases being 5-meC (159, 271). In differentiated vertebrate cells, 99.98% of 5-meC is found within CpG sequences and in stem cells 25% 5-meC may be found at CA sequences (205, 290). Sequence analysis shows that gene regulatory regions in nearly all constitutively expressed, and 60-70% of total, human genes are enriched in CpG sequences (referred to as CpG islands) (276, 360). Although the majority of CpGs in mammals are methylated (153), 91.8% of CpG islands exhibit low levels of methylation (less than 20%), while 4.8% show intermediate methylation (20-80%), and only 3.4% are highly methylated (greater than 80%). In contrast, 5-meC is largely found within repetitive DNA sequences such as Alu repeats and at non-island CpGs within coding and non-coding regions (205). Methylation of cytosine contributes to chromosomal organization as methyl binding domain (MBD) bearing proteins are recruited to methylated CpGs and subsequently

recruit histone deacetylases (HDACs) which deacetylate local histone tails, promoting chromatin condensation (26). Accordingly, methylation in CpG island promoters is generally associated with gene silencing (158, 305); however, methylation within gene bodies has been found in certain transcribed genes, suggesting that context may be important to 5-meC function in gene regulation (305). Recently, base-pair resolution mapping of human fibroblast methylation sites has shown that sites of DNA-protein interaction correlate with reduced CpG methylation and furthermore, that large tracts of DNA may be partially methlylated, resulting in reduced activity of genes 5' of these methylated cytosines, indicating that methylated cytosines are generally refractory to protein binding and suggesting that they promote formation of a chromatin structure inhibitory to DNA metabolism (205).

Although CpG methylation is a powerful gene-silencing mechanism (175), it also contributes to genome instability by promoting spontaneous hydrolytic deamination of cytosine which generates thymine (292). Unmethylated cytosine also undergoes deamination at lower rates and generates uracil. Left unrepaired, the resulting G:U and G:T mispairs these mispairs will give rise to G:C to A:T transitions upon replication. Such alterations to genomic coding sequences may alter gene regulatory regions as well as coding sequences. Analysis of cytosine methylation in non-pathologic human tissue samples has uncovered variations in methylation significantly correlating to age and exposure to environmental toxins such as cigarette smoke (54). High resolution mapping of DNA methylation in lung cancers has shown that misregulation of this epigenetic mark is a common feature of human cancers and can potentially affect expression of thousands of genes (276, 277).

The p53 tumor suppressor gene is an ideal mutation reporter to investigate the role of DNA damage in carcinogenesis (272). Mutations of the p53 gene are one of the most

common characteristics of human cancers (144, 261, 328) occurring in approximately 50% of all cancers. The majority of these (approximately 90%) are single point missense mutations which inactivate the p53 protein DNA binding domain, preventing it from enacting a transcriptional program which causes cell cycle arrest and apoptosis (269, 328, 330). Other cancers show altered p53 expression and nuclear localization (236). The IARC p53 mutation database is a repository for mutations found in the human *p53* gene which have been found in tumor tissue samples. Examination of this database shows that almost one half of mutations in colon and rectal tumors are attributable to G:C to A:T transitions at CpG sequences. This grows to over 60% when all G:C to A:T transitions are considered (269).

Measurements of the spontaneous rates of deamination of cytosine and 5-methylcytosine in double stranded DNA have shown that 5-methylcytosine undergoes spontaneous deamination at a rate 2-3 times higher than cytosine with average rates of 2.6×10^{-13}/s and 5.8×10^{-13}/s for 5-methylcytosine and cytosine respectively (292). These rates are consistent with a role for spontaneous hydrolytic deamination of cytosine and 5-methylcytosine as major contributors to genome instability and carcinogenesis (202, 292). Importantly however, observations indicate that CpGs are 12-42 fold more prone to mutation than other nucleotide sequences as measured in cells, rates much higher than can be accounted for by spontaneous processes alone (59, 177, 297, 306). This may be due to inefficient repair G:T mispairs (36, 201) and/or malfunction of enzyme-mediated deamination mechanisms which have been reported to play a role in transcriptional regulation (172, 238).

Thymine DNA Glycosylase is a multifunctional DNA repair enzyme

There are two known enzymes possessing an activity which excises mispaired thymine and uracil in a CpG context – thymine DNA glycosylase (TDG) and methyl binding

domain protein 4 (MBD4). Although TDG and MBD4 process similar lesions by initiating the DNA base excision repair pathway (see above), they lack sequence homology (13, 81, 136, 137, 249, 311, 352). TDG has orthologs in bacteria, yeast, insects, frogs, and vertebrates (5, 93, 125, 126). Sequence analysis shows that TDG posses a highly conserved central region containing the active site, and more divergent amino and carboxy-terminal regions (Fig 1.3, adapted from Cortazar et al. (2007)) (60). Structural studies have shown that this highly conserved TDG central region forms a relatively large substrate pocket (Fig 1.4) (13). Accordingly, TDG processes a large number of aberrant bases in addition to oxidative damage of cytosine and methylcytosine, including the products of base damage due to alkylation, halogenation, and lipid peroxidation (17, 82, 123, 245, 352). Interestingly, TDG also processes halogenated bases such as 5-fluorouracil, contributing to the DNA-directed cytotoxicity of this chemotherapeutic agent (187). Crystallographic analysis of the TDG core, along with kinetic studies of both core and full-length proteins have shown that the amino terminus is critical for full TDG glycosylase function as it forms hydrogen bonds with guanine bases which are essential for mispair recognition specificity and mediating tight DNA interactions necessary to process thymine mispairs (222). Without this region, TDG binds less stably to DNA and loses the ability to excise thymine from G:T mispairs while retaining G:U processing activity (244, 301). For the purposes of this thesis, we have used mouse TDG (or mTDG). Human and mouse TDG are biochemically indistinguishable and highly conserved both in sequence and structure (See multiple sequence alignment Fig. S.3.1). In NIH 3T3 mouse fibroblast cells MBD4 largely associates with transcriptionally silent heterochromatic regions of the genome while TDG is largely associated with euchromatic, or transcriptionally active regions (316) suggesting that TDG, rather than MBD4 is the predominant protein responsible for maintenance of transcriptionally active regions of the genome. A small portion of TDG has also been shown to associate with heterochromatin in complex with DNA

methyltransferase 3b (Dnmt3b) in P19 embryonic carcinoma stem cells (30) and in NIH 3T3 cells upon coexpression with DNA methyltransferase 3a (Dnmt3a) (198), suggesting that TDG, in complex with these regulators of DNA methylation, may play a role in maintenance of these transcriptionally silent regions of the genome.

Figure 1.3. Schematic of mouse (m) TDG indicating the relative organization of important regulatory regions and surfaces mediating specific protein-protein interactions. Abbreviations: estrogen receptor α (ERα), retinoic acid receptor α (RARα), retinoid x receptor (RXR), CREB binding protein (CBP) – specifically CBP histone acetyltransferase (HAT) and CH3 domain interactions, protein kinase C α (PKCα), jun oncogene (C-Jun), DNA methyltransferase 3a (Dnmt3a). Adapted from Cortazar et al., 2007 (60).

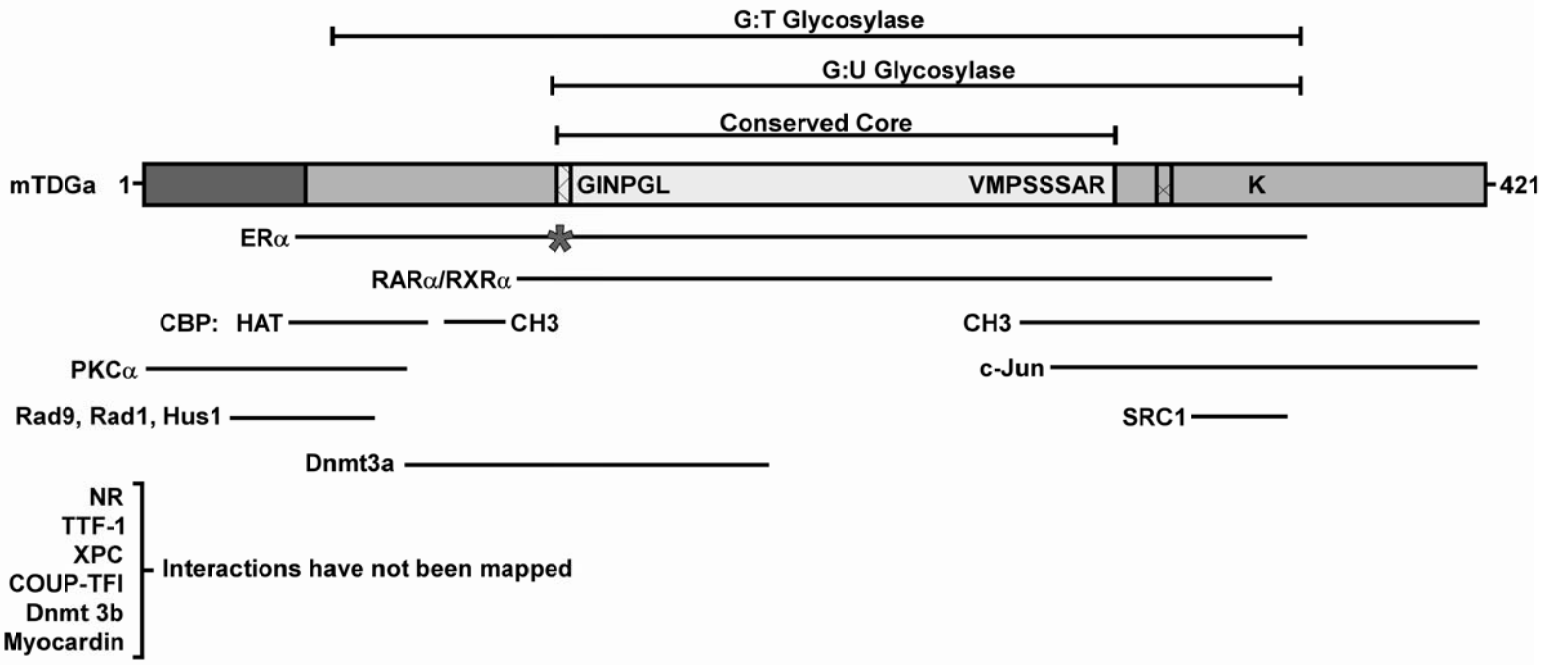
G:T Glycosylase
G:U Glycosylase
Conserved Core
mTDGa 1
GINPGL
VMPSSSAR
K
421
ERα
RARα/RXRα
CBP: HAT
CH3
CH3
PKCα
c-Jun
Rad9, Rad1, Hus1
SRC1
Dnmt3a
NR
TTF-1
XPC
COUP-TFI
Dnmt 3b
Myocardin
Interactions have not been mapped
SUMO binding motifs: residues 144-148, and 319-322
LDIVI motif for ERα interaction: residues 143-147
K Sumoylated lysine: residue 341
GINPGL & VMPSSSAR critical residues of the active site: residues 149-154 and 279-286
NR (Nuclear Receptors); androgen receptor, glucocorticoid receptor, progesterone receptor, peroxisome proliferator activated receptor a, thyroid hormone receptor a, vitamin D3 receptor.

Figure 1.3

Figure 1.4. Overview of the structure of TDG. (A) The hTDG catalytic domain (hTDGcat, residues 111–308) binds a 22-bp DNA containing a tetrahydrofuran nucleotide (THF), a chemically stable mimic of the natural AP product, in a 2:1 complex: one subunit at the abasic site (product complex) and the other at an undamaged site (nonspecific complex). DNA shown includes a full 22-bp duplex and part of the adjacent duplex joined by 3' A/T overhangs (blue arrow, see B). Overall, the two subunits are highly similar (rms deviation of 0.8 Å for Cα positions). (B) Schematic overview of the enzyme–DNA interactions and the dimer interface. The 22-bp DNA is yellow with phosphates shown as orange circles. The adjoining DNA fragment (purple) shows contacts with K246 and K248 from the NS subunit. The arrows represent hydrogen bonds involving side-chain or main-chain (mc) atoms of the enzyme. In the product complex, the flipped abasic nucleotide (THF) is a red pentagon, the "opposing G" is magenta, and the "3′-G" is cyan. A277 intercalates the complementary strand, disrupting base-stacking interactions between the opposing G and its 5′ neighbor. Contacts involving N157, S273, and A274 for hTDGcat are topologically conserved with contacts in the eMUG product complex (13), and the N157, K232, S271, and S273 contacts are conserved with those in the UDG product complex (264). (C) Close-up view of the dimer interface, with the G·THF-bound subunit in green and the nonspecific subunit in cyan. The N termini of each subunit (T123) are indicated. Adapted from Maiti A et al., 2008 (222). PDB ID: 2RBA.

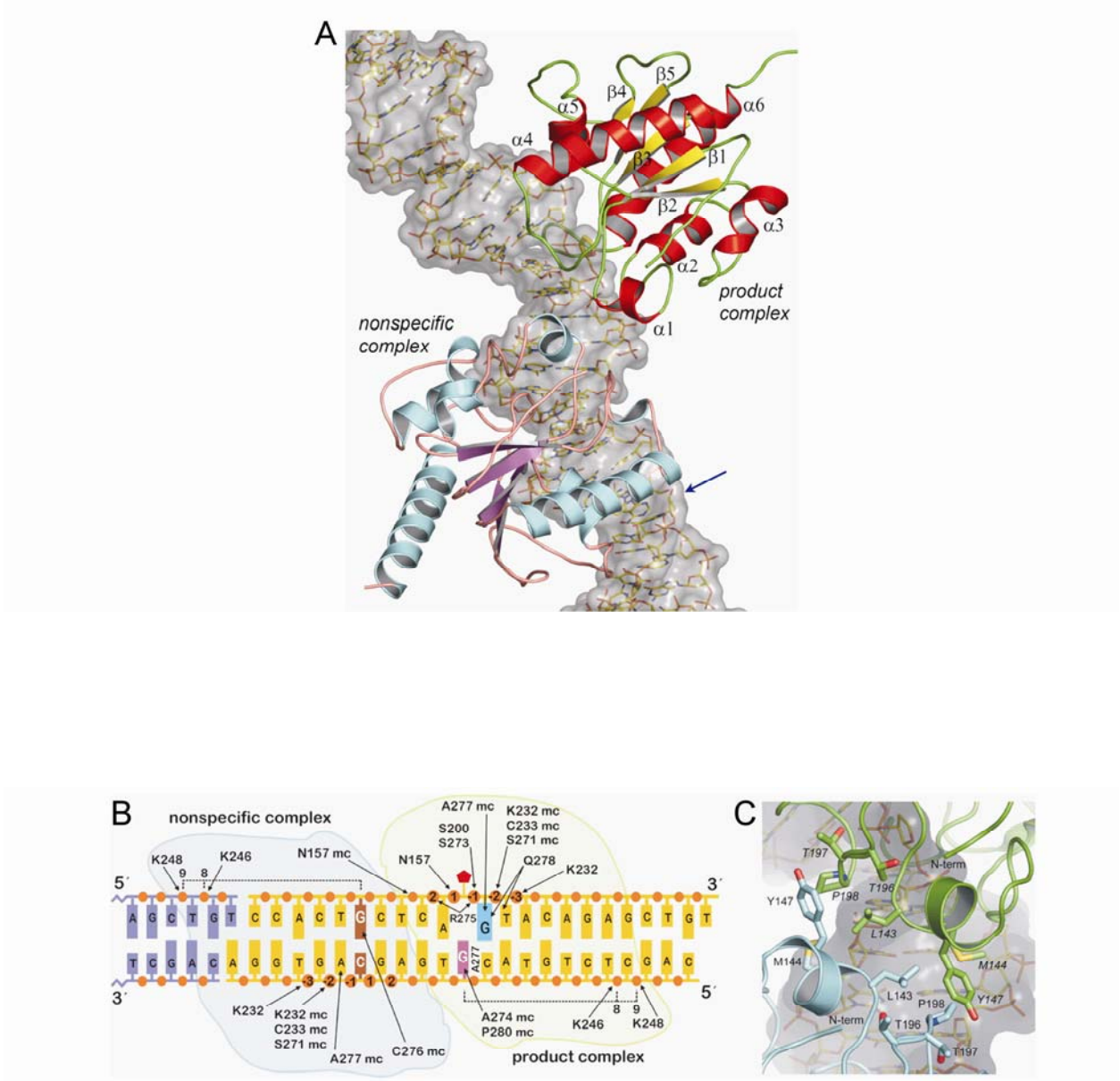
A
β5
β4
α5
α6
α4
β3
β1
β2
α3
α2
product complex
nonspecific complex
α1
B
nonspecific complex
A277 mc
K232 mc
C233 mc
S271 mc
S200
S273
N157
N157 mc
Q278
K232
K248
K246
5′
3′
R275
A277
K232
K232 mc
C233 mc
S271 mc
A277 mc
C276 mc
A274 mc
P280 mc
K246
K248
product complex
C
T197
N-term
T196
P198
Y147
L143
M144
M144
L143
P198
Y147
N-term
T196
T197

Figure 1.4

Interestingly, TDG also associates with subnuclear structures called promyelocytic leukemia protein (PML) oncogenic domains (PODs) (244, 307). PODs are subnuclear structures comprised mainly of promyelocytic leukemia (PML), SP100, and Daxx proteins (231). PML is a necessary component for assembly of PODs and comprises their main structural component (191, 355). Distinct in composition from other nuclear bodies, the PODs may be detected by labeling PML by methods such as immunohistochemistry or tagging with fluorescent proteins (244). Interestingly, PML has been shown to bind a diversity of protein partners and PODs have been shown to be a site of localization and putative site of storage for many proteins which function in transcription and the maintenance of genome stability (197, 200, 356). Interestingly, certain DNA repair factors such as the Rad50, MRE11 and NBS1 proteins associate dynamically with PODs, accumulating in these structures until DNA damage is induced, at which time they move into the nucleus where they presumably perform a repair function, after which they return to the PODs (239). PODs may also be a site of nucleation for protein complexes, creating local accumulations of proteins with high effective concentrations promoting assembly of these multi-subunit complexes. Additionally, proteins may be placed in close proximity within PODs to facilitate interactions post-translational modifications (200). Accordingly, numerous acetyltransferases, kinases, proteases, and transcription factors have been found localized to PODs and these structures are surrounded by regions of active transcription (112, 344). Although deletion of the PML gene in mice results in viable and fertile animals, misregulation of PML by fusion with the retinoic acid receptor (PML-RAR) has been shown to cause promyelocytic leukemia (200).

Interestingly, TDG has been shown to functionally interact with a number of transcription factors and transcriptional coactivators including estrogen receptor alpha (ERα) (48, 238), retinoic acid receptors (RAR and RXR) (320), thyroid transcription factor 1 (TTF1) (240), p53 tumor suppressor family members p53 and p73α (171), the p160 coactivator of

nuclear receptors SRC1 (213), serum response factor (SRF) co-activator myocardin (358), as well as the transcriptional coactivators and acetyltransferases CBP/p300 (316). Finally a novel role for TDG has recently been reported linking TDG to the mechanism of reversible DNA methylation.

Finally, a role for TDG in the regulation of transcription through demethylation of cytosine had been proposed by Zhu et al., who demonstrated that overexpression of TDG reactivated a transgene silenced by CpG methylation (359). Recently, Metivier et al. identified a critical role for TDG-mediated BER when they reported that TDG plays a role in the cyclical DNA methylation of the transcriptionally active estrogen responsive pS2/TFF1 gene (238). This process takes place when TDG is recruited to the promoter along with the methyltransferases (Dnmt) 3a and 3b as well as p68 and the BER proteins APE, DNA ligase, and DNA polymerase β. In the presence of low concentrations of co-factor S-adenosyl methionine (SAM) these methyltransferases promote hydrolytic deamination of methylated cytosine (291, 361). Interestingly, the methyltransferase activity of Dnmt3a is inhibited in the presence of TDG (198). The product of this targeted deamination event is a G:T mispair. However, the presence of Dnmt3a promotes TDG base excision activity *in vitro* (198). Accordingly, TDG recognizes and excises the mispaired thymine base, and through completion of the BER pathway it is replaced with cytosine. This mechanism of transcriptional regulation through cyclical methylation has also been described for another ERα responsive gene, *Wisp-2*, raising the possibility that this may be a mechanism of transcriptional regulation on other promoters (238). Importantly, this was the first mechanistic explanation to indicate that DNA methylation is reversible and involves BER. Soon afterward, it was shown that BER-mediated demethylation of cytosine also occurs in Zebrafish (275) and recently, MBD4 was also shown to participate in cytosine demethylation facilitating transcription although MBD4 is capable of direct excision of methylated cytosine (172).

TDG is a coactivator and substrate for the CBP and p300 acetyltransferases

CBP/p300 are coactivators that participate in transcription through various mechanisms to integrate signaling pathways for a large number of sequence specific transcription factors such as CREB , AP-1, p53, and steroid receptors (89, 148, 154, 162, 211). For example, when recruited to chromatin by p53 as well as other transcription factors, CBP/p300 acetylate histone proteins, promoting an open chromatin conformation more amenable to DNA metabolism (77). CBP/p300 have also been shown to acetylate numerous non-histone proteins (consensus sequence G/SK (9)). For example, CBP/p300 mediated acetylation of sequence-specific transcription factors p53, p73, and Sp3, result in increased DNA binding activity (89, 154, 155, 161, 180, 248). CBP/p300 have also been shown to act as bridging proteins between sequence specific transcription factors and basal transcription machinery apparatus (108). Additionally, the large surface area and multiple protein-protein interaction sites of CBP/p300 may facilitate use of these acetyltransferases as scaffolds for assembly of multi-protein complexes. Such an arrangement has been observed on the β-interferon gene promoter in response to viral infection (237). Some of these proteins may have additional enzymatic activities not limited to acetyltransferase or histone deacetylase activity seen in CBP/p300 interactors pCAF (254) and HDAC1 (254) respectively.

Evidence indicates that there is a role for CBP/p300 in tumor suppression. Breakpoints, microdeletions, and point mutations in the *CBP* and *p300* genes are associated with Rubenstein Taybi Syndrome in humans (14, 270). This autosomal dominant syndrome is characterized by physical abnormalities including skeletal deformities, mental retardation, and high risk for malignancy. Somatic mutations in CBP/p300 have been found in a number of malignancies and translocations in the CBP and p300 genes occur in acute myeloid leukemia. Additionally, CBP/p300 are targets for transforming viruses,

suggesting that disruption of their function permits carcinogenesis (155). Importantly, mouse models lacking *CBP/p300* do not survive embryogenesis (257, 258, 351), and those heterozygous for *CBP* develop hematological failures and malignancies in which the second *CBP* allele had been inactivated (185, 257).

Intriguingly, TDG is capable of forming ternary complexes with CBP and DNA which are competent for base excision (316). Additionally, TDG has been shown to be a potent stimulator of CBP-mediated transcription and is substrate for CBP-mediated acetylation on amino terminal lysine residues 70, 94, 95, and 98 (316). Proteomics-based analysis of non-histone protein acetylation has shown that important mediators of longevity, carcinogenesis, tumor suppression, and metabolism are all substrates for acetylation (25, 173).

TDG interacts covalently and non-covalently with SUMO

In addition to acetylation, TDG is also subject to modification by conjugation of the small ubiquitin-like modifier (SUMO) proteins (sumoylation) to lysine residue 341 (consensus conjugation site ψKXE) and approximately 5-50 percent of the cellular pool of mTDG is sumoylated at any time, depending on the tissue or cell line examined (M. Tini, unpublished). This 97 amino acid protein is distantly related to ubiquitin and despite sharing only 18% EST cDNA and amino acid sequence identity, establishes a common three-dimensional structure (15, 221). There are four SUMO isoforms termed SUMO-1 (referred to as SUMO), -2, -3, and -4. Although SUMO-1 is only 50% related to SUMO-2/3, SUMO-2/3 differ by only 3 amino-terminal residues and are functionally identical (133). Interestingly, SUMO-2/3 are able to form multimeric chains while SUMO-1 does not (246) although SUMO-2/3 may be sumoylated by SUMO-1 (229). TDG is subject to sumoylation by SUMO-1, -2, and -3 (6, 7, 128) and also possess a non-covalent SUMO-binding activity (7, 244). The effect of covalent modification, or noncovalent interaction,

with SUMO is protein-specific, and has been shown to regulate diverse biological processes such as subcellular localization, chromosome segregation, DNA repair, transcription, proteolysis (103, 133). The mechanism underlying this regulation occurs through any combination of alterations to a sumoylated or SUMO-bound protein function through differential subcellular localization, protein-protein interactions, intramolecular interactions, and crosstalk between sumoylation and subsequent posttranslational covalent modifications (132, 133). For example, sumoylation of RanGAP1 promotes translocation to the cytoplasmic fibrils of the nuclear pore complex protein through increased binding to RanBP2 (5). Sumoylation of CBP/p300 causes recruitment of Daxx and HDAC6 respectively, negatively affecting transcription (104, 188). Sumoylation of PML assists in assembly of PODs and promotes recruitment of POD components Daxx, sp100, and CBP (8). Additionally, sumoylation of HSF1 and HSF2 alters the ability of these proteins to interact with DNA (109, 128, 146).

TDG is a phosphoprotein

TDG was first identified as a phosphoprotein in experiments showing that phosphatase treatments could alter the mobility of TDG as resolved by SDS-PAGE (320). In chapter 4 we identify the kinase responsible for these marks as protein kinase C alpha (PKCα). First identified as a histone protein kinase in rats (253), the PKC family consists of 11 family members which share a common catalytic domain recognizing the consensus sequence S/T-X-[R/K] (174), but distinct regulatory domains which have different activation requirements. Classical PKCs (α, βI, βII, and γ) require either diacylglycerol or phorbol esters, calcium, and an acidic phospholipid, such as phosphatidylserine, for activation. Additional PKCs δ, ε, η, and θ are classified as "novel PKCs" and require either diacylglycerol or phorbol esters, and an acidic phospholipid for activation, but are calcium independent. The ζ, and ι/λ PKCs are classified as "atypical PKCs" and require

only an acidic phospholipid for activation. In addition to specific cofactor requirements for activation, PKC isoforms possess distinct tissue expression and subcellular localization which contributes to effective separation of biological function (334). PKCα is the only PKC which is ubiquitously expressed and exhibits both cytoplasmic and nuclear localization (225). Activation of PKCα with phorbol esters causes autophosphorylation and relocalization to lipid membranes and nuclear compartments (314). Interestingly, PKCα is also activated by H_2O_2 (179) and products of lipid peroxidation, agents known to cause DNA damage that may be repaired by TDG (91, 195). Phosphorylation appears to be a major mediator of the DNA damage response as proteomic analysis has identified over 700 proteins which phosphorylated by the kinases ATM and ATR in response to DNA damage (230). Furthermore, PKC phosphorylates BER proteins APE (150) and DNA polymerase β (317). PKCα signaling has also been implicated in transcriptional control at various stages of transcription through phosphorylation of proteins such as PPARα (114), thyroid hormone receptor-α1 (170) and histones (100). Interestingly, PKCα phosphorylation of CBP is required for coactivation of transcription on AP1 responsive promoters (353).

1.2 Hypothesis and experimental aims

The observations described above place the lysine- and serine-rich amino terminus of TDG in focus as a hot-spot for PTM. Importantly, these PTM and the mediators of these marks have been implicated in transcriptional regulation and carcinogenesis. Interestingly, the TDG amino terminus also participates in mediating numerous protein-protein interactions, including those with APE and CBP (316). Furthermore, mechanisms regulating TDG enzymatic function may consequently play an integral role in regulation of gene expression and removal of epigenetic marks (279).

We hypothesize that PTM of TDG may act in concert to regulate TDG function. In this thesis we aim to identify novel TDG PTM, determining the modifying enzymes as well as the substrate residues on TDG. We will then determine the effect of these PTM on regulating TDG intramolecular and intermolecular interactions. Furthermore, we aim to investigate whether these PTM play a role in regulating TDG subcellular localization. Importantly, we will determine the effect of these PTM on TDG substrate interactions and base excision activity. Lastly, we will elucidate a possible interplay between TDG PTM and determine the nature of any relationship between PTM.

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Chapter 2: SUMO-1-dependent allosteric regulation of thymine DNA glycosylase alters subnuclear localization and CBP/p300 recruitment

2.1 Introduction

In vertebrate genomes, methylation of cytosine within CpG dinucleotides constitutes an important mechanism regulating transcription and chromatin structure (35). CpG methylation also contributes to genome instability by promoting spontaneous hydrolytic deamination of methylated cytosines to generate thymine residues (27), which in the absence of DNA repair give rise to cytosine-to-thymine transition mutations believed to have a causative role in cancer (17). For example, these CpG mutations are the most prevalent genetic alterations in the p53 tumour suppressor gene detected in many human tumors (36). The incidence of CpG mutations is also dramatically increased in aging mouse tissues and, therefore, may contribute significantly to cellular aging (11).

Thymine DNA glycosylase (TDG) is one of two enzymes mediating the excision of mispaired thymine (G:T) and uracil (G:U) in the CpG context (23, 32, 33). TDG processes thymine, uracil, 5-hydroxymethyluracil, and 3,N^4-ethenocytosine mispaired with guanine (18) to generate an abasic site that is subsequently repaired by other base excision repair (BER) enzymes (41). Interestingly, TDG has also been shown to interact with a number of transcription factors, including Jun and members of the nuclear receptor family, suggesting a link between transcription and BER (7, 8, 28, 46, 47).

Previous studies have revealed a functional association between TDG and transcriptional coactivators CREB-binding protein (CBP) and p300 (46). CBP/p300 are essential proteins that potentiate diverse transcription factor signaling pathways in part by mediating acetylation of chromatin and chromatin-associated proteins (16). Notably, CBP/p300-TDG complexes are recruited to DNA *in vitro* and have the potential to participate in both

transcriptional regulation and DNA repair (46). Accordingly, TDG was shown to be both a potent activator of CBP/p300-dependent transcription and a substrate for CBP/p300 acetylation (46).

TDG is posttranslationally modified by covalent conjugation to SUMO (small ubiquitin-like modifier) proteins (SUMO-1, -2, and -3), resulting in inhibition of DNA binding and altered DNA repair kinetics (20). SUMO-1 is a 97-amino-acid peptide that is covalently attached to proteins at lysine residues (consensus ΨKXE), thereby affecting subcellular localization and molecular interactions (22). Importantly, SUMO modification plays important roles in transcriptional regulation and maintenance of genomic integrity (22). Sumoylation, in some instances, promotes localization to nuclear compartments, known as promyelocytic leukemia protein (PML) oncogenic domains (PODs) (6, 26, 37). The dynamic association of transcription and DNA repair factors with PODs suggests that these nuclear structures play important roles in regulating gene expression and genome stability (26).

We have investigated the role of sumoylation and noncovalent SUMO-1 binding in the regulation of subcellular localization and biochemical properties of TDG. Our studies have mapped SUMO-1 binding activity to two separate SUMO binding motifs (SBMs) located in the amino- and carboxy-terminal regions. We show that both SBMs are essential for normal POD localization and activation of CBP-dependent transcription. Furthermore, the SBMs are regulated by DNA interactions mediated via an amino-terminal hydrophilic domain. Interestingly, we have shown that sumoylation of TDG promotes intramolecular interactions that dramatically alter the biochemical properties of TDG, thereby preventing association with CBP and POD translocation.

2.2 Materials and Methods

Plasmids

Plasmid constructs were verified by sequencing, and details are available on request. GAL-CBP and TDG were expressed in pCMX mammalian expression vectors (46). FLAG-tagged constructs lacking the amino-terminal region of TDG were fused to the simian virus 40 (SV40) nuclear localization signal (NLS) to replace the natural NLS contained within this region. Carboxy-terminal deletions of TDG were constructed by directional cloning of PCR-amplified fragments into the pCMX-FLAG vector. TDG and CBP point mutants were constructed using the QuikChange mutagenesis kit (Stratagene) according to the manufacturer's directions. Cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) fusions of TDG and PML were constructed using the pCMX-CFP or pCMX-YFP expression vectors. Renilla green fluorescent protein (GFP) fusion constructs were made using the phrGFP-N1 vector from Stratagene. Other expression vectors have been previously described (10, 31, 46).

Cell culture, transfections, and heat shock treatment

MCF-7 cells were maintained in Dulbecco's minimal essential medium containing penicillin-streptomycin and supplemented with 10% fetal bovine serum. Cells were seeded onto 24-well dishes and transfected using Lipofectamine 2000 transfection reagent (Invitrogen). Approximately 250 ng of luciferase-based reporter plasmid, 100 ng of Gal-CBP, and 100 to 500 ng of pCMX-based expression vectors were used per well. Transfection efficiency was normalized by cotransfection of *Renilla* luciferase reporter vector phRL-SV40 (Promega). Transfection experiments were performed at least three times in duplicate, and results are shown with standard error plotted (standard error = standard deviation/sqrt of n, where n= the number of replicates). Heat shock treatments

(42°C) were performed on MCF-7 cells seeded on six-well dishes transfected with expression vectors for TDG (200 ng) and PML (100 ng). At 0, 15, or 30 min, cells were lysed in 300 µl Laemmli buffer containing 3 units Benzonase (Novagen), and the modification state of TDG was analyzed by immunoblotting with a TDG-specific antibody.

Preparation of whole-cell extracts

MCF-7 whole-cell extracts for glutathione *S*-transferase (GST)-based interaction assays were prepared from 10-cm dishes of cells transfected with 7.5 µg of TDG expression vector. Cell pellets were resuspended in 500 µl of lysis buffer (50 mM Tris-HCl pH 7.9, 300 mM NaCl, 1 mM EDTA, 1 mM EGTA 10% glycerol, 0.5% Triton X-100, 1 mM dithiothreitol [DTT], proteinase inhibitors) and incubated on ice for 30 min. Subsequently, the cell lysate was diluted with 500 µl of dilution buffer (50 mM Tris-HCl pH 7.9, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1 mM DTT), and insoluble products were removed by centrifugation. Whole-cell extracts for the analysis of sumoylation were prepared from transfected MCF-7 cells lysed in Laemmli buffer containing Benzonase.

Protein purification and *in vitro* interaction assays

Protein purification and GST-based interaction assays using *in vitro*-translated and recombinant proteins have been previously described (46). For ethidium bromide treatments, *in vitro*-translated proteins were treated with 100 µM ethidium bromide for 20 min at 4°C prior to use in pull-down experiments. Binding reaction mixtures and washing buffers also contained ethidium bromide. For interaction assays performed in the presence of duplex oligonucleotides containing a G:T mispair, recombinant TDG was preincubated with increasing amounts of the oligonucleotides for 15 min at room temperature. Whole-cell extracts for pull-down experiments were precleared twice with 25 µl (packed bead

volume) of glutathione-Sepharose beads (Amersham) for 30 min at 4°C. Total protein concentration of the precleared lysate was determined by bicinchoninic acid assay (Pierce), and the relative expression of transfected proteins was determined by immunoblotting with a TDG-specific antibody. Subsequently, the amount of expressed protein in each lysate used for the pull-down was equalized by addition of untransfected cellular lysates. Pull-downs were performed using 3 μg of GST-SUMO and bound proteins detected by immunoblotting with a TDG-specific antibody. FLAG-epitope-based interaction assays were performed with baculovirus-expressed FLAG-CBP (400 ng) and recombinant sumoylated (400 ng) or mock-sumoylated HIS-TDG (400 ng). Proteins were incubated with 10 μl packed commercial anti-FLAG affinity matrix (Sigma-Aldrich) in NETN buffer (50 mM Tris-HCl pH 7.9, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.2% NP-40, 1 mM DTT) in a final volume of 150 μl for 1 h (4°C). The beads were subsequently washed with NETN buffer, and bound proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by immunoblotting with an anti-HIS antibody. Recombinant GST-p53 (2.5 μg) was sumoylated as described below and bound to glutathione-Sepharose affinity matrix (25 μl packed volume). Beads were washed three times with NETN buffer, including one wash with NETN containing 500 mM NaCl and subsequently resuspended in 150 μl of NETN. Binding reactions were carried out with 40-μl aliquots as described above. A portion of the slurry was analyzed by immunoblotting with anti-p53 and anti-GMP-1 monoclonal antibodies. Interaction assays with baculovirus-expressed FLAG-CBP (1 μg) were carried out as described above, but bound complexes were immunoprecipitated with CBP polyclonal antibody.

Oligonucleotide cleavage assays

Cleavage assays were performed essentially as previously described (32). Approximately 25 ng of recombinant TDG or 5 μl of *in vitro*-translated TDG was incubated at 30°C with

5 ng of radiolabeled duplex oligonucleotide containing either a G:T or G:U mispair in 20 µl of cleavage buffer (25 mM HEPES-KOH [pH 7.8], 1 mM EDTA, 0.1 mg/ml bovine serum albumin, and 1 mM DTT). Reactions were carried out for 30 min for recombinant TDG and 2 h for *in vitro*-translated protein. Subsequently, the DNA was precipitated, resuspended in 100 mM NaOH, and incubated at 90°C for 30 min. The cleavage products were fractionated by denaturing polyacrylamide gel electrophoresis and visualized by autoradiography and phosphorimaging. Assays on sumoylated TDG were carried out using 10 ng of duplex oligonucleotide.

ABCD assays

Duplex oligonucleotides containing either no mispairs or a single G:T or G:U mispair were generated by annealing the following complementary oligonucleotides: 5'-[biotin]-TAG ACA TTG CCC TCG AGG TAC CAT GGA TCC GAT GTC GAC CTC AAA CCT AGA CGA ATT CCG -3' and 5'-CGG AAT TCG TCT AGG TTT GAG GT[C, T, or U] GAC ATC GGA TCC ATG GTA CCT CGA GGG CAA TGT CTA -3'). Approximately 500 ng annealed oligonucleotide was incubated for 30 min at room temperature with 10 µl of streptavidin-coated paramagnetic beads (MagneSphere; Promega) and 500 ng of purified bacterially expressed TDG in avidin-biotin complex DNA (ABCD) buffer (50 mM Tris-HCl [pH 7.9], 150 mM NaCl, 10% glycerol, 5 mM $MgCl_2$, 0.1% NP-40, and 0.5 mM DTT). Total reaction volume was 50 µl. Beads were washed five times with 200 µl of ABCD buffer, and bound proteins were analyzed by immunoblotting. In some experiments, TDG was preincubated on ice with 2 µg GST-SUMO or GST for 30 min prior to analysis.

Protein acetylation assays

Sumoylated or mock-sumoylated TDG (400 ng) was incubated with approximately 100 ng of purified, full-length CBP in a total volume of 30 μl in acetylation buffer (20 mM HEPES [pH 7.8], 1 mM EDTA, 1 mM DTT, 10 mM sodium butyrate, and 10% glycerol) in the presence of 1.5 μM [^{14}C]acetyl coenzyme A (AcCoA) and incubated for 30 min at 30°C followed by electrophoresis on an 8% sodium dodecyl sulfate-polyacrylamide gel. The gel was subsequently fixed with a 30% methanol, 10% acetic acid solution and treated with amplifying solution (Amersham) before exposure to film. Western blotting was performed to confirm equal loading of protein and the maintenance of SUMO modification of TDG.

In vitro sumoylation

Sumoylation was performed as previously described (10). Briefly, recombinant GST-SAE1, GST-SAE2, polyhistidine (His)-tagged UBC9 (1 μg each), and SUMO-1 (1.5 μg) proteins were incubated with 5 μg of His-TDG in SUMO conjugation buffer (20 mM HEPES [pH 7.4], 5 mM $MgCl_2$, 1 mM creatine phosphate, 0.35 units/ml of creatine kinase [Roche], 1 mM ATP). Mock sumoylation reactions were performed in the absence of SUMO-1. Modified TDG was purified using Ni-nitrilotriacetic acid Superflow affinity resin (QIAGEN) and dialyzed against NETN at 4°C for 12 h. Copurified His-UBC9 was removed by centrifugal membrane separation using a 10-kDa molecular weight cut-off cellulose filter (Centricon).

Antibodies and immunostaining

TDG-specific antibody was raised in rabbits immunized with recombinant full-length TDG. Immunoglobulin G (IgG) was purified from immune sera by protein A chromatography. Human PML-specific monoclonal (PG-M3, sc-966), CBP-specific polyclonal (sc-369), and anti-p53 (DO-1, sc-126) monoclonal antibodies were obtained

from Santa Cruz Biotechnology. Mouse PML-specific monoclonal antibody (05-718) was from Upstate/Chemicon. SUMO-1-specific monoclonal antibody was purchased from Zymed (clone 21C7). Anti-FLAG monoclonal antibody (M2) was obtained from Sigma-Aldrich. For immunostaining, cells were fixed with 4% formaldehyde for 15 min followed by a 10-min incubation with 0.1 M glycine in phosphate-buffered saline. Cells were then permeabilized with 0.5% Triton X-100. Alternatively, cells expressing FLAG-tagged proteins were fixed with methanol-acetone (1:1) for 1 minute at room temperature. Immunostaining was performed with the appropriate primary antibody and fluorophore-conjugated donkey secondary antibody (CY3 and fluorescein isothiocyanate [FITC]; Jackson ImmunoResearch Laboratories).

Microscopy

Epifluorescence imaging was performed on an Axiovert 200 M inverted microscope equipped with an Apotome (Carl Zeiss) using appropriate fluorophore-specific filter sets. Z-series images (x63 magnification) of 0.5-μm thickness were acquired and processed with Axiovision software and Adobe Photoshop.

2.3 Results

SUMO-dependent translocation of TDG to PML oncogenic domains.

Previous studies have demonstrated that TDG is acetylated by CBP/p300 and can act as a potent activator of CBP-dependent transcription (46). CBP is recruited to PODs by association with PML (12), and there is considerable evidence suggesting that these nuclear structures play important roles in transcription and DNA repair (9, 26, 50). We investigated the subnuclear localization of TDG in human breast carcinoma cells (MCF-7) by indirect immunofluorescence using TDG-specific antibodies and by transient expression of YFP-tagged TDG. YFP-TDG localized throughout the nucleoplasm with the exception of nucleoli (Fig. 2.1A, panel II); accentuated fluorescence was observed in nuclear PODs, as demonstrated by colocalization of YFP-TDG with PML (Fig. 2.1A, panel III). Furthermore, coexpression of YFP-TDG and PML dramatically increased POD localization of TDG (panels V to VIII). Since these observations suggested that TDG associates with PML, we determined whether a bacterially expressed GST-PML fusion protein bound TDG in whole-cell lysates derived from transfected MCF-7 cells. While we did not detect binding of TDG to GST-PML, binding to GST-SUMO-1 was readily observed (Fig. 2.1B). In light of the SUMO-1 binding properties of TDG, we investigated whether this activity is required for POD targeting. Mild hyperthermic stress causes rapid desumoylation of PML and another POD component, SP100, without affecting the structural integrity of the PODs (34). We subjected MCF-7 cells expressing YFP-TDG and CFP-tagged PML (CFP-PML) to heat shock at 42°C for 15 min and monitored protein localization in live cells by fluorescence microscopy. A dramatic loss of POD accumulation of YFP-TDG was observed without detectable changes in CFP-PML localization (Fig. 2.1C). Immunoblotting analysis of cell lysates, using a TDG-specific antibody, indicated that heat shock did not alter the levels of TDG sumoylation (Fig.

2.1C). In light of the reported desumoylation of PML and SP100 following hyperthermic stress (34), these observations suggest that the SUMO-1 binding activity of TDG mediates POD targeting.

To rule out the potential influence of overexpression on the subcellular distribution of TDG, we determined whether native TDG is found in PODs by immunostaining MCF-7 cells with purified TDG-specific rabbit IgG and commercial PML-specific mouse monoclonal antibodies. TDG-specific rabbit antibody raised against recombinant mouse TDG also recognizes human TDG (Fig. 2.1D) but does not cross-react with PML (data not shown). In untransfected MCF-7 cells, endogenous TDG staining was observed in a granular pattern throughout the nucleoplasm: a subpopulation of cells consistently displayed increased staining within the PODs (Fig. 2.1E, panels I to III). Similar results were obtained with immortalized mouse NIH 3T3 cells (Fig. 2.1E, panels IV to VI). These findings indicate that a small fraction of endogenous TDG localizes to the PODs, consistent with our transient-expression studies.

Figure. 2.1. SUMO-1-dependent recruitment of TDG to PML oncogenic domains. (A) Subcellular localization of YFP-TDG with and without coexpression of PML. MCF-7 cells were transfected with 300 ng of YFP-TDG expression vector alone or in combination with 300 ng of PML expression vector. PODs were detected by immunostaining fixed cells with PML-specific antibody. Fluorescence microscopy was performed using appropriate filters (CY3, YFP). Representative 0.5-µm optical sections are shown. (B) *In vitro* interaction of MCF-7-expressed TDG with recombinant GST-SUMO-1 and GST-PML. Cellular lysates were incubated with GST fusion proteins, and bound TDG was detected by immunoblotting. (C) Hyperthermic stress releases TDG from PODs. Live MCF-7 cells expressing YFP-TDG and CFP-PML were imaged initially at 37°C and following incubation at 42°C for 15 min. Lysates of control and heat-shocked cells (15 and 30 min) were immunoblotted with a TDG-specific antibody to reveal unmodified and sumoylated (S) YFP-TDG. (D) TDG-specific antibody recognizes mouse and human TDG. Whole-cell extracts from MCF-7 cells transfected with empty vector (control) or mouse TDG expression vector were immunoblotted with purified TDG-specific rabbit IgG. (E) Nuclear colocalization of endogenous TDG and PML. Untransfected MCF-7 and NIH 3T3 cells were immunostained with TDG- and PML-specific antibodies and fluorophore-conjugated (FITC, CY3) secondary antibodies. The fluorescence intensity plot illustrates the coincidence of peak fluorescence for TDG (CY3, red) and PML (FITC, green). Measurements were obtained by performing a line scan across three PODs using Axiovision software. Representative 0.5-µm optical sections are shown.

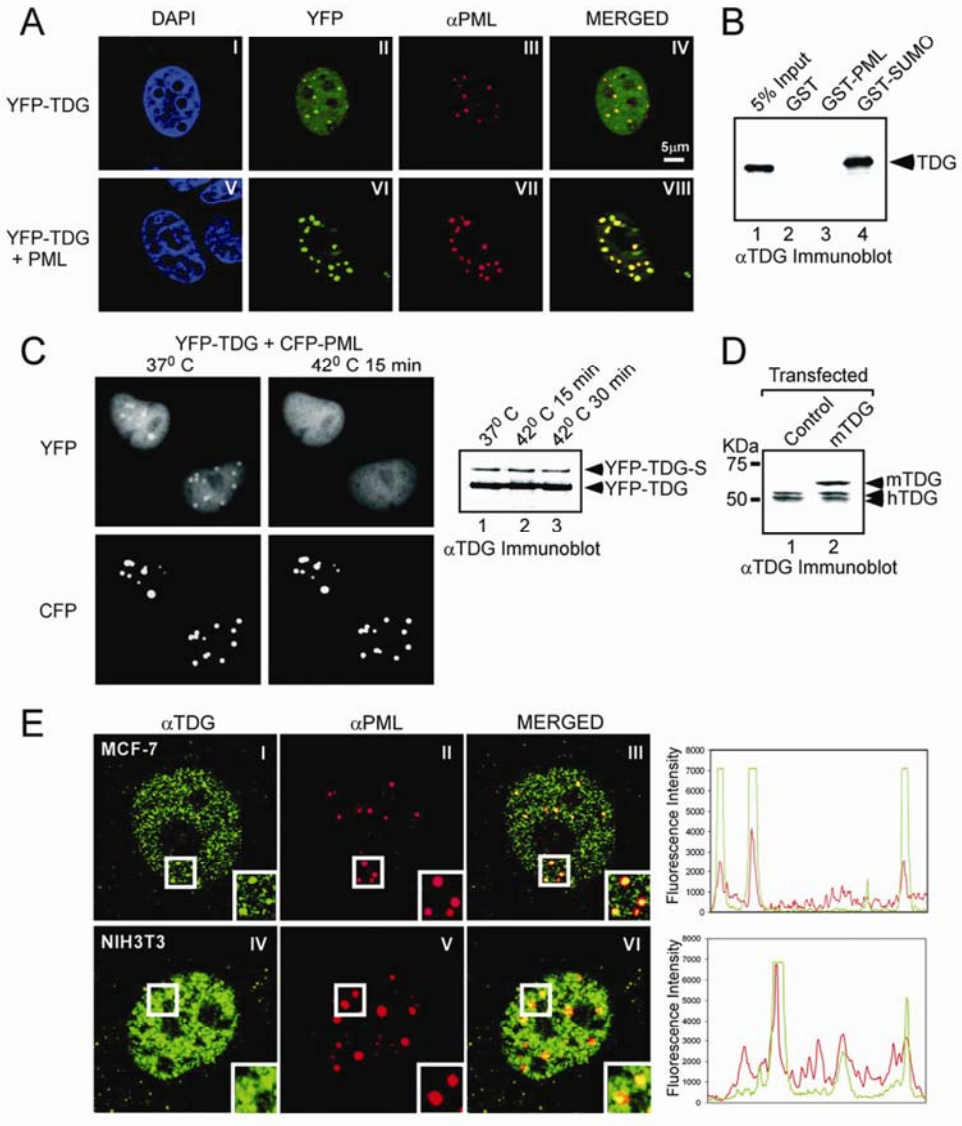
A
DAPI
YFP
αPML
MERGED
YFP-TDG
YFP-TDG + PML
5μm
B
5% Input
GST
GST-PML
GST-SUMO
TDG
1 2 3 4
αTDG Immunoblot
C
YFP-TDG + CFP-PML
37⁰ C
42⁰ C 15 min
YFP
CFP
37⁰ C
42⁰ C 15 min
42⁰ C 30 min
YFP-TDG-S
YFP-TDG
1 2 3
αTDG Immunoblot
D
Transfected
Control
mTDG
KDa
75
50
mTDG
hTDG
1 2
αTDG Immunoblot
E
αTDG
αPML
MERGED
MCF-7
NIH3T3
Fluorescence Intensity

Figure 2.1

Identification of POD targeting and SUMO-1 binding domains.

In order to identify protein domains within TDG essential for POD localization, we generated a series of CFP-tagged amino- and carboxy-terminal deletions and examined their cellular localization following coexpression with PML. Our analysis indicated that amino-terminal residues were required for nuclear targeting of TDG (Fig. 2.2A). Deletion of residues 1 to 156 shifted localization predominantly to the cytoplasm, whereas deletion of residues 1 to 121 resulted in similar levels of nuclear and cytoplasmic fluorescence. In order to assess the contribution of this region to POD localization, we engineered deletion constructs containing the SV40 NLS. FLAG epitope-tagged amino- and carboxy-terminal deletions were coexpressed with YFP-PML, and subcellular localization was examined by immunostaining with anti-FLAG antibody. Remarkably, we found that TDG lacking the first 121 residues (i.e., NLS122-421) accumulated preferentially in the PODs compared to wild-type TDG (Fig. 2.2B, compare panels I and II). Consequently, in a large fraction of cells (40 to 50%) nuclear fluorescence was predominantly associated with PODs, whereas in the case of wild-type TDG, substantial nucleoplasmic localization was observed. A lysine-rich regulatory domain (LRD; residues 70 to 118) previously shown to be acetylated by CBP/p300 is contained within this deleted region (46). Further removal of residues 123 to 156 (NLS157-421) led to a dramatic decrease in the number of expressing cells, with the majority of the tagged protein being found in large aberrant nucleoplasmic foci that also contained PML. Loss of carboxy-terminal residues 346 to 421 did not affect POD targeting; however, further deletion to residue 307 completely abrogated TDG accumulation in these structures. These data suggest that both amino- and carboxy-terminal regions of TDG contribute to POD localization.

In light of evidence suggesting a SUMO-1-dependent mechanism in POD targeting, we also tested amino- and carboxy-terminal deletions of TDG produced by *in vitro*

transcription/translation for binding to GST-SUMO-1. While full-length TDG displayed only weak SUMO binding activity, removal of residues 1 to 121 dramatically stimulated binding (Fig. 2.2C). In contrast, deletion of residues 1 to 156 or 307 to 346 resulted in complete loss of SUMO binding activity. These findings suggest that two distinct regions of TDG (residues 122 to 156 and 307 to 346) are essential for SUMO-1 binding, whereas a third region (residues 1 to 121) containing the LRD appears to suppress binding activity. Notably, these domains are also involved in POD targeting, suggesting that the SUMO-1 binding activity of TDG may be required for targeting to these nuclear structures.

Figure 2.2. Deletion mapping of SUMO-1 binding and POD-targeting domains in mouse TDG. (A) Subcellular localization of amino- and carboxy-terminal deletions of TDG. CFP-tagged deletions of TDG were expressed in MCF-7 cells and analyzed by direct fluorescence microscopy. (B) Amino- and carboxy-terminal deletions of TDG (depicted in the upper panel) containing the FLAG epitope were coexpressed with YFP-PML in MCF-7 cells. Approximately 300 ng of TDG and 500 ng of YFP-PML expression vectors were used. TDG was detected by immunostaining with anti-FLAG monoclonal antibody and CY3-conjugated secondary antibody. Representative 0.5-μm optical sections are shown. The location of the LRD is indicated. Note that FLAG-tagged amino-terminal deletions include the SV40 NLS. (C) *In vitro* interaction of TDG with GST-SUMO-1 is enhanced by deletion of residues 1 to 121. *In vitro*-translated ^{35}S-radiolabeled full-length TDG and the indicated deletion mutants were used in binding assays with GST-SUMO-1 and GST. Bound proteins were detected by autoradiography.

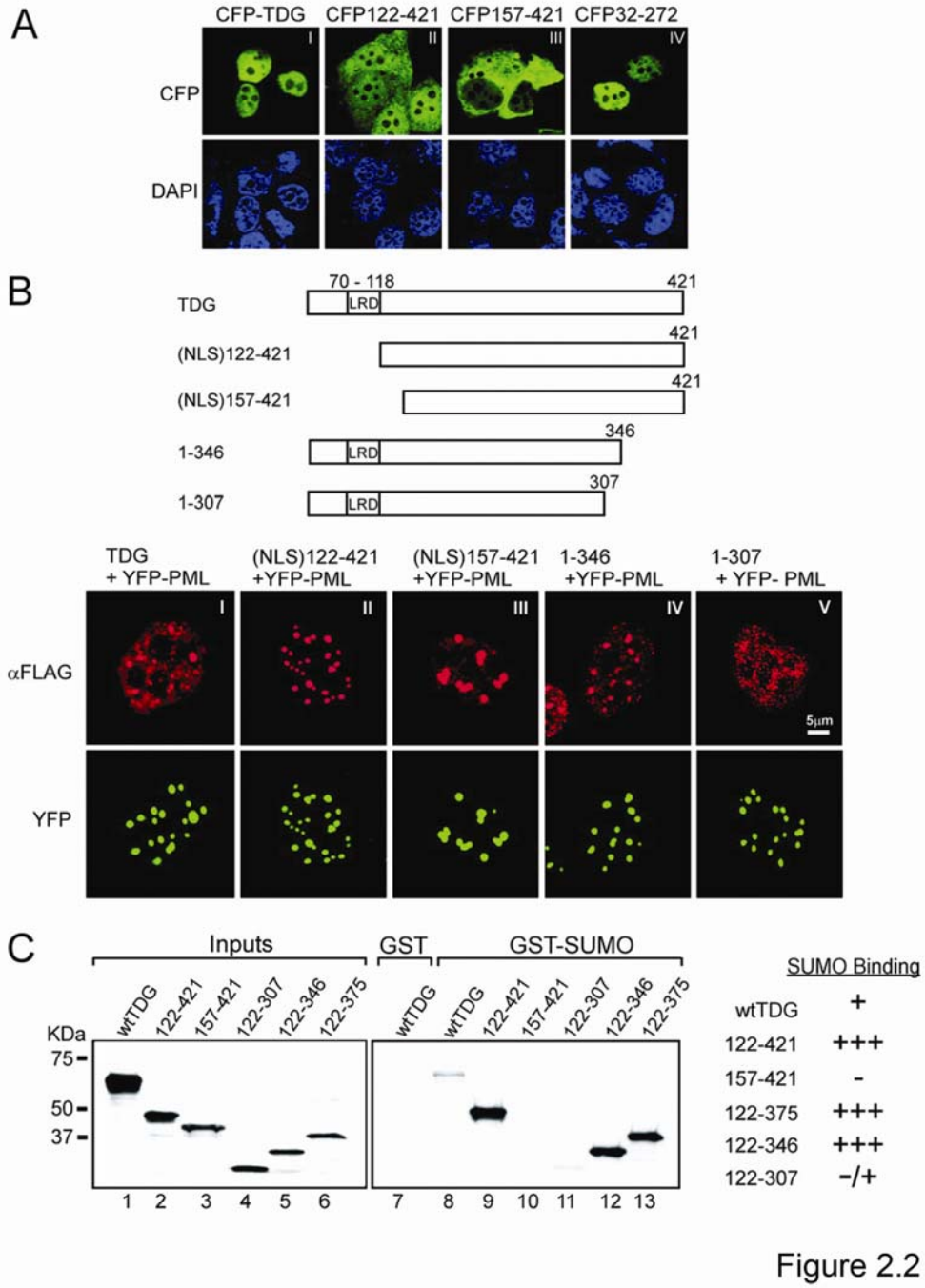
A
CFP-TDG
CFP122-421
CFP157-421
CFP32-272
I
II
III
IV
CFP
DAPI
B
70 - 118
421
TDG
LRD
(NLS)122-421
421
(NLS)157-421
421
1-346
346
LRD
1-307
307
LRD
TDG
+ YFP-PML
(NLS)122-421
+YFP-PML
(NLS)157-421
+YFP-PML
1-346
+YFP-PML
1-307
+ YFP- PML
I
II
III
IV
V
αFLAG
5μm
YFP
C
Inputs
GST
GST-SUMO
KDa
wtTDG
122-421
157-421
122-307
122-346
122-375
75
50
37
1 2 3 4 5 6 7 8 9 10 11 12 13
SUMO Binding
wtTDG +
122-421 +++
157-421 -
122-375 +++
122-346 +++
122-307 -/+

Figure 2.2

DNA interactions regulate SUMO-1 binding activity.

In vitro mapping studies suggested that the amino-terminal region (residues 1 to 121) modulated interactions with SUMO-1. In human TDG, this region has been found to be essential for nonspecific DNA binding and interactions with abasic sites (44). Since *in vitro* translation reaction mixtures contain plasmid DNA, we wanted to establish whether the DNA binding properties of the amino-terminal region could interfere with SUMO-1 binding *in vitro*; therefore, we performed SUMO binding experiments in the presence of ethidium bromide to effectively prevent DNA binding (25). A marked stimulation in SUMO-1 binding from full-length TDG was observed, while binding of the 122-421 protein was not affected (Fig. 2.3A). These data suggested that DNA binding by the amino-terminal region of TDG may prevent SUMO-1 recognition.

Human TDG has been shown to bind both G:T/U-mispaired and normally paired DNA (19). The mouse and human TDG orthologs are highly conserved within the central enzymatic core and less well conserved in the amino- and carboxy-terminal regions. Using an electrophoretic mobility shift assay (13), we confirmed that bacterially expressed mouse TDG has similar DNA binding specificity and could form complexes with normally paired (G:C) as well as G:T/U-mispaired duplex oligonucleotides (Fig. 2.3B). The requirement of the 1-121 region in DNA interactions was confirmed using the ABCD binding assay (15). In these assays, recombinant full-length TDG bound to a G:T-mispaired oligonucleotide, while an amino-terminal-truncated protein fragment (122-421) did not detectably associate with DNA (Fig. 2.3C). To determine whether residues 1 to 121 contained a modular DNA binding domain, we assayed a GST fusion protein containing this region for binding to G:T duplex oligonucleotide using the ABCD assay (data not shown). The fact that DNA binding was not observed suggests that the amino-terminal region does not independently associate with DNA. Interestingly, the NLS122-

421 protein displayed preferential POD localization upon coexpression with PML (Fig. 2.2B), suggesting that loss of DNA interactions promotes POD targeting. We tested whether the DNA and SUMO binding activities of TDG are mutually exclusive by performing binding studies with GST-SUMO-1 and recombinant TDG in the presence of increasing amounts of duplex oligonucleotide containing a G:T mispair. A dose-dependent reduction in SUMO binding was consistently observed in the presence of DNA (Fig. 2.3D); in contrast, preincubation of TDG with SUMO-1 did not affect binding to G:T or G:C duplex oligonucleotides (see Fig. S.2.1).

Figure 2.3. The amino-terminal DNA binding domain of TDG regulates SUMO-1 binding activity. (A) Binding of full-length *in vitro*-translated TDG to GST-SUMO-1 is sensitive to ethidium bromide. *In vitro*-translated ^{35}S-radiolabeled full-length TDG or the 122-421 truncated protein was bound to GST-SUMO-1 in the presence or absence of ethidium bromide. (B) Recombinant mouse TDG binds normally paired as well as G:T or G:U mispaired duplex oligonucleotides. An electrophoretic mobility shift assay was performed using the indicated radiolabeled duplex oligonucleotides. Approximately 100-fold molar excess of the same unlabeled oligonucleotides was used as competitor DNA. (C) Residues 1 to 121 of mouse TDG are essential for DNA binding. A biotin-tagged duplex oligonucleotide (500 ng) containing a G:T mispair was bound to recombinant full-length TDG or the 122-421 protein (500 ng each). DNA-protein complexes were isolated using streptavidin-Sepharose and analyzed by immunoblotting with a monoclonal antihistidine antibody. (D) DNA binding suppresses the SUMO-1 binding activity of TDG. Recombinant TDG (200 ng) was bound to GST-SUMO-1 in the presence of increasing amounts (70, 210, and 420 ng) of duplex oligonucleotides containing a G:T mispair.

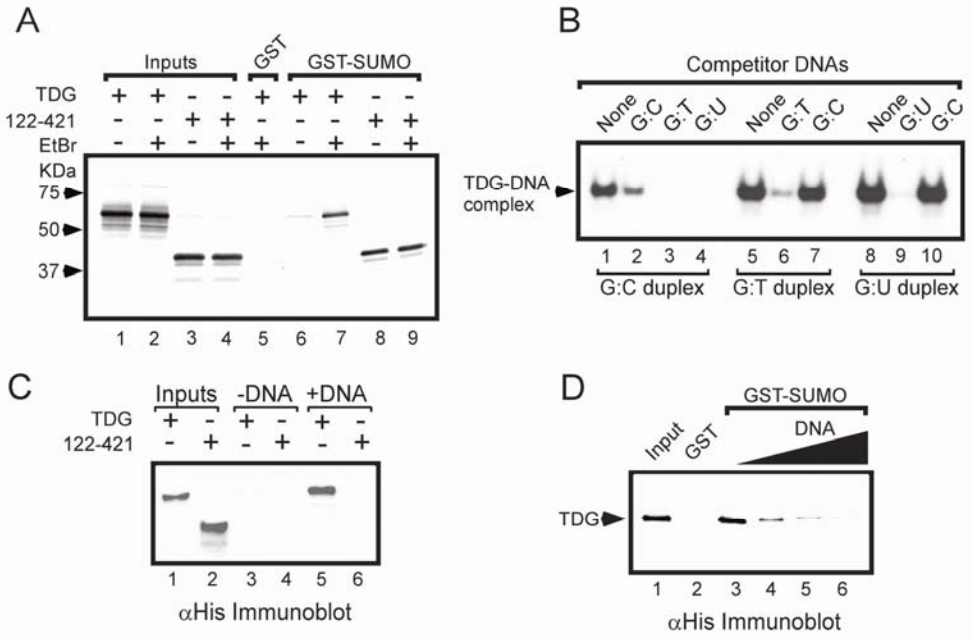
A
Inputs
GST
GST-SUMO
TDG
122-421
EtBr
KDa
75
50
37
1 2 3 4 5 6 7 8 9
B
Competitor DNAs
None
G:C
G:T
G:U
None
G:T
G:C
None
G:U
G:C
TDG-DNA complex
1 2 3 4 5 6 7 8 9 10
G:C duplex
G:T duplex
G:U duplex
C
Inputs
-DNA
+DNA
TDG
122-421
1 2 3 4 5 6
αHis Immunoblot
D
GST-SUMO
Input
GST
DNA
TDG
1 2 3 4 5 6
αHis Immunoblot

Figure 2.3

Identification of a novel amino-terminal SUMO-1 binding motif.

In vitro mapping studies indicated that residues 122 to 156 are required for SUMO-1 binding; therefore, we examined the amino acid sequence within this region and identified four residues (IVII; amino acids 145 to 148) which are identical to the recently characterized SUMO-1 binding consensus motif (I/V-X-I/V-I/V) (42). Furthermore, this motif is flanked by an aspartic acid (i.e., DIVII) residue also present adjacent to the SUMO-1 binding motifs of the RanBP2/NUP358 and SUMO activating enzyme 2 (SAE2) proteins (42). The DIVII residues are conserved in mammalian, chicken, and *Drosophila melanogaster* TDG orthologs and are contiguous with the conserved GINPGL glycosylase motif (2, 18) (Fig. 2.4A). Previous structural studies using a truncated form of human TDG have identified a carboxy-terminal SUMO-1 binding motif (VQEV) (1) that is conserved in mouse, human, and chicken TDG, but not in the *Drosophila* ortholog (Fig. 2.4A). In order to establish whether the putative amino- and carboxy-terminal SBMs in mouse TDG bind SUMO-1, we generated a series of mutant proteins with single amino acid substitutions and measured their ability to bind to GST-SUMO-1 (Fig. 2.4B). Alanine substitution mutants were generated for each residue in the DIVII motif, whereas a single glutamic acid-to-glutamine (E321Q) substitution in the VQEV motif was analyzed, as this had been previously reported to abrogate SUMO-1 binding in human TDG (1). Substitution of specific residues within each putative SBM independently abrogated SUMO-1 binding, suggesting that in the context of full-length TDG both motifs are essential for stable SUMO-1 interactions. Specifically, within the DIVII motif, the I145A and V146A substitutions produced small but consistent reductions in binding, whereas the D144A and I147A substitutions displayed more pronounced loss of binding. In contrast, the I148A substitution appeared to stimulate binding. The E321Q substitution in the carboxy-terminal SBM completely abrogated binding. To rule out gross effects of the amino acid substitutions on protein folding, we performed DNA glycosylase assays

using radiolabeled duplex oligonucleotides containing a single G:T mispair (see Fig. S.2.2). Comparable levels of base excision were observed with *in vitro*-translated wild-type TDG, D144A, and E321Q, consistent with proper folding. Similar results on SUMO binding were obtained when we expressed the respective TDG mutants in MCF-7 cells and used whole-cell lysates in interaction studies (Fig. S.2.3).

In order to determine whether the SBMs are required for binding to SUMO-1 conjugated to a target protein, we employed an *in vitro* sumoylation system (10) reconstituted with bacterially expressed enzymes (SAE1, SAE2, and UBC9) and SUMO-1 to sumoylate a purified bacterially expressed GST fusion of tumor suppressor p53 protein (39). As a control, mock sumoylation reactions were carried out in the absence of SUMO-1. Analysis of the reaction products by immunoblotting revealed the presence of a protein band reactive with both p53 and SUMO-1 antibodies only in the sumoylation reaction (Fig. 2.4C). The products of both the mock and sumoylation reactions were bound to glutathione affinity beads and used in interaction studies with *in vitro*-translated TDG and SBM mutants (D144A and E321Q). We observed appreciable binding of wild-type TDG on beads containing sumoylated GST-p53, while only marginal binding was detected with mock-sumoylated GST-p53 (Fig. 2.4D). The D144A mutant displayed substantially reduced binding, while the E321Q substitution almost completely abrogated binding. Since the sumoylation reaction mixtures contain GST fusions of the SAE1 and SAE2 enzymes, we also performed binding reactions with the products of sumoylation reactions lacking GST-p53. In this case, binding of TDG was not detected. These findings indicate that the DIVII residues in TDG constitute a bona fide SBM and that the SUMO-1 binding activity of TDG resides within two separate motifs.

Figure 2.4. SUMO-1 binding activity of TDG resides in amino-terminal and carboxy-terminal motifs. (A) Amino acid sequence alignments of the mouse (47), human (32), chicken (51), and *Drosophila melanogaster* (18) TDG orthologs showing putative SBMs. The coordinates for the mouse sequences are indicated. Complete sequences were aligned using Clustal W software, but only pertinent regions are shown (136 to 155 and 312 to 326 of mouse TDG). The location of the conserved DIVII and VQEV motifs (boxed) as well as the active site glycosylase motif (GINPGL) and the substrate recognition motif (VMPSSSAR) (19) are shown. Asterisks indicate identical residues, while colons indicate conserved residues. The different engineered substitution mutants of the DIVII and VQEV motifs are indicated. (B) Single amino acid substitutions within the conserved DIVII and VQEV motifs abrogate SUMO-1 binding. Radiolabeled *in vitro*-translated wild-type TDG and the indicated substitution mutants were analyzed for binding to GST-SUMO-1 in the presence of ethidium bromide. Binding was measured by phosphorimaging. (C) *In vitro* sumoylation of recombinant GST-p53. *In vitro* sumoylation reactions were performed by incubating GST-p53 with sumoylation enzymes (GST-SAE1, GST-SAE2, and UBC9) and SUMO-1. Reaction products were immunoblotted with p53 or SUMO-1 antibodies. (D) SBMs are required for optimal binding to conjugated SUMO-1. The products of the GST-p53 sumoylation and mock sumoylation reactions were bound to glutathione affinity beads and used for interaction assays with *in vitro*-translated TDG and SBM mutants (D144A and E321Q). Binding experiments were also carried out with sumoylation reaction mixtures lacking GST-p53 to exclude interactions of TDG with GST-SAE1/SAE2. The results of three independent experiments are plotted, showing the mean percent binding of input proteins to beads containing sumoylated GST-p53. The standard error is shown.

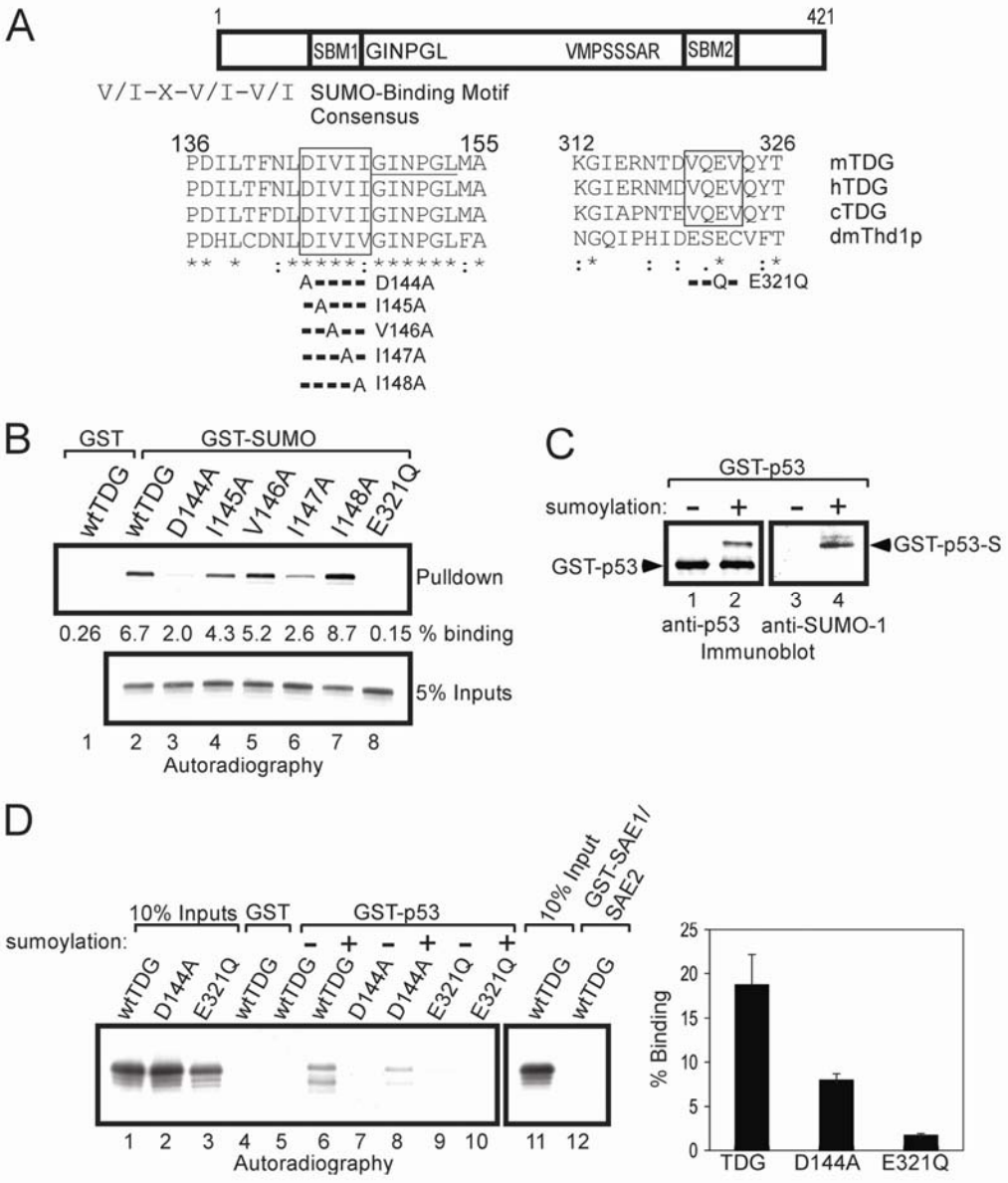
A
1
421
SBM1
GINPGL
VMPSSSAR
SBM2
V/I-X-V/I-V/I SUMO-Binding Motif Consensus
136
155
312
326
PDILTFNLDIVIIGINPGLMA
PDILTFNLDIVIIGINPGLMA
PDILTFDLDIVIIGINPGLMA
PDHLCDNLDIVIVGINPGLFA
KGIERNTDVQEVQYT
KGIERNMDVQEVQYT
KGIAPNTEVQEVQYT
NGQIPHIDESECVFT
mTDG
hTDG
cTDG
dmThd1p
A---- D144A
-A--- I145A
--A-- V146A
---A- I147A
----A I148A
--Q- E321Q
B
GST
GST-SUMO
wtTDG
wtTDG
D144A
I145A
V146A
I147A
I148A
E321Q
Pulldown
0.26 6.7 2.0 4.3 5.2 2.6 8.7 0.15 % binding
5% Inputs
1 2 3 4 5 6 7 8
Autoradiography
C
GST-p53
sumoylation: - + - +
GST-p53
GST-p53-S
1 2 3 4
anti-p53
anti-SUMO-1
Immunoblot
D
10% Inputs
GST
GST-p53
10% Input
GST-SAE1/ SAE2
sumoylation:
- + - + - +
wtTDG
D144A
E321Q
wtTDG
wtTDG
wtTDG
D144A
D144A
E321Q
E321Q
wtTDG
wtTDG
1 2 3 4 5 6 7 8 9 10 11 12
Autoradiography
% Binding
25
20
15
10
5
0
TDG
D144A
E321Q

Figure 2.4

To assess whether the amino- and carboxy-terminal SBMs are required for POD translocation, we expressed the SUMO-1 binding-deficient mutants (D144A, I147A, and E321Q) with YFP-PML and monitored cellular localization by immunostaining with an anti-FLAG antibody (Fig. 2.5A and B). This analysis showed that mutations within the amino-terminal SBM substantially reduced the number of transfected cells displaying POD accumulation of TDG; in contrast, the E321Q substitution completely abolished POD accumulation. These findings indicate that both SBMs in TDG are involved in POD targeting.

Previous studies have demonstrated a potent stimulatory function of TDG on CBP-dependent transcription (46). To assess whether the SUMO binding activity of TDG is involved in stimulating CBP-dependent transcription, we tested the activation potential of the D144A, I147A, and E321Q mutants using a chimeric fusion of CBP and the GAL4 DNA binding domain on a GAL4-responsive reporter gene (Fig. 2.5C and D). The amounts of transfected expression vectors were adjusted to achieve approximately equivalent levels of expression of wild-type TDG and mutants. In accordance with the SUMO-1 binding and POD localization studies, the D144A, I147A, and E321Q mutants were found to be defective in CBP activation. Previous studies have shown that TDG interacts with the histone acetyltransferase and a carboxy-terminal domain (CH3) of CBP (46). Since the amino acid substitutions may affect interactions with CBP, we tested *in vitro*-translated wild-type, D144A, and E321Q proteins for binding to full-length recombinant CBP (see Fig. S.2.4 in the supplemental material). However, no differences in binding were detected, suggesting that abrogation of the CBP activation properties in the SBM mutants is likely due to loss of SUMO binding activity. We also investigated whether the previously reported sumoylation sites in CBP (14, 24) are required for activation by TDG. Accordingly, we examined the ability of TDG to activate a CBP mutant containing lysine-to-arginine substitutions at sumoylation sites K999, K1034, and

K1057. The transcriptional activity of this mutant was also robustly stimulated by TDG, indicating that CBP sumoylation is not essential for this effect (see Fig. S.2.5).

Figure 2.5. SUMO-1 binding activity of TDG is essential for CBP activation and normal POD recruitment. (A) SUMO-1 binding mutants are defective in POD recruitment. FLAG-tagged wild-type TDG and the indicated mutants were coexpressed with YFP-PML, and POD recruitment was analyzed by indirect immunofluorescence using anti-FLAG antibody. Representative 0.5-μm optical sections are shown. (B) Incidence of POD accumulation for wild-type TDG and mutants coexpressed with YFP-PML. An average of 120 cells were counted to determine POD accumulation of wild-type TDG or SUMO-1 binding mutants. Error bars represent standard deviations of determinations from three independent experiments. (C) Activation of CBP-dependent transcription is abrogated by E321Q substitution in the VQEV motif. (D) Mutations in DIVII motif also abrogate CBP activation. Expression vectors for GAL-CBP, wild-type TDG, and point mutants of TDG were cotransfected into MCF-7 cells. The luciferase reporter plasmid contains five copies of the GAL4 DNA binding site fused to the core ß-globin promoter. The amounts of transfected TDG expression vectors were titrated to obtain approximately equal levels of protein expression (immunoblot, lower panel).

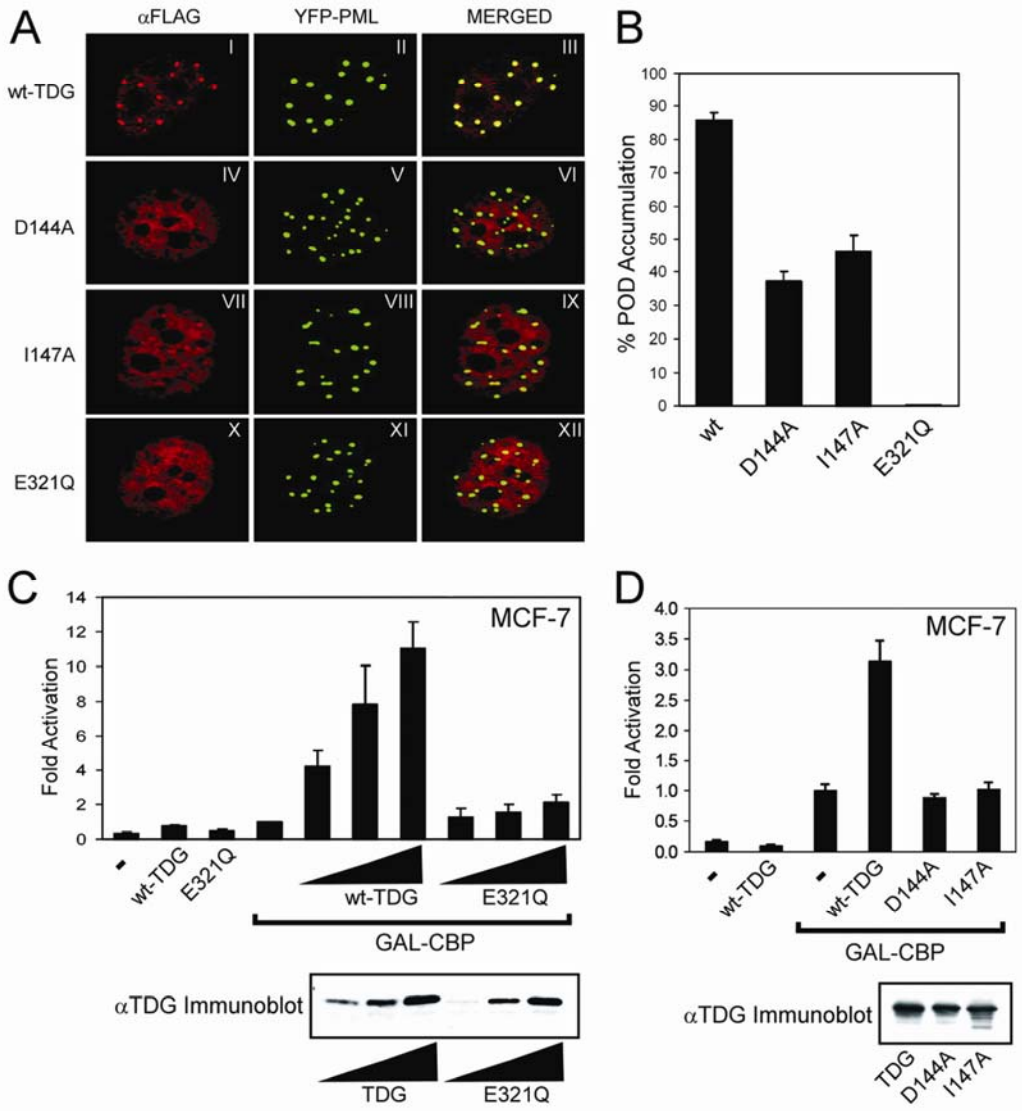
A
αFLAG
YFP-PML
MERGED
wt-TDG
D144A
I147A
E321Q
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
B
% POD Accumulation
wt
D144A
I147A
E321Q
C
MCF-7
Fold Activation
wt-TDG
E321Q
GAL-CBP
αTDG Immunoblot
TDG
E321Q
D
MCF-7
Fold Activation
wt-TDG
D144A
I147A
GAL-CBP
αTDG Immunoblot
TDG
D144A
I147A

Figure 2.5

Sumoylation of TDG regulates association with CBP and subnuclear localization.

Human TDG has been reported to be sumoylated at a single carboxy-terminal lysine residue (20). Consistent with this, we have observed a higher-molecular-weight band in immunoblots of mouse cell extracts and in transfected human cells expressing mouse TDG (Fig. 2.6 and data not shown). To determine whether the higher-molecular-weight band corresponds to sumoylated TDG, we cotransfected FLAG-TDG and HA-SUMO-1 expression vectors into MCF-7 cells and analyzed cellular lysates by immunoblotting with anti-FLAG and anti-HA antibodies (Fig. 2.6A). This analysis indicated that the higher-molecular-weight band detected with the anti-FLAG antibody corresponds to SUMO-1-modified TDG, since it was also detected with the anti-HA antibody. The sumoylated form of TDG is not observed when lysine 341, located within the SUMO consensus conjugation site (V<u>K</u>EE), is mutated to arginine (Fig. 2.6B).

In vitro sumoylation experiments, using recombinant TDG as a substrate, confirmed that the sumoylation machinery is present in both nuclear and cytoplasmic extracts (data not shown). We assessed whether TDG sumoylation occurred within the nucleus by expressing CFP-tagged amino-terminal deletions (CFP122-421, CFP157-421) of TDG defective in nuclear targeting and determining the level of sumoylation by immunoblotting cellular lysates with a TDG-specific antibody (Fig. 2.6C). The CFP157-421 fusion protein that localized preferentially to the cytoplasm was not efficiently sumoylated, since a higher-molecular-weight band corresponding to sumoylated TDG was not readily detectable. A truncated form of TDG (CFP32-272) lacking the sumoylation site was also not sumoylated. These data suggest that TDG sumoylation takes place in the nuclear compartment and/or at the nuclear membrane. The observation that SUMO-1 binding mutants (E321Q, D144A, and I147A), defective in POD localization, were sumoylated efficiently *in vivo* indicates that noncovalent SUMO binding is not

required for sumoylation (Fig. 2.6D). Furthermore, these findings also suggest that sumoylation is likely not occurring within the PODs.

Figure 2.6. TDG sumoylation occurs in the nucleus and does not require noncovalent SUMO-1 binding activity. (A) FLAG-TDG and HA-SUMO-1 expression vectors were cotransfected into MCF-7 cells, and lysates were analyzed by immunoblotting using anti-FLAG or anti-HA antibody. Sumoylated TDG (S-TDG), TDG, and nonspecific (NS) bands are indicated. (B) *In vivo* sumoylation occurs at lysine 341 of mouse TDG. Expression vectors for TDG and K341R were transfected into MCF-7 cells, and cellular lysates were blotted with TDG-specific antibody. (C) TDG is sumoylated predominantly in the nucleus. Whole-cell lysates of MCF-7 cells transfected with expression vectors for the indicated CFP fusions were analyzed by immunoblotting with TDG-specific antibody. CFP157-421 is localized preferentially in the cytoplasm (Fig. 2.2A). (D) SUMO-1-binding-deficient mutants are sumoylated efficiently in MCF-7 cells. Cellular lysates from MCF-7 cells transfected with the indicated expression vectors were analyzed by immunoblotting with a TDG-specific antibody.

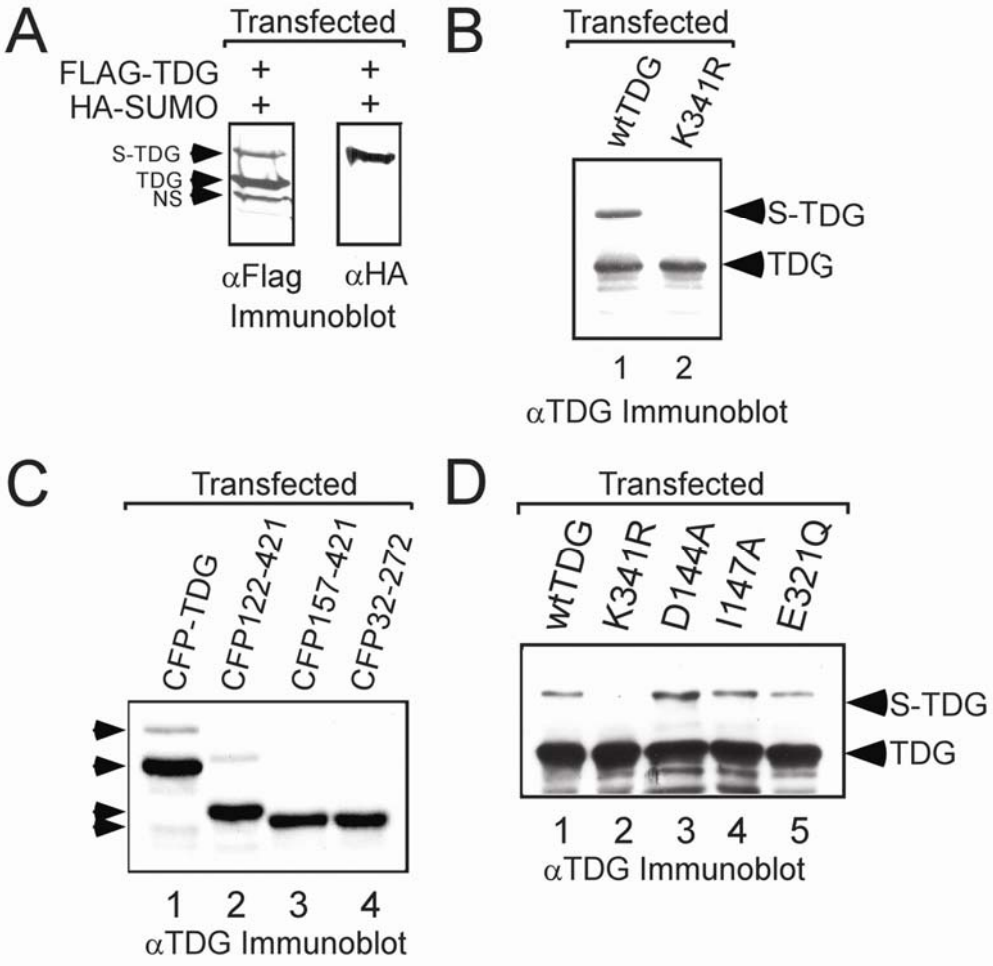
A
Transfected
FLAG-TDG + +
HA-SUMO + +
S-TDG
TDG
NS
αFlag
αHA
Immunoblot
B
Transfected
wtTDG
K341R
S-TDG
TDG
1 2
αTDG Immunoblot
C
Transfected
CFP-TDG
CFP122-421
CFP157-421
CFP32-272
1 2 3 4
αTDG Immunoblot
D
Transfected
wtTDG
K341R
D144A
I147A
E321Q
S-TDG
TDG
1 2 3 4 5
αTDG Immunoblot

Figure 2.6

Sumoylation has been shown to regulate cellular partitioning of PML and other proteins (49); we therefore compared the subcellular localization of *Renilla* GFP fusions of the sumoylation-deficient mutant K341R and wild-type TDG. Notably, the K341R mutant protein was found to accumulate exclusively in PODs in approximately 30% of transfected cells, whereas wild-type TDG exhibited its characteristic nucleoplasmic distribution (Fig. 2.7A). The enhanced ability of the K341R mutant to localize to the PODs suggests that sumoylation of TDG negatively regulates POD translocation.

The conjugation of SUMO-1 to lysine 341 covalently links a bulky peptide to a region that has been previously shown to interact with CBP/p300 (46). Moreover, the presence of SUMO binding motifs within the amino- and carboxy-terminal regions suggests that sumoylation of TDG may promote intramolecular interactions that drastically alter the conformation of this protein. To investigate the functional consequences of sumoylation, we produced sumoylated recombinant TDG *in vitro* along with a mock-sumoylated control (Fig. 2.7B). Mock-sumoylated TDG and sumoylated TDG proteins were analyzed for DNA binding to a duplex oligonucleotide containing a single G:T mispair using the ABCD assay. As reported for the human ortholog, sumoylated mouse TDG failed to interact with DNA (Fig. 2.7C). We performed interaction studies with purified recombinant baculovirus-expressed CBP bearing a FLAG epitope tag. Protein complexes were captured using anti-FLAG affinity resin. While binding of mock-sumoylated TDG to CBP was readily observed, only weak interactions were observed with sumoylated TDG (Fig. 2.7D). Consequently, sumoylated TDG was not appreciably acetylated by CBP in the presence of ^{14}C-AcCoA (Fig. 2.7E). To establish whether intramolecular SUMO binding in sumoylated TDG occludes both SBMs, we tested whether sumoylated TDG can interact with GST-SUMO-1 *in vitro*. In contrast to mock-sumoylated TDG, sumoylated TDG failed to interact with GST-SUMO-1 (Fig. 2.7F). To confirm that sumoylated TDG is enzymatically active, we performed DNA glycosylase assays using

radiolabeled duplex oligonucleotides containing a single G:U mispair. As previously reported (20), sumoylated TDG displayed enhanced G:U processing activity compared to unmodified TDG and mock-sumoylated TDG (see Fig. S.2.6). Therefore, abrogation of the interaction of sumoylated TDG with CBP is unlikely due to aberrant misfolding but likely involves sumoylation-induced conformational changes.

Figure 2.7. Sumoylation of TDG abrogates interaction with CBP *in vitro* and negatively regulates POD translocation. (A) A sumoylation-defective TDG mutant localizes preferentially to PODs. Expression vectors for GFP-TDG or GFP-TDG(K341R) were transfected into MCF-7 cells, and subcellular localization was analyzed following immunostaining with PML-specific antibody. Representative 0.5-μm optical sections are shown. (B) *In vitro* sumoylation of TDG using recombinant SUMO-1 conjugation enzymes (UBC9, SAE1, and SAE2). Sixty nanograms of purified reaction products was analyzed by immunoblotting with TDG- and SUMO-1 specific antibodies. Mock-sumoylated TDG was produced by performing sumoylation reactions without SUMO-1. (C) Sumoylated mouse TDG does not bind to DNA. Approximately 400 ng of either mock-sumoylated or sumoylated TDG was analyzed for DNA binding using the ABCD assay. Supernatants of each binding reaction mixture were also immunoblotted to demonstrate the stability of the modification during the assay (SUP-M and SUP-S). (D) TDG sumoylation abrogates CBP-TDG interactions. Mock or sumoylated TDG produced *in vitro* (400 ng) was incubated with recombinant FLAG-CBP (100 ng). Anti-FLAG resin was used to immunoprecipitate CBP, and the presence of TDG was detected by immunoblotting. Supernatants of each binding reaction mixture were also immunoblotted (SUP-M and SUP-S). (E) Sumoylated TDG is not acetylated efficiently by CBP. Mock-sumoylated or sumoylated TDG (400 ng) was incubated with CBP (100 ng) in the presence of ^{14}C-acetyl-CoA. Reaction products were separated by electrophoresis, and acetylation was detected by autoradiography. (F) Sumoylated TDG does not bind GST-SUMO-1. Mock-sumoylated or sumoylated TDG was analyzed for binding to GST-SUMO-1.

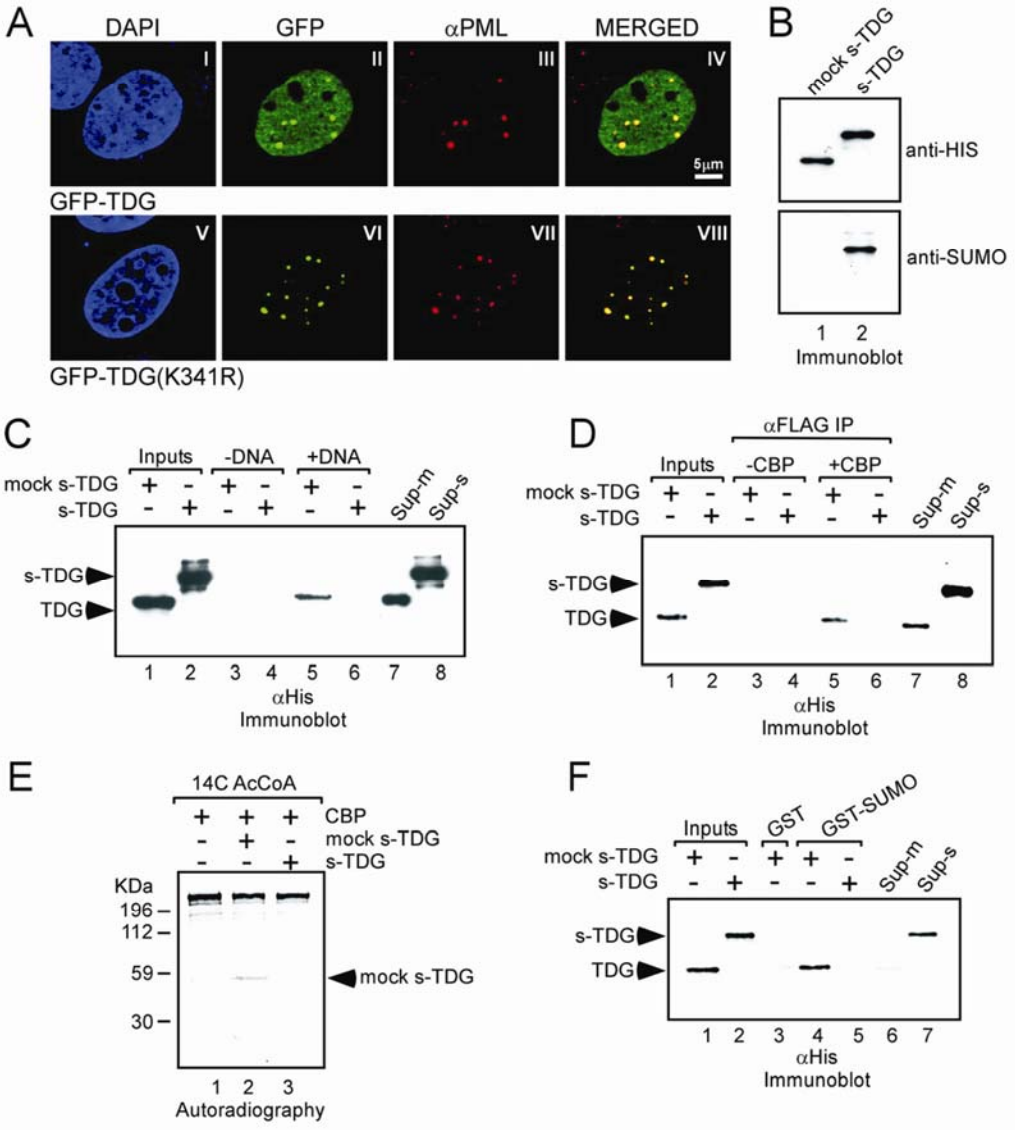
A
DAPI
GFP
αPML
MERGED
I
II
III
IV
5μm
GFP-TDG
V
VI
VII
VIII
GFP-TDG(K341R)
B
mock s-TDG
s-TDG
anti-HIS
anti-SUMO
1 2
Immunoblot
C
Inputs
-DNA
+DNA
mock s-TDG
s-TDG
Sup-m
Sup-s
s-TDG
TDG
1 2 3 4 5 6 7 8
αHis
Immunoblot
D
αFLAG IP
Inputs
-CBP
+CBP
mock s-TDG
s-TDG
Sup-m
Sup-s
s-TDG
TDG
1 2 3 4 5 6 7 8
αHis
Immunoblot
E
14C AcCoA
CBP
mock s-TDG
s-TDG
KDa
196
112
59
30
mock s-TDG
1 2 3
Autoradiography
F
Inputs
GST
GST-SUMO
mock s-TDG
s-TDG
Sup-m
Sup-s
s-TDG
TDG
1 2 3 4 5 6 7
αHis
Immunoblot

Figure 2.7

2.4 Discussion

We have investigated the role of sumoylation and noncovalent SUMO-1 binding in the regulation of subcellular localization and biochemical properties of thymine DNA glycosylase. Our studies have mapped the regions of TDG necessary for non-covalent SUMO-1 interactions to two separate motifs (SBMs) located in the amino- and carboxy-terminal regions that are essential for POD localization and activation of CBP-dependent transcription. The activities of the SBMs are regulated by DNA interactions, and uncoupling of TDG from DNA appears to be an essential step in POD translocation. In addition, we have established that TDG sumoylation regulates molecular interactions with CBP as well as translocation to PODs.

PODs contain regulatory proteins involved in different nuclear processes, including DNA repair and transcription (9, 50). Diverse models of POD function have been proposed, including their potential role as sites of storage and modification of nuclear factors (reviewed in reference 26). For example, tumour suppressor p53 acetylation by CBP in PODs constitutes a critical step in p53 activation during RAS-induced premature senescence (38). Interestingly, there is evidence that PODs associate with transcriptionally active genomic loci, and transcription has been detected at the periphery of these structures (5, 48). We have established the presence of endogenous TDG in PODs using a TDG-specific antibody and demonstrated the SUMO-dependent recruitment of exogenously expressed TDG to these nuclear structures (Fig. 2.1). While recent studies have reported a direct interaction of mouse TDG with PML (45), we have not observed significant binding of these proteins *in vitro*. It is unlikely that the lack of interaction is due to misfolding of bacterially expressed PML, since we have obtained similar results with baculovirus-expressed FLAG-tagged PML (data not shown). Furthermore, no appreciable binding of sumoylated TDG with PML *in vitro* was observed

(data not shown). Our findings suggest that TDG may be recruited to PODs by sumoylated PML (30) and/or other sumoylated POD components. We base this assertion on the requirement of SUMO-1 binding activity of TDG for POD recruitment and the fact that mild hyperthermic stress, previously shown to cause rapid desumoylation of PML and SP100 (34), abrogates POD accumulation of TDG without affecting PML localization.

Deletion analysis of TDG indicated the presence of two regions (residues 122 to 156 and 308 to 346) essential for SUMO-1 binding *in vitro*, as well as a third region containing the LRD that exerted an inhibitory function (Fig. 2.2B). We have identified a novel SUMO-1 binding motif (DIVII) within the 122-156 region conforming to the recently reported consensus SUMO-1 binding site (I/V-X-I/V-I/V) that mediates recognition of SUMO-modified proteins (42). The observation that single amino acid substitutions within the DIVII motif (particularly D144A and I147A) substantially reduce SUMO-1 binding in the context of full-length TDG clearly demonstrates that this is a bona fide SBM (Fig. 2.4B) (see also Fig. S.2.3).

Recent structural analysis of amino- and carboxy-terminal-truncated sumoylated human TDG identified a SUMO-1 binding motif (VQEV) located within the carboxy terminus near the SUMO conjugation site (1). Mutational analysis of mouse TDG revealed that both the amino- and carboxy-terminal SBMs (i.e., DIVII and VQEV) are required for stable interactions with both free and conjugated SUMO-1 (Fig. 2.4). Consequently, these observations suggest that SUMO-1 may bind concurrently to both SBMs. However, as both motifs have been reported to make nearly identical contacts (1, 42, 43) with residues on SUMO-1, we infer that there is considerable plasticity in these interactions. Accordingly, the I/V-X-I/V-I/V motif has recently been shown to interact bidirectionally with SUMO-1 (43). In contrast to a recent report (45), we have found that the SUMO-1 binding activity of TDG is not required for covalent conjugation of SUMO-1 (Fig. 2.6).

This discrepancy could be explained by the reliance of previous studies on the analysis of a TDG mutant containing an 11-amino-acid deletion, whereas more subtle single amino acid substitutions were employed in our studies.

The essential role of SUMO binding in POD translocation is demonstrated by the observation that single amino acid substitutions that decrease or abolish SUMO-1 binding also disrupt POD targeting (Fig. 2.4 and 2.5). Notably, both SBMs, in the context of full-length TDG, appeared to be required for optimal POD translocation. In contrast, similar analysis of deletion mutants provided some discrepant results. Deletion of the 122-156 region, containing the amino-terminal SBM, did not prevent POD localization *in vivo*. This may result from an inhibitory effect of the amino terminus (residues 1 to 121) on the carboxy-terminal SBM; removal of this region in the 157-421 deletion mutant may relieve inhibition and promote POD targeting. The TDG amino terminus (residues 1 to 121) is required for tight interaction with DNA and abasic sites (44) (Fig. 2.3C). Our findings suggest that uncoupling of TDG from DNA is necessary to unmask SUMO binding activity and promote POD translocation (Fig. 2.8). This may occur following excision of base mispairs via apurinic/apyrimidinic endonuclease (APE)-mediated displacement of TDG and/or following sumoylation of TDG (20). In the latter case, our findings suggest that sumoylation would prevent POD targeting by occluding the SBMs via intramolecular interactions; consequently, translocation to the PODs would require removal of SUMO-1 by isopeptidases (22).

A number of DNA repair factors have been shown to transit in PODs prior or following DNA damage (reviewed in reference 9), including enzymes involved in repair of double-stranded DNA breaks (DSB). For example, MRE11 and NBS1 associate with PODs in unirradiated cells and relocate to sites of DNA damage following gamma irradiation (4, 29). These observations suggest that PODs may act as sites of storage and/or assembly of

DNA repair protein complexes. Consistent with a role of PODs in genome maintenance, *PML* null mice display increased susceptibility to tumors following exposure to carcinogens (40). The association of PODs with transcriptionally active genomic loci (5, 48) provides an attractive model for TDG function (Fig. 2.8). While DSB occur infrequently, it is estimated that several hundred DNA mispairs occur daily per cell as a result of cytosine deamination (27), suggesting that the DNA repair functions of TDG would be required more frequently to maintain genome integrity. The well-documented interactions of TDG with transcriptional coactivators and sequence-specific transcription factors suggest that the genome surveillance functions of this enzyme are linked to transcription (8, 46, 47). *In vitro* studies have demonstrated that CBP/p300 and TDG form stable ternary complexes with DNA containing G:T/U mispairs (46). The recruitment of CBP/p300 to repair sites *in vivo* may be required to promote local chromatin remodelling and/or regulate the functions of BER enzymes, such as TDG, APE, and DNA polymerase ß, previously shown to be acetylated by these factors (3, 21, 46). On the basis of the association of PODs with genomic loci (48), it is plausible that transient association of TDG with these nuclear structures is required to deliver this DNA repair enzyme to sites of active transcription, ensuring efficient repair of damaged CpG dinucleotides. Alternatively, as reported for p53 (38), POD localization of TDG may serve as a regulatory step to promote acetylation by CBP (46).

Our studies indicate that the SUMO-1 binding activity of TDG is essential for activation of CBP-dependent transcription. Using reporter gene assays, we have demonstrated that SBM mutants (D144A, I147A, and E321Q), defective in SUMO-1 binding, do not mediate CBP activation (Fig. 2.5). Given that SUMO-1 binding is also required for POD recruitment, we are not able to resolve whether POD recruitment is required for CBP activation. However, this seems unlikely, since coexpression of TDG with PML does not produce greater levels of CBP activation despite increasing POD localization of TDG

(data not shown). Accordingly, we believe that SUMO-1 binding activity per se, and not POD targeting, is essential for CBP activation. The presence of a sumoylation-dependent transcriptional repressor domain in CBP/p300 that recruits histone deacetylases (14, 24) suggests a plausible role of TDG binding in promoting derepression by displacement of histone deacetylases. However, a CBP mutant containing lysine-to-arginine substitutions at characterized sumoylation sites (24) was also robustly activated by TDG (Fig. S.2.5), indicating that CBP sumoylation is not essential.

Figure 2.8. Model for SUMO-1-dependent regulation of TDG subcellular localization and function. TDG associated with transcriptionally active euchromatin (46) initiates repair of G:T/U mispairs within CpG dinucleotides in a process that is likely linked to transcription. TDG-mediated repair may require CBP/p300 acetylase for local chromatin remodeling and/or regulation of repair enzymes via acetylation (3, 21, 46). Transcription has been detected in the periphery of PODs, and there is evidence for association of these structures with transcriptionally active genomic loci (5, 48). POD localization of TDG is dependent on its intrinsic SUMO binding activity and may be required to deliver this enzyme to transcriptionally active loci. For this purpose, TDG would require regulatory switches to control transit to PODs. Chromatin-associated TDG may not translocate to these nuclear structures due to DNA interactions that suppress SUMO binding activity. On the basis of our experimental findings, we propose that POD translocation is contingent upon uncoupling TDG from DNA, which may occur following base excision as a result of displacement by APE and/or sumoylation. Sumoylated TDG may not translocate to the PODs due to occlusion of the SBMs by intramolecular SUMO-1 interactions. In this case, desumoylation would be required to permit POD translocation. Within these structures, TDG may be posttranslationally modified by CBP, as previously demonstrated for p53 (38), and/or assembled into functional complexes for delivery to transcriptionally active genomic loci.

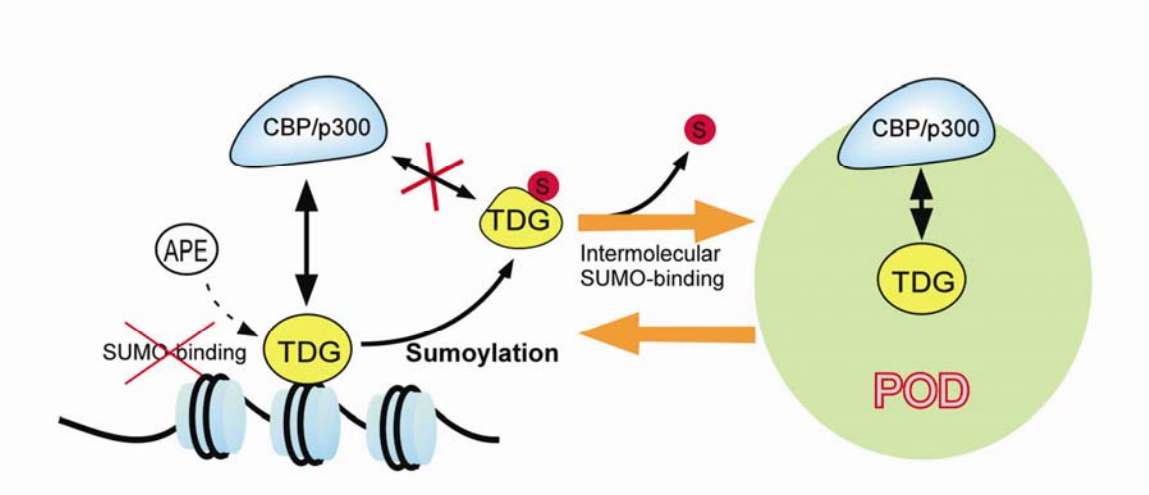
CBP/p300
S
TDG
APE
SUMO-binding
TDG
Sumoylation
S
Intermolecular
SUMO-binding
CBP/p300
TDG
POD

Figure 2.8

TDG sumoylation has been shown to abrogate DNA binding activity and has been proposed as a mechanism to promote the turnover of TDG from abasic sites following base excision (20). We have confirmed that sumoylated mouse TDG is also defective in DNA binding. Our studies suggest that TDG sumoylation also plays an important role in regulating POD translocation and protein-protein interactions. Recent structural studies of sumoylated human TDG have revealed important insights on the conformational changes resulting from this covalent modification (1, 20). In addition, partial proteolysis studies have shown that sumoylation induces conformational changes involving interactions between the amino- and carboxy-terminal regions of human TDG (44). We have now identified a conserved amino-terminal SBM that in concert with a carboxy-terminal SBM may account for the observed sumoylation-induced conformational changes. Indeed, binding of the amino- and carboxy-terminal domains to SUMO-1 likely interferes with the DNA binding functions associated with the amino terminus (residues 1 to 121) (44). We have examined the effect of sumoylation on interactions with CBP and intermolecular SUMO-1 recognition in mouse TDG. Remarkably, TDG sumoylation abrogates both CBP interaction as well as intermolecular SUMO binding (Fig. 2.7). Based on these observations, loss of intermolecular SUMO binding should prevent POD translocation. Corroborating evidence for a role of sumoylation in regulating POD translocation comes from the analysis of the sumoylation-deficient mutant GFP-K341R, which displays exclusive POD localization in a subpopulation of cells. In view of our biochemical studies, we believe that loss of negative regulation (i.e., sumoylation) dramatically favors POD translocation. In view of the documented interactions of CBP/p300 with the TDG amino terminus and the resulting acetylation of the lysine-rich regulatory domain (46), we cannot exclude a role for CBP/p300 and acetylation in regulating POD targeting. Nevertheless, CBP is not likely to be a direct intermediary in POD recruitment since, when coexpressed with PML and TDG, it did not consistently accumulate with TDG in the PODs (data not shown).

In conclusion, we have elucidated the roles of sumoylation and noncovalent SUMO-1 binding in regulating the subcellular localization and biochemical properties of TDG. Although the significance of POD localization remains to be established, our findings suggest a key role for these nuclear structures in regulating the functions of TDG in transcription and/or genome maintenance.

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2.6 Supplemental information

Figure S.2.1. SUMO-1 binding does not interfere with DNA binding. Recombinant full-length TDG (500 ng) was preincubated with 2 μg of GST or GST-SUMO-1 prior to binding to biotin tagged duplex oligonucleotides (500 ng) containing either no mispairs (G:C) or a G:T mispair. DNA-protein complexes were isolated using streptavidin sepharose and analyzed by immunoblotting with an anti-histidine antibody.

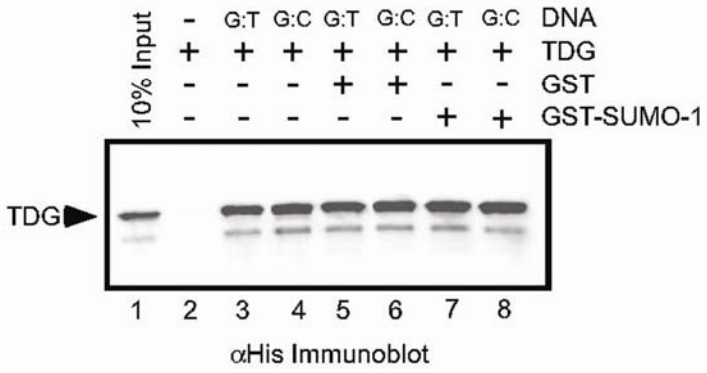
10% Input
-
G:T G:C G:T G:C G:T G:C DNA
+ + + + + + + TDG
- - - + + - - GST
- - - - - + + GST-SUMO-1
TDG
1 2 3 4 5 6 7 8
αHis Immunoblot

Figure S.2.1

Figure S.2.2. Analysis of DNA glycosylase activity of *in vitro* translated TDG and SUMO binding motif point mutants. The indicated *in vitro* translated proteins were incubated with radiolabelled duplex oligonucleotide containing a single G:T mispair. Excision of the mispaired thymine produces a 12-nucleotide fragment following alkali treatment and analysis by denaturing PAGE. The control reaction contained substrate only, while the reaction in lane 2 contained unprogrammed rabbit reticulocyte lysate. Phosphorimaging was performed on dried gels.

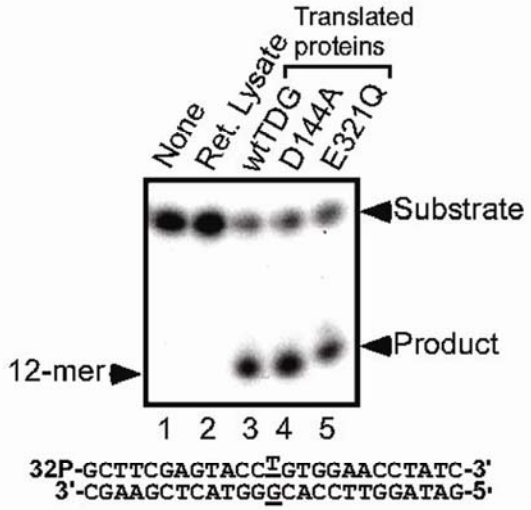
Translated proteins
None
Ret. Lysate
wtTDG
D144A
E321Q
Substrate
Product
12-mer
1 2 3 4 5
32P-GCTTCGAGTACCTGTGGAACCTATC-3'
3'-CGAAGCTCATGGGCACCTTGGATAG-5'

Figure S.2.2

Figure S.2.3. SUMO-1 binding activity of MCF-7 expressed TDG resides in amino- and carboxy-terminal motifs. The D144A, I147A and E321Q mutant proteins as well as wild-type TDG were expressed in MCF-7 cells and whole cell extracts were bound to GST-SUMO-1. Protein complexes were captured with glutathione sepharose, fractionated by SDS-PAGE and immunoblotted with a TDG-specific antibody.

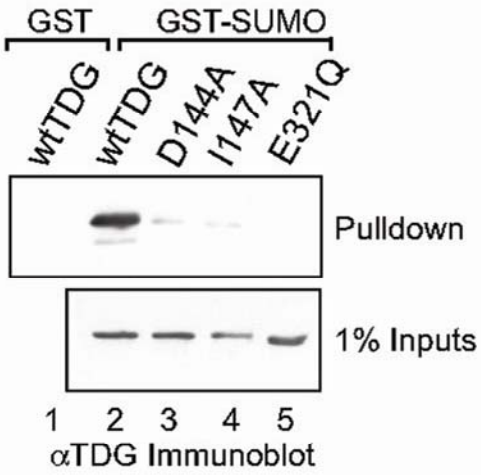
GST
GST-SUMO
wtTDG
wtTDG
D144A
I147A
E321Q
Pulldown
1% Inputs
1 2 3 4 5
αTDG Immunoblot

Figure S.2.3

Figure S.2.4. SUMO binding motif mutants bind CBP *in vitro*. Recombinant CBP (1 μg) was incubated with *in vitro* translated wild-type TDG, D144A and E321Q proteins. Protein complexes were isolated by immunoprecipitation with a CBP-specific antibody, fractionated by SDS-PAGE and visualized by autoradiography.

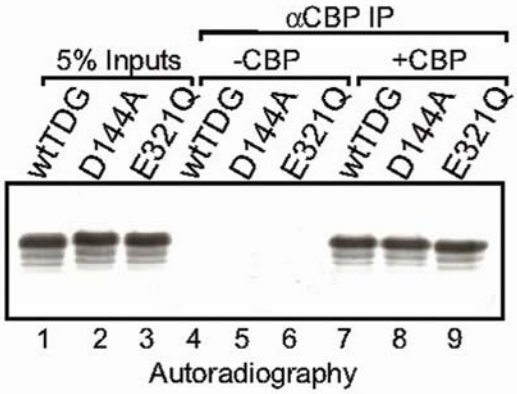
αCBP IP
5% Inputs
-CBP
+CBP
wtTDG
D144A
E321Q
wtTDG
D144A
E321Q
wtTDG
D144A
E321Q
1 2 3 4 5 6 7 8 9
Autoradiography

Figure S.2.4

Figure S.2.5. Sumoylation sites in CBP are not required for activation by TDG. Lysine residues in CBP previously shown to be sumoylated (K999, K1034, K1057) (24) were substituted with arginines. This mutant also contained an arginine substitution at K1087. GAL DNA-binding domain fusions of wild-type CBP (GAL-CBP) and the above mutant (GAL-CBP4KR) were analyzed for transcriptional activation upon coexpression with TDG using a reporter plasmid consisting of five copies of the GAL4 binding site fused to the core β-globin promoter.

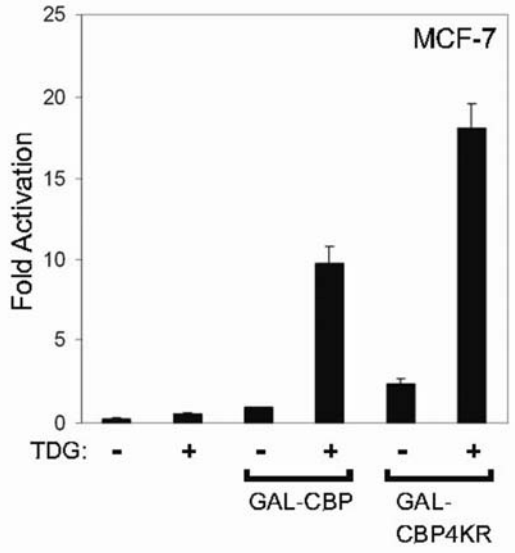

Figure S.2.5

Figure S.2.6. Sumoylated TDG possesses robust G:U processing activity. DNA glycosylase assays were performed by incubating proteins with radiolabelled duplex oligonucleotide containing a single G:U mispair. Excision of the mispaired uracil produces a 12-nucleotide fragment following alkali treatment and analysis by denaturing PAGE. The control reaction contained substrate only.

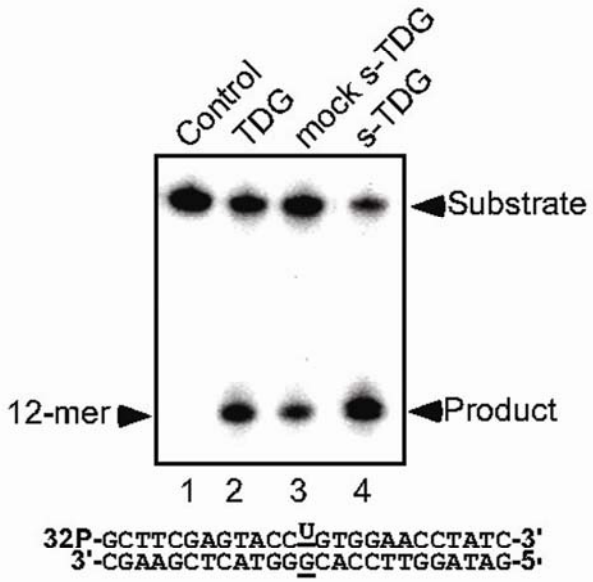
Control
TDG
mock s-TDG
s-TDG
Substrate
12-mer
Product
1 2 3 4
32P-GCTTCGAGTACCUGTGGAACCTATC-3'
3'-CGAAGCTCATGGGCACCTTGGATAG-5'

Figure S.2.6

Chapter 3: Opposing regulatory roles of phosphorylation and acetylation in DNA mispair processing by thymine DNA glycosylase

3.1 Introduction

Cytosine methylation in vertebrates is an important epigenetic mechanism regulating gene expression (24) that also contributes to CpG dinucleotide instability by promoting spontaneous base damage and increased susceptibility to endogenous and environmental mutagens (42). Hydrolytic deamination of cytosine and 5-methylcytosine respectively generates uracil (U) and thymine (T) moieties mispaired with guanine (G) (29). If these G:T/U mispairs remain unrepaired, they give rise to C to T transition mutations associated with oncogenic transformation and genetic diseases (43). In fact, approximately 25% of all somatic mutations in the p53 tumor suppressor gene in human tumors involve C to T transitions at CpG and in some tumors this figure rises to almost 50% (41).

Thymine DNA glycosylase (TDG) and methyl-CpG binding domain protein 4 (MBD4) mediate excision of mispaired thymine (G:T) and uracil (G:U) in the CpG context (20, 38, 39) and also process various modified pyrimidines (5). Excision of the aberrant base generates a cytotoxic abasic site that is subsequently processed by the coordinated action of other base excision repair (BER) enzymes, including apurinic/apyrimidinic endonuclease (APE) and DNA polymerase β (Polβ) (46). MBD4 localizes predominantly to transcriptionally inactive heterochromatin while TDG is mostly excluded from these regions and associates with sequence specific transcription factors and cofactors (2, 3, 30, 35, 50). These findings suggest that TDG is targeted to transcriptionally active regions of the genome and that BER may be coupled to transcription. Interestingly, overexpression of TDG was shown to reactivate a hormone regulated transgene silenced by CpG methylation, suggesting a role for TDG in epigenetic regulation (56). Recent studies have

demonstrated that recruitment of TDG, in concert with other BER enzymes and DNA methyltransferases 3a/b, to the promoter regions of estrogen-responsive genes is essential to establish cyclic methylation/demethylation patterns in transcriptionally active chromatin (22).

TDG contains a highly conserved central glycosylase domain flanked by divergent amino- and carboxy-terminal regions (12). The amino-terminal region of mammalian TDG contains a hydrophilic lysine-rich region (residues 70-118) that is acetylated by CREB-binding protein and p300 (CBP/p300) while the carboxy-terminal region is modified by covalent conjugation of small ubiquitin-like modifier (SUMO) protein (17, 50). The amino-terminal region is essential for non-specific DNA interactions, as well as tight binding to abasic sites and processing of G:T mispairs (12, 36, 48). The tight association of TDG to the abasic site following base excision prevents enzyme turnover thereby limiting mispair processing efficiency (48). TDG contains two separate SUMO-binding motifs located in the amino- and carboxy-terminal regions that mediate noncovalent binding to SUMO, which is required for translocation to PML oncogenic domains (PODs) (36).

CBP/p300 and TDG form ternary complexes with DNA *in vitro* that retain the ability to mediate base excision and histone acetylation, suggesting that the recruitment of CBP/p300 *in vivo* may promote chromatin remodeling at the site of repair (50). Furthermore, TDG potentiates CBP-dependent transcription by means of intrinsic SUMO-binding activity (36). Covalent SUMO conjugation to a carboxy-terminal lysine residue effectively abrogates DNA binding and association with CBP, while acetylation of the amino-terminal region may regulate interactions with accessory factors (36, 48, 50). The reported effects of CBP/p300-mediated acetylation on the activities of BER

enzymes (TDG, Polβ, flap endonuclease 1) (18, 19, 50), suggest an important role for CBP/p300 in coordinating BER.

In light of the critical role of the amino-terminus in G:T processing, we have undertaken to identify additional covalent modifications in this region with potential regulatory functions. Previous studies have shown that TDG is phosphorylated in living cells (52) and *in silico* analysis identified several putative protein kinase C (PKC) α/β/γ phosphorylation sites in the amino-terminal lysine-rich regulatory domain. PKC comprises a family of 11 related signaling proteins with different tissue distributions and cofactor requirements that participate in diverse cellular processes such as proliferation, apoptosis, and differentiation (16). PKC signaling is activated by oxidative stress (15) and there is evidence that Polβ and mismatch repair proteins Msh2 and Msh6 are regulated by PKC phosphorylation (4, 51). In the present study, we identify a novel mechanism of crosstalk between CBP and PKCα that regulates the DNA mispair processing functions of TDG through mutually exclusive covalent modification of the amino-terminal region. We demonstrate that acetylation of lysine residues not directly involved in DNA binding, selectively and potently abrogates G:T processing whereas phosphorylation of adjacent serine residues by PKCα may preserve this function by preventing CBP-mediated acetylation. These findings highlight the importance of covalent modifications in regulating a DNA repair enzyme integral to the maintenance of CpG dinucleotides and epigenetic regulation.

3.2 Materials and Methods

Plasmids

pCMX-based mammalian expression vectors for FLAG epitope tagged TDG have been previously described (50). Amino acid substitution mutants of TDG were constructed using the QuikChange mutagenesis kit (Stratagene) according to the manufacturer's directions and were verified by sequencing. Other expression vectors have been previously described (6, 36, 50).

Antibodies and peptides

TDG-specific rabbit polyclonal antibody has been previously described (36). Human PKCα-specific polyclonal antibodies (sc-208, or sc-208-G) were obtained from Santa Cruz Biotechnology. Anti-polyhistidine (HS1) and anti-FLAG monoclonal antibodies (M2) were obtained from Sigma-Aldrich. TDG peptides obtained from GenScript Corporation were analyzed by mass spectrometry and quantified by reverse phase HPLC.

Cell culture, and metabolic labeling

NIH 3T3 fibroblasts were maintained in Dulbecco's minimal essential medium (D-MEM) containing penicillin/streptomycin and supplemented with 10% fetal calf serum. For subcellular localization experiments, cells were grown under serum-free conditions for 4 hours and then treated with either 100 nM PMA or vehicle (DMSO) for 15 minutes in serum containing media. For metabolic labeling experiments, 150 mm dishes of NIH 3T3 or HEK 293T cells were transfected with TDG expression vectors using the Polyfect transfection reagent (Qiagen). Approximately 24 hours later, the culture medium was replaced with serum and phosphate-free D-MEM and the cells were incubated at 37° C

for 2 hours. Subsequently, ^{32}P-orthophosphate (200 μCi/ml) was added to the media followed by a 2.5-hour incubation at 37° C which included treatment with PMA or vehicle (DMSO) during the final 30 minutes. Cells were then washed with PBS and whole cell extracts were prepared for immunoprecipitation as described below. Immunoprecipitates were separated by SDS-PAGE and incorporation of ^{32}P was detected by phosphorimaging. Equal loading of TDG was verified by immunoblotting.

Preparation of whole cell extracts and immunoprecipitation

Whole cell extracts for immunoprecipitation were prepared from 100 mm dishes of NIH 3T3 cells stably expressing FLAG-TDG and control cells transduced with the empty expression vector (pLNCX). Cells grown to 80% confluency were harvested by scraping, then pelleted by centrifugation and resuspended in 500 μl of Lysis buffer (50 mM Tris HCl pH 7.9, 300 mM NaCl, 1 mM EDTA, 1mM EGTA 10% glycerol, 0.5% Triton X-100, 1 mM DTT, 50 mM sodium fluoride, 200 μM sodium orthovanadate and proteinase inhibitors - 20 μg/mL Pepstatin A, 10 μg/ml Aprotinin, 1 μg/ml Leupeptin, 0.5 mM PMSF) and incubated on ice for 30 minutes. Subsequently, the cell lysate was diluted with 500 μl of dilution buffer (50 mM Tris-HCl pH 7.9, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1 mM dithiothreitol (DTT), 50 mM sodium fluoride, 200 μM sodium orthovanadate and proteinase inhibitors) and insoluble material removed by centrifugation. Whole cell extracts were precleared twice with 50 μl (50% v/v) rabbit IgG-agarose (Sigma-Aldrich) for 30 minutes at 4° C and immunoprecipitated with 50 μl (50% v/v) anti-FLAG affinity resin (M2 agarose, Sigma-Aldrich). Immunoprecipitated proteins were separated by SDS-PAGE and detected by immunoblotting. In the case of immunoprecipitation of radiolabelled *in vivo* phosphorylated TDG, detection following SDS-PAGE was carried out by phosphorimaging and equal loading of TDG was verified by immunoblotting.

2D-PAGE

Cell lysates and 2D-PAGE analysis was performed as previously described (7). Cells were grown to 80-90% confluency in 150 mm dishes and treated with PMA as described above. Approximately, 150 μg of cell lysate was fractionated by isoelectric focusing using a 7 cm Immobiline DryStrip gel (pH 6.2-7.5 – GE Healthcare). Subsequently, proteins on the Immobiline Dry strip were fractionated by SDS-PAGE and TDG was detected by immunoblotting.

Protein purification and *in vitro* interaction assays

Protein purification, GST-based interaction assays, nickel-based pull-downs, and immunoprecipitation procedures have been previously described (36, 50).

Base excision and electrophoretic mobility shift assays

Base excision assays and electrophoretic mobility shift assays (EMSA) were performed as previously described using identical oligonucleotide substrates (36). Either 25 or 12 ng of recombinant TDG was incubated at 30° C with 10 ng of radiolabeled duplex oligonucleotide (25 base pairs) containing a single G:U or G:T mispair in 20 μl of buffer (25 mM HEPES-KOH [pH 7.8], 1 mM EDTA, 0.1 mg/ml BSA, and 1 mM DTT). Reactions were carried out for 30 minutes followed by the addition of 2.5 μl 50% glycerol and 10 μl of the reaction was used directly for EMSA. EMSA samples were fractionated using 6% polyacrylamide gels cast in 0.25xTBE and pre-run for 1.5 hours in 0.5xTBE. Protein-DNA complexes were detected by autoradiography or phosphorimaging. The remainder of the reaction was treated with alkali to cleave the abasic sites generated by base release and subsequently the samples were analyzed by denaturing polyacrylamide electrophoresis as described by Hardeland et al (2002).

Avidin-biotin coupled DNA binding (ABCD) assays

The ABCD assay was performed as described in Tini et al (2002) using identical biotinylated duplex oligonucleotides. Briefly, streptavidin-coated paramagnetic beads (MagneSphere, Promega) were prepared by washing 0.6 ml (1 mg/ml) with ABCD assay buffer (50 mM Tris-HCl (pH 7.9), 150 mM NaCl, 10% glycerol, 5 mM MgCl2, 0.1% NP40, and 0.5 mM DTT) and resuspending in a final volume of 200 µl. Approximately, 250 to 500 ng of annealed oligonucleotide was incubated for 30 minutes at room temperature with 10 µl of the streptavidin-coated paramagnetic bead slurry and 100-500 ng of purified bacterially expressed TDG in a total volume of 50 µl of ABCD buffer. Following incubation, beads were washed five times with 200 µl of ABCD buffer, and bound proteins were recovered by resuspending in 12 µl Laemmli buffer and incubation at 90° C for 5 minutes. Proteins were fractionated by SDS-PAGE and detected by immunoblotting with an anti-polyhistidine antibody. In experiments addressing the displacement of TDG from abasic sites, we first incubated TDG with DNA at room temperature for 10 minutes and subsequently GST-APE was added with a further 30-minute incubation.

Preparation of acetylated and phosphorylated TDG for biochemical studies

Acetylated TDG was prepared by incubating recombinant TDG (750 ng) with 1 µg recombinant CBP and 1.25 mM acetyl coenzyme A (AcCoA) in acetylation buffer (20 mM HEPES [pH 7.8], 1 mM EDTA, 1mM DTT, 10 mM sodium butyrate, and 10% glycerol) at 30° C for 90 minutes. Subsequently, acetylation reactions were dialyzed for 60 minutes against Nickel Binding Buffer (NiBB) (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 5% glycerol, 0.1% NP40, supplemented with 200 µM sodium orthovanadate, 50 mM sodium fluoride, and 10 mM sodium butyrate). NiBB (500 µl) and 60 µl (50% v/v)

Ni-NTA agarose were then added to the dialyzed reactions and they were incubated at 4° C for 90 minutes. The Ni-ATA agarose was then washed three times with 200 µl NiBB and TDG was eluted 3 times with 50 µl of NiBB containing 1 M imidazole. Eluted TDG was then dialyzed against NETN (50 mM Tris [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.1% NP-40, 10% Glycerol, and 1 mM DTT) for 60 minutes. The final concentration of TDG was determined by immunoblotting. Phosphorylated TDG was prepared by incubating recombinant TDG (750 ng) with 50 mU recombinant PKCα (Calbiochem) and 1 mM ATP in 50 µl PKC phosphorylation buffer (20 mM HEPES, pH 7.2, 1 mM sodium orthovanadate, 25 mM glycerol-3-phosphate, 1 mM dithiothreitol, 1 mM CaCl2, 15 mM $MgCl_2$, 10 mg/ml 1,2-dioleyl-sn-glycerol, 100 mg/ml phosphatidylserine) for 90 minutes at 30° C. Mock reactions were performed in the absence of ATP. Subsequently, TDG was purified as described above.

Protein acetylation and phosphorylation assays

Purified phosphorylated or mock-phosphorylated TDG (100 ng) was incubated with approximately 100 ng of purified, recombinant CBP in 30 µL of acetylation buffer in the presence of 1.5 µM ^{14}C acetyl CoA (AcCoA) and incubated for 30 minutes at 30° C prior to stopping of the reaction by addition of 30 µl of Laemmli buffer and incubation at 95° C for 5 minutes. Reaction components were then separated by SDS-PAGE. After separation, the gel was fixed in a 30% methanol, 10% acetic acid solution and treated with amplifying solution (Amersham) prior to imaging by autoradiography or phosphorimaging. Mock-acetylated or acetylated TDG were incubated with 0.25 mU of recombinant PKCα in phosphorylation buffer with 1 µl $\gamma^{32}P$-ATP. Reactions were incubated at 30° C for 30 minutes then stopped by addition of Laemmli buffer and incubation at 95° C for 5 minutes. Reaction products were then separated by SDS-PAGE. The gel was fixed with a 30% methanol, 10% acetic acid solution and ^{32}P incorporation

was detected by autoradiography. To examine the effect of DNA on acetylation or phosphorylation, we preincubated TDG with DNA for 30 minutes on ice before proceeding with the reaction.

Immunostaining and microscopy

NIH 3T3 cells were fixed for 15 minutes with 4% formaldehyde in phosphate buffered saline (PBS) followed by a 10 minute incubation with 0.1 M glycine in PBS. Cells were then permeabilized with 0.5% Triton X-100 in PBS for 10 minutes. Immunostaining was performed with the appropriate primary antibodies and fluorophore-conjugated Donkey secondary antibodies (CY3, FITC) (Jackson ImmunoResearch Laboratories). Epifluorescence imaging was performed on an Axiovert 200M inverted microscope equipped with an Apotome (Carl Zeiss) using appropriate fluorophore-specific filter sets. Z-series images (63X magnification) were acquired at 0.5 μm intervals and processed with Axiovision software and Adobe Photoshop. Fluorescence intensity plots were obtained by performing a line scan bisecting the cell using Axiovision software.

3.3 Results

Phorbol ester-stimulated phosphorylation of TDG in living cells

We have previously shown that TDG is acetylated by CBP/p300 at four lysine residues located within an amino-terminal region essential for DNA binding and G:T processing (Fig. 3.1A) (36, 48, 50). *In silico* analysis using the Scansite algorithm (40) revealed several potential protein kinase C (PKC) α/β/γ phosphoacceptor residues flanking acetyl-acceptor lysines, suggesting possible functional interplay between PKC and CBP/p300 signaling in TDG regulation. These residues are located within a short sequence motif (^{93}SKKSGKS^{99}) that is conserved in mouse, rat and human TDG. We initially investigated whether endogenous TDG is phosphorylated in living cells in response to treatment with PKC agonist phorbol 12-myristate 13-acetate (PMA); serum-starved NIH 3T3 mouse fibroblasts were treated for 15 minutes and whole-cell lysates were subjected to 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Fractionated cellular proteins were immunoblotted with TDG specific antibodies. Theoretically, each phosphorylation event leads to a 78 Dalton gain in molecular weight and a 0.11-0.14 decrease in isoelectric point. We observed increases in apparent molecular weight and discrete changes in isoelectric point in response to PMA treatment consistent with phosphorylation of TDG *in vivo* (Fig. 3.1B). We next verified that TDG is phosphorylated in living cells by metabolic labeling of NIH 3T3 cells transiently transfected with FLAG epitope tagged TDG. Cells grown under serum free conditions were labeled with ^{32}P inorganic phosphate for 2.5 hours which included a 30 minute treatment with either PMA or DMSO. TDG was immunoprecipitated from cell lysates

Figure 3.1. Phorbol ester-stimulated phosphorylation of TDG in living cells. (A) Illustration of the functional domains of mouse TDG and sites of posttranslational modification. The central conserved glycosylase domain is sufficient for processing of G:U mispairs while a more divergent amino-terminal extension is required for tight DNA binding and G:T processing (36, 48). Two SUMO binding motifs (SBM1, SBM2) and the sumoylation site (K341) are shown. A lysine-rich regulatory region located in the amino-terminus is acetylated by CBP/p300 at four distinct lysines (K70, K94, K95, K98) (50). Putative protein kinase C (PKC) α/β/γ phosphorylation sites (consensus [S/T-X-[R/K]) within a sequence (boxed) conserved in mouse, rat and human TDG, are indicated by asterisks. Complete sequence alignments and accession numbers are found in Figure S.3.1. (B) 2D-PAGE analysis of cellular TDG demonstrating PMA-dependent alterations in apparent molecular weight and isoelectric point (pI). Cell lysates were prepared from NIH 3T3 cells stimulated with PMA and then separated by 2D-PAGE. TDG was detected by immunoblotting with a TDG-specific antibody. (C) *In vivo* metabolic labeling of transiently expressed TDG with ^{32}P-orthophosphate. Transfected NIH 3T3 fibroblasts were grown in serum-free media and metabolically labeled with or without PMA treatment. One population of transfected cells was pretreated with a PKCα/β inhibitor (Gö6976, 100 nM) prior to PMA stimulation. Immunoprecipitated TDG was fractionated by SDS-PAGE and analyzed by phosphorimaging (upper panel) and immunoblotting (lower panel).

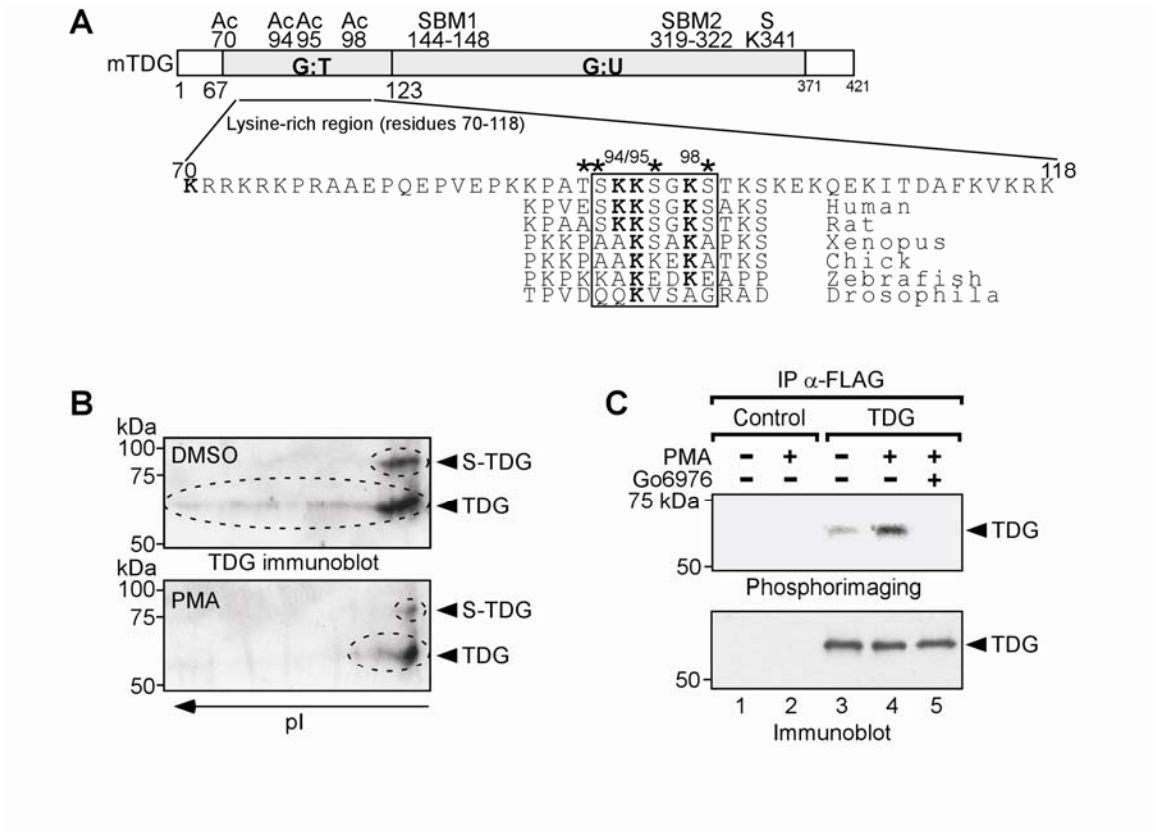
A
Ac 70
Ac 94
Ac 95
Ac 98
SBM1 144-148
SBM2 319-322
S K341
mTDG
G:T
G:U
1 67
123
371 421
Lysine-rich region (residues 70-118)
70
118
94/95
98
KRRKRKPRAAEPQEPVEPKKPATSKKSGKSTKSKEKQEKITDAFKVKRK
KPVESKKSGKSAKS Human
KPAASKKSGKSTKS Rat
PKKPAAKSAKAPKS Xenopus
PKKPAAKKEKATKS Chick
PKPKKAKEDKEAPP Zebrafish
TPVDQQKVSAGRAD Drosophila
B
kDa
100
75
50
DMSO
S-TDG
TDG
TDG immunoblot
PMA
pI
C
IP α-FLAG
Control
TDG
PMA
Go6976
75 kDa
Phosphorimaging
Immunoblot
1 2 3 4 5

Figure 3.1

with anti-FLAG resin and quantified by immunoblotting. Subsequently, comparable levels of TDG were analyzed by SDS-PAGE and incorporation of ^{32}P was detected by phosphorimaging. We observed a basal level of phosphorylation which was enhanced by PMA treatment (Fig. 3.1C, top panel). Pretreatment of cells with a PKCα/β specific inhibitor (Gö6976) (33) prior to PMA stimulation abolished phosphorylation. Since the ubiquitous expression of PKCα (53) is more consistent with the wide tissue distribution of TDG, we focused our investigations on this isozyme. To obtain corroborating evidence for a link between PKCα and TDG, we examined the subcellular distribution of the endogenous proteins in NIH 3T3 fibroblasts and undifferentiated P19 embryonal carcinoma cells by indirect immunofluorescence. In NIH 3T3 cells cultured in the absence of serum, PKCα displayed predominantly cytoplasmic distribution while TDG was found almost exclusively in the nucleus (Fig. 3.2A panels I, III, V). Treatment with PMA for fifteen minutes in the presence of serum, triggered nuclear translocation of PKCα as previously reported (47), and colocalization with TDG (Fig. 3.2C, panel II, IV, VI). We observed mainly nuclear staining for PKCα (54) in untreated P19 cells and strong colocalization with TDG (Fig. 3.2B). These findings are consistent with numerous studies demonstrating phosphorylation of nuclear proteins by PKCα (32).

Figure 3.2. Subcellular localization of TDG and PKCα in NIH 3T3 fibroblast and P19 EC cells. (A) Subcellular localization of endogenous TDG and PKCα in NIH 3T3 cells. Serum starved cells were treated with PMA or DMSO and subsequently immunostained for TDG and PKCα. (B) Undifferentiated P19 embryonic carcinoma cells (not treated) were immunostained to detect endogenous TDG and PKCα. Representative optical sections generated by epifluorescence microscopy are shown. The fluorescence intensity plot illustrates the coincidence of peak fluorescence for TDG (CY3, red) and PKCα (FITC, green).

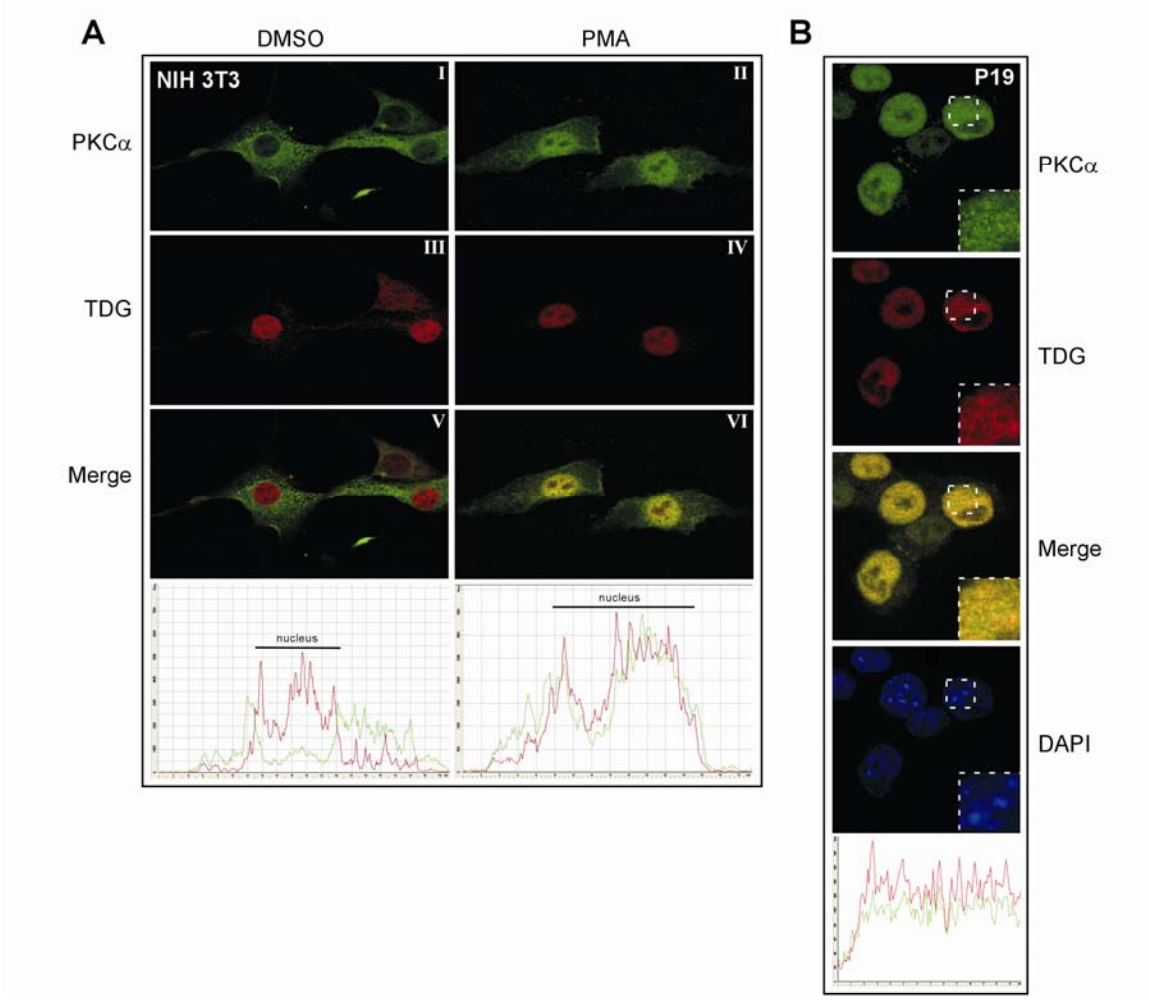
A
DMSO
PMA
NIH 3T3
I
II
III
IV
V
VI
PKCα
TDG
Merge
nucleus
nucleus
B
P19
PKCα
TDG
Merge
DAPI

Figure 3.2

PKCα interacts directly with TDG and phosphorylates the amino-terminal region

To determine whether TDG and PKCα associate in living cells we carried out immunoprecipitations with anti-FLAG affinity resin on cell lysates derived from PMA treated NIH 3T3 fibroblasts stably expressing FLAG-tagged TDG. The amount of full length FLAG-tagged TDG in these cells is comparable to that of endogenous TDG (Fig. 3.3A, compare lanes 1 and 2). Immunoprecipitates were separated by SDS-PAGE and immunoblotted for TDG and PKCα. PKCα was only detected in immunoprecipitates derived from FLAG-TDG expressing cells but not from control cells, consistent with the association of these proteins in living cells (Fig. 3.3A, compare lanes 3 and 4). Similar results were obtained using transiently expressed epitope tagged proteins (Fig. S.3.2). To examine whether these proteins interact directly, we carried out *in vitro* interaction studies using commercially available recombinant PKCα and poly-histidine tagged TDG or a truncated variant lacking the amino-terminal region (residues 1 to 121) (Fig. 3.3B) Following incubation of these proteins, nickel affinity resin was used to pull down TDG and PKCα was detected by immunoblotting (Fig. 3.3C). PKCα was retained on the nickel affinity resin in the presence of full-length TDG but not with the amino terminal variant. Coomassie staining of an aliquot of the binding reaction confirmed that both TDG and TDG(122-421) were bound to the affinity resin. These findings establish a direct interaction between TDG and PKCα that is dependent on the amino-terminal region of TDG.

Figure 3.3. PKCα associates directly with TDG. (A) PKCα coimmunoprecipitates with stably expressed TDG in NIH 3T3 fibroblasts. Immunoprecipitations using anti-FLAG affinity resin were carried out on whole-cell extracts prepared from PMA treated cells stably expressing FLAG- TDG or control cells transduced with the empty expression vector. Aliquots of the cell lysates and immunoprecipitated proteins were immunoblotted for PKCα and TDG. (B) Coomassie staining of polyhistidine tagged TDG and TDG(122-421) used for *in vitro* protein interaction studies. (C) *In vitro* association of recombinant TDG and PKCα requires amino-terminal residues 1 to 121 of TDG. Approximately 1 μg polyhistidine-tagged TDG or TDG(121-421) were incubated with 10 ng of PKCα and subjected to pull-down with nickel-affinity resin. As a control, PKCα was also incubated with beads alone. Bound proteins were subjected to SDS-PAGE and immunoblotting with a PKCα-specific antibody (upper panel). Binding of poly-histidine tagged TDG and TDG(122-421) to the nickel-affinity beads was confirmed by Coomassie staining (lower panel).

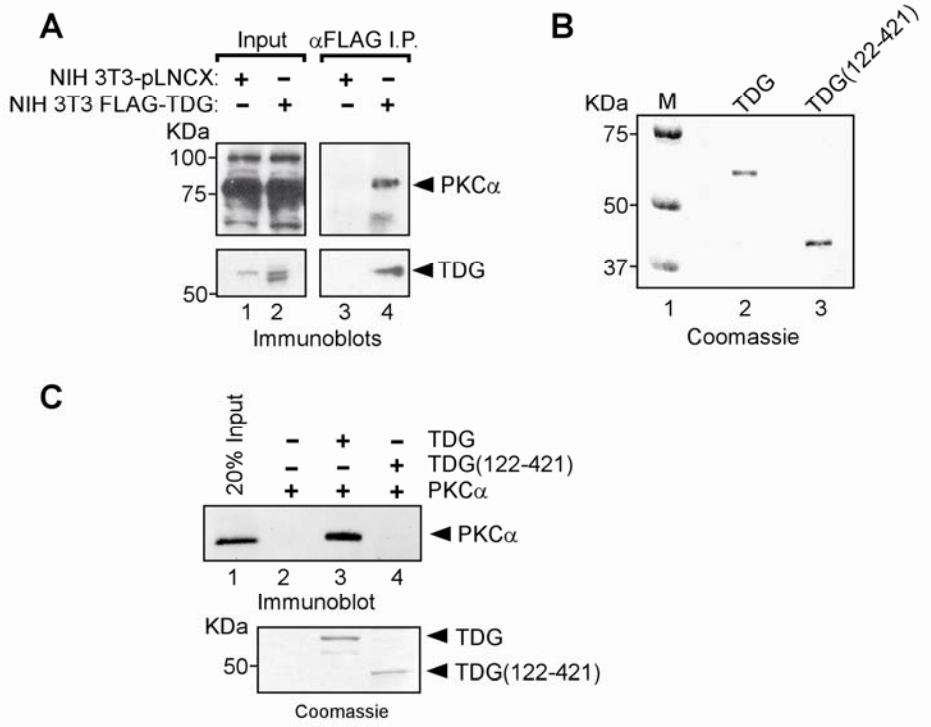
A
Input
αFLAG I.P.
NIH 3T3-pLNCX: + − + −
NIH 3T3 FLAG-TDG: − + − +
KDa
100
75
50
PKCα
TDG
1 2 3 4
Immunoblots
B
KDa
M
TDG
TDG(122-421)
75
50
37
1 2 3
Coomassie
C
20% Input
TDG
TDG(122-421)
PKCα
PKCα
1 2 3 4
Immunoblot
KDa
50
TDG
TDG(122-421)
Coomassie

Figure 3.3

We next assessed whether TDG is a direct substrate for PKCα phosphorylation *in vitro*, by incubating recombinant TDG or TDG(122-421) and PKCα in the presence of γ^{32}P-ATP and essential cofactors; subsequently, the reaction products were fractionated by SDS-PAGE and incorporation of ^{32}P was measured by phosphorimaging (Fig. 3.4A). Incorporation of radioactivity was observed only with full-length TDG, indicating that the amino-terminal region is essential for phosphorylation by PKCα. Considering that PKCα/β/γ consensus phosphorylation sites are located adjacent to acetyl-acceptor lysines 94, 95 and 98 (see Fig. 3.1A), we employed two short peptides consisting of amino acid residues 68-91 and 91-107 in phosphorylation reactions to further delineate the location of phosphoacceptor serines or threonines. Analysis of the reaction products by SDS-PAGE and phosphorimaging revealed that the peptide containing residues 91-107 was robustly phosphorylated *in vitro* while the second peptide (residues 68-91) was not appreciably radiolabelled (Fig. 3.4B). Phosphorylated residues were identified by examining the effect of alanine substitutions of potential phosphoacceptor residues in the context of the 91-107 peptide. This analysis indicated that substitution of serine 96 (S96) and serine 99 (S99) substantially decreased ^{32}P incorporation, thereby identifying these residues as the principal phosphoacceptor sites (Fig. 3.4C). In the context of full length bacterially-expressed TDG, dual substitution of these residues with either alanine (S96-99A) or aspartate (S96-99D) resulted in approximately 60% and 80% reduction in phosphorylation, respectively (Fig. 3.4D). To assess whether these serines are phosphorylated *in vivo*, we transiently expressed FLAG-tagged wild-type TDG and TDG(S96-99D) in NIH 3T3 fibroblasts and carried out metabolic labeling with radiolabelled inorganic phosphate with and without PMA stimulation. We observed that PMA treatment increased phosphorylation of wild-type TDG by approximately 80% but had no detectable effects on the S96-99D mutant (Fig. 3.4E, upper panel) confirming that these are the major phosphoacceptor sites. Immunoblotting analysis indicated the

presence of comparable amounts of TDG in the different immunoprecipitates (Fig. 3.4E, lower panel). These data confirm that phosphorylation of serine 96 and 99 of TDG is induced by phorbol ester stimulation in living cells.

Figure 3.4. PKCα phosphorylates TDG on serines 96 and 99. (A) PKCα-mediated phosphorylation of TDG requires the amino-terminal region. *In vitro* phosphorylation reactions were performed in the presence of γ^{32}P-ATP using 2 μg of TDG (lane 1) or TDG(122-421) (lane 2) and 0.3 ng (0.25 mU) of recombinant PKCα. Reaction products were fractionated by SDS-PAGE and incorporation of ^{32}P was detected by phosphorimaging. (B) Delineation of the phosphorylated region using peptide probes. Equimolar amounts of TDG peptides (residues 68-91 and 91-107) and PKCα peptide substrate from glycogen synthetase (residues 1-8, designated GS 1-8) along with the FLAG peptide were reacted with PKCα and analyzed as indicated above. (C) Identification of phosphoacceptor residues by alanine substitution. *In vitro* phosphorylation of the TDG(91-107) (1 μg) peptide and alanine substituted derivatives was performed and analyzed by SDS-PAGE and phosphorimaging (top panel). Quantification of signal intensity is displayed in the bottom panel. Lysines acetylated by CBP/p300 are indicated with asterisks. (D) Dual alanine (lane 2) or aspartate (lane 3) substitutions of serine 96 and 99 reduces PKCα-mediated phosphorylation in full-length TDG. Recombinant TDG and the indicated substitution mutants (2 μg) were phosphorylated *in vitro* and analyzed by SDS-PAGE and autoradiography. (E) *In vivo* metabolic labeling of transiently expressed TDG and S96-99D mutant with ^{32}P-orthophosphate. Transfected NIH 3T3 fibroblasts grown in serum-free media were metabolically labeled for 2.5 hours which includes treatment with either vehicle or PMA during the final 30 minutes of labeling. Proteins were immunoprecipitated with anti-FLAG resin and then fractionated by SDS-PAGE and phosphorimaged (upper panel). Aliquots of the immunoprecipitates were analyzed by immunoblotting to ensure equal loading of TDG (lower panel).

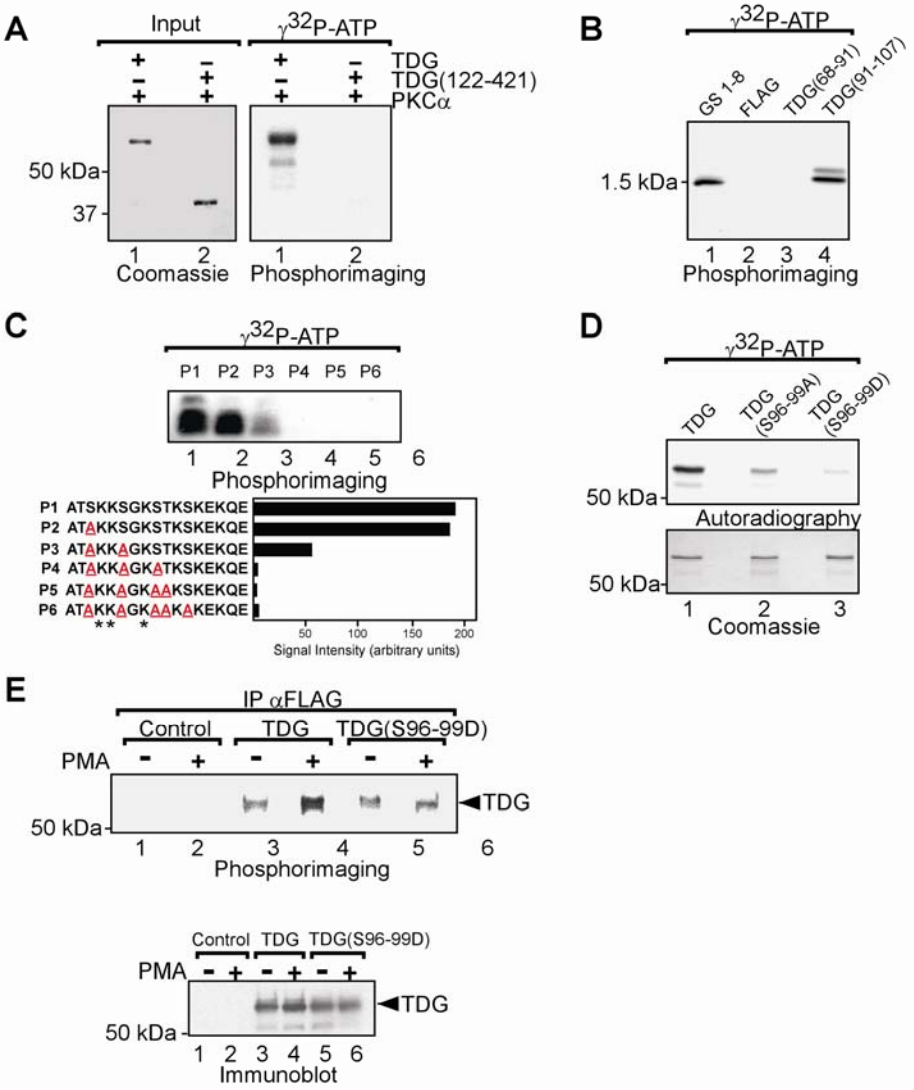
A
Input
γ32P-ATP
TDG
TDG(122-421)
PKCα
50 kDa
37
1 2 1 2
Coomassie
Phosphorimaging
B
γ32P-ATP
GS 1-8
FLAG
TDG(68-91)
TDG(91-107)
1.5 kDa
1 2 3 4
Phosphorimaging
C
γ32P-ATP
P1 P2 P3 P4 P5 P6
1 2 3 4 5 6
Phosphorimaging
P1 ATSKKSGKSTKSKEKQE
P2 ATAKKSGKSTKSKEKQE
P3 ATAKKAGKSTKSKEKQE
P4 ATAKKAGKATKSKEKQE
P5 ATAKKAGKAAKSKEKQE
P6 ATAKKAGKAAKAKEKQE
50 100 150 200
Signal Intensity (arbitrary units)
D
γ32P-ATP
TDG
TDG (S96-99A)
TDG (S96-99D)
50 kDa
Autoradiography
50 kDa
1 2 3
Coomassie
E
IP αFLAG
Control
TDG
TDG(S96-99D)
PMA
TDG
50 kDa
1 2 3 4 5 6
Phosphorimaging
Control
TDG
TDG(S96-99D)
PMA
TDG
50 kDa
1 2 3 4 5 6
Immunoblot

Figure 3.4

Amino-terminal acetylation and phosphorylation are mutually exclusive.

The proximity of the acetylated residues to the PKCα phosphorylation sites (see Fig. 3.1A) suggested that each modification may affect the other by altering the effective charge in this region. To investigate this, we established an assay whereby we initially acetylated or phosphorylated recombinant TDG *in vitro* and then used nickel-affinity chromatography to purify TDG from the modifying enzymes. Following quantification by immunoblotting, we assessed whether acetylated TDG could be phosphorylated by PKCα and vice versa. We found that acetylated TDG was not appreciably phosphorylated by PKCα (Fig. 3.5A) and similarly, when phosphorylated TDG was used as a substrate for CBP, acetylation was greatly reduced (Fig. 3.5B). Quantification by phosphorimaging revealed a 10-fold and 3-fold reduction in phosphorylation and acetylation, respectively. The more moderate decrease in acetylation following phosphorylation is consistent with the presence of an additional acetyl-acceptor lysine (K70) which may not be affected by phosphorylation. To determine whether loss of positive charges at acetyl-acceptor lysines was responsible for inhibition of phosphorylation we introduced charge neutralizing alanine substitutions at lysines 94, 95 and 98 (K94-95-98A triple mutant), which constitute the major acetylation sites *in vitro* and carried out *in vitro* phosphorylation (Fig. 3.5C). Reduced levels of phosphorylation were observed with the mutant consistent with lysine residues being essential for optimal phosphorylation by PKCα. Serine to aspartate substitutions (S96 and S99) which mimic phosphorylation also reduced acetylation consistent with the mutually exclusive relationship (Fig. 3.5D). We next examined the relationship between these modifications in living cells by carrying out *in vivo* phosphorylation experiments in HEK 293T cells. This cell line was chosen due to the high transfection efficiency that can be routinely achieved and the fact that TDG acetylation can be readily observed by metabolic labeling (data not shown). As with NIH 3T3 fibroblasts, we observed PMA-dependent

phosphorylation of TDG which could be attenuated by coexpression of CBP (60% reduction) (Fig 3.5E). Furthermore, the TDG mutant with alanine substitutions at acetyl-acceptor lysines was not phosphorylated in a PMA-dependent manner. These findings are in agreement with the *in vitro* data and consistent with a mutually exclusive relationship between acetylation by CBP/p300 and PKC mediated phosphorylation. When we performed *in vivo* acetylation by metabolic labeling with ^{3}H sodium acetate, we found surprisingly that both the S96-99D mutant and wild-type TDG were acetylated at comparable levels (data not shown). These findings are consistent with our unpublished data indicating that TDG is acetylated by other acetylases in addition to CBP/p300,

Figure 3.5. Acetylation and phosphorylation are mutually exclusive. (A) Acetylated TDG (acTDG) is refractory to phosphorylation by PKCα. Recombinant polyhistidine-tagged TDG was acetylated *in vitro* with CBP and then purified by nickel affinity chromatography. Acetylated TDG was quantified by immunoblotting and approximately 100 ng was used in phosphorylation reactions that included γ^{32}P-ATP. Reaction products were analyzed by SDS-PAGE and phosphorimaging. (B) Phosphorylated TDG (pTDG) is refractory to acetylation by CBP. Phosphorylated TDG was purified as indicated above and reacted with CBP and in the presence of ^{14}C-acetyl coenzyme A (AcCoA). (C) Reduced *in vitro* phosphorylation of the K94-95-98A mutant. (D) Reduced *in vitro* acetylation of the S96-99D mutant. Recombinant proteins (1 μg) were phosphorylated or acetylated *in vitro* as described above. (E) Inhibition of *in vivo* TDG phosphorylation by coexpression of CBP or substitution of positively charged acetyl-acceptor lysines. HEK 293T cells were transfected with the indicated expression vector and metabolically labeled with and without stimulation with PMA.

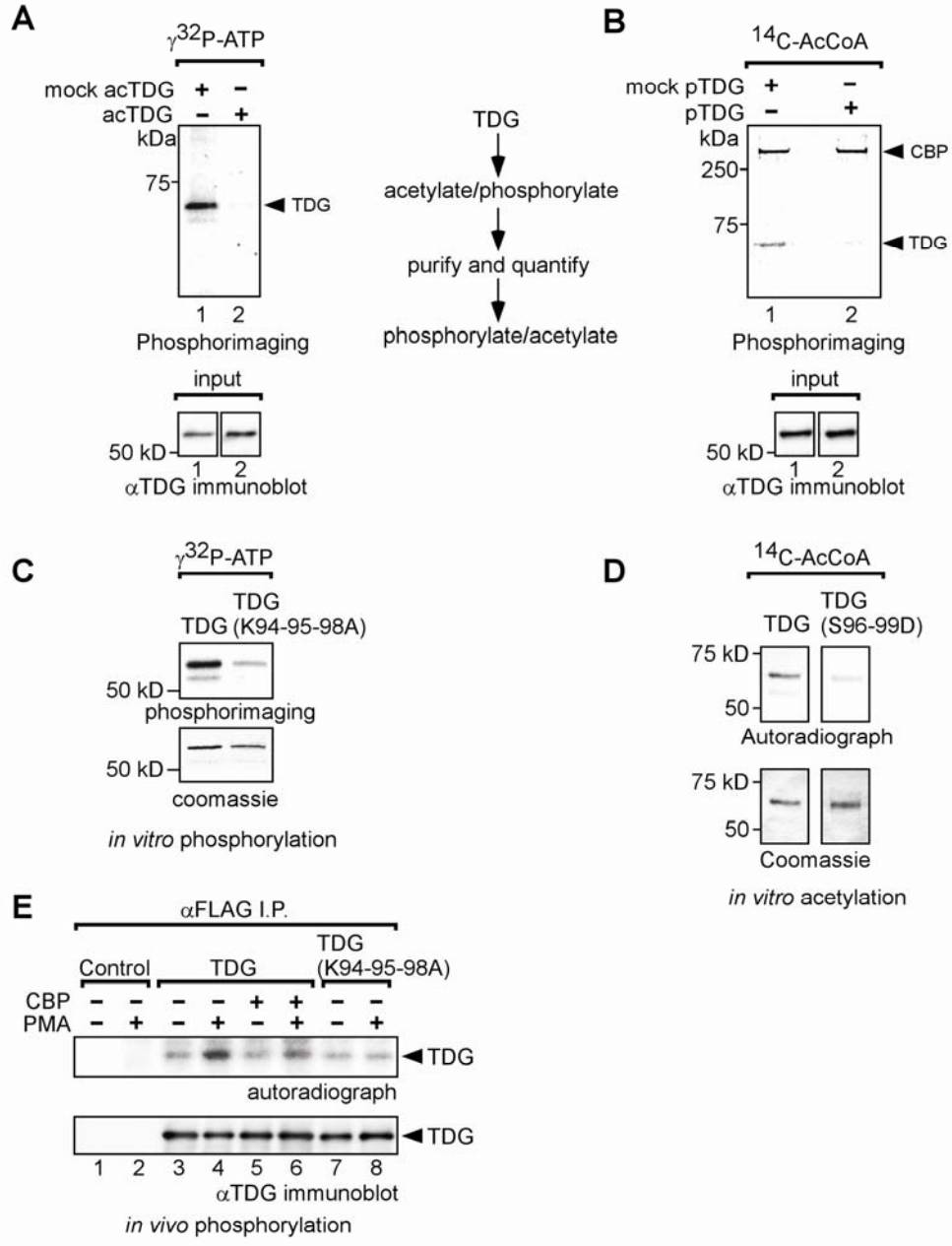
A
γ32P-ATP
mock acTDG + −
acTDG − +
kDa
75
TDG
1 2
Phosphorimaging
input
50 kD
1 2
αTDG immunoblot
TDG
acetylate/phosphorylate
purify and quantify
phosphorylate/acetylate
B
14C-AcCoA
mock pTDG + −
pTDG − +
kDa
250
75
CBP
TDG
1 2
Phosphorimaging
input
50 kD
1 2
αTDG immunoblot
C
γ32P-ATP
TDG
TDG (K94-95-98A)
50 kD
phosphorimaging
50 kD
coomassie
in vitro phosphorylation
D
14C-AcCoA
TDG
TDG (S96-99D)
75 kD
50
Autoradiograph
75 kD
50
Coomassie
in vitro acetylation
E
αFLAG I.P.
Control
TDG
TDG (K94-95-98A)
CBP − − − − + + − −
PMA − + − + − + − +
TDG
autoradiograph
TDG
1 2 3 4 5 6 7 8
αTDG immunoblot
in vivo phosphorylation

Figure 3.5

Divergent effects of TDG acetylation and phosphorylation on DNA mispair processing

DNA binding has been reported to promote conformational changes in TDG (48). In order to address whether TDG could be acetylated or phosphorylated when bound to DNA, we carried out acetylation reactions with CBP in the presence of increasing amounts of either normally paired or G:T mispaired duplex oligonucleotides (Fig. 3.6A). A considerable reduction in acetylation of TDG was observed in the presence of either oligonucleotide, while CBP autoacetylation was not significantly affected. These findings suggest that *in vivo* TDG bound to DNA is unlikely to be acetylated by CBP/p300. In contrast, the presence of DNA had little effect on the acetylation of GST-p53 and SET/TAF1β/I_2^{pp2A} proteins (Fig. 3.6B). Additionally, the presence of duplex oligonucleotides caused a more moderate reduction in PKCα mediated phosphorylation (Fig. 3.6C) suggesting that phosphorylation of TDG may occur on DNA.

The marked reduction in acetylation following DNA binding suggests that the acetyl-acceptor lysines may directly contact DNA. To investigate this possibility, we compared the DNA binding activity of bacterially expressed recombinant wild-type TDG and the K94-95-98A mutant using the electrophoretic mobility shift assay (11), We found that the alanine substitution mutant displayed moderately enhanced binding to duplex oligonucleotides containing either G:U or G:T mispairs (Fig. 3.6D). Interestingly, we found that the mutant bound to an abasic site was resistant to displacement by APE compared to wild-type TDG (Fig. S.3.3). These findings suggest that lysine 94, 95 and 98 are critical determinants of the DNA binding properties of TDG and that these positively charged residues are not directly interacting with DNA but may be conformationally important.

Figure 3.6. DNA binding prevents CBP-mediated acetylation of TDG. (A) Dose dependent inhibition of TDG acetylation by duplex oligonucleotides. TDG (150 ng) was pre-incubated with the indicated duplex oligonucleotides for 30 minutes on ice and then acetylated *in vitro* with CBP (100 ng) and ^{14}C-AcCoA. Reaction products were analyzed by SDS-PAGE and autoradiography. (B) DNA-dependent inhibition of CBP-mediated acetylation is specific to TDG. *In vitro* acetylation was performed with TDG (150 ng), GST-p53 (150 ng), and SET/TAF-1β/I_2^{pp2A} (150 ng) recombinant proteins in the presence or absence of 200 ng of G:T mispaired oligonucleotide. (C) Phosphorylation of TDG in the presence of duplex oligonucleotides. *In vitro* phosphorylation reactions were performed as described in Figure 3.4 using TDG pre-incubated with 200 ng of the indicated oligonucleotides. (D) Alanine substitution of acetyl acceptor lysines enhances DNA binding. Electrophoretic mobility shift assay was carried out with radiolabeled duplex oligonucleotides bearing either a G:U or a G:T mispair with 25 ng of recombinant wild-type TDG or TDG(K94-95-98A).

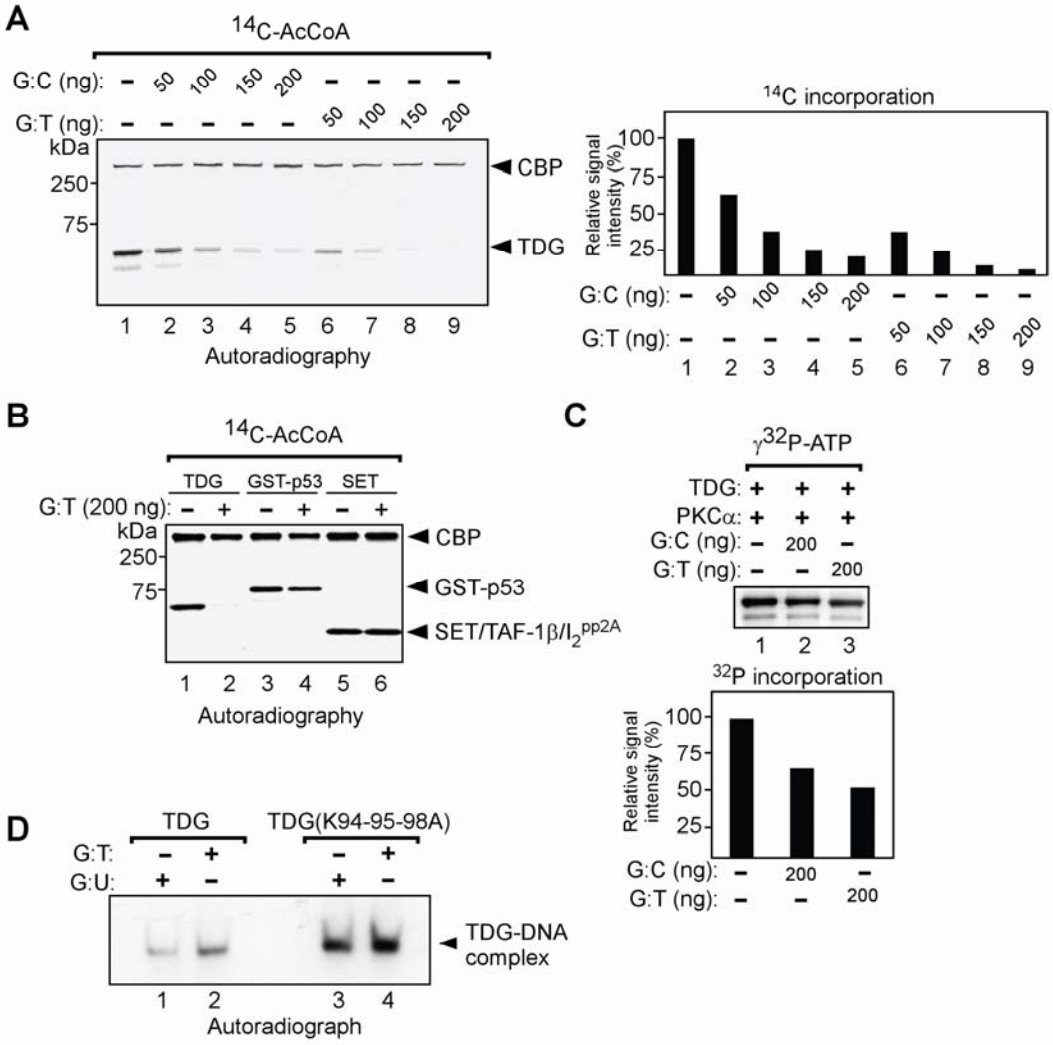
A
14C-AcCoA
G:C (ng): – 50 100 150 200 – – – –
G:T (ng): – – – – – 50 100 150 200
kDa
250
75
CBP
TDG
1 2 3 4 5 6 7 8 9
Autoradiography
14C incorporation
Relative signal intensity (%)
100
75
50
25
G:C (ng): – 50 100 150 200 – – – –
G:T (ng): – – – – – 50 100 150 200
1 2 3 4 5 6 7 8 9
B
14C-AcCoA
TDG
GST-p53
SET
G:T (200 ng): – + – + – +
kDa
250
75
CBP
GST-p53
SET/TAF-1β/I2pp2A
1 2 3 4 5 6
Autoradiography
C
γ32P-ATP
TDG: + + +
PKCα: + + +
G:C (ng): – 200 –
G:T (ng): – – 200
1 2 3
32P incorporation
Relative signal intensity (%)
100
75
50
25
G:C (ng): – 200 –
G:T (ng): – – 200
D
TDG
TDG(K94-95-98A)
G:T: – + – +
G:U: + – + –
TDG-DNA complex
1 2 3 4
Autoradiograph

Figure 3.6

In light of the critical role of the amino-terminal region of TDG in G:T processing and the observed inhibition of CBP-mediated acetylation by DNA, we investigated whether acetylation or phosphorylation could modulate DNA interactions. To address this, we produced purified covalently modified (acetylated or phosphorylated) recombinant TDG *in vitro* using nickel affinity chromatography (see Fig. 3.5). As controls, we also carried out mock acetylation and phosphorylation reactions using heat-denatured CBP or by omission of ATP, respectively. We assayed *in vitro* modified TDG for DNA-binding and G:T/U processing activity using asymmetrically radiolabelled duplex oligonucleotides containing the indicated DNA mispairs. Remarkably, we observed that acetylated TDG retained robust G:U processing activity but displayed severely reduced G:T processing (Fig. 3.7A, compare lane 4 and 6). Consistent with these findings, we observed using both the avidin-biotin coupled DNA (ABCD) binding assay (data not shown) and electrophoretic mobility shift assay (EMSA) (Fig. 3.7B) that the binding of acetylated TDG to G:U mispaired oligonucleotides was comparable to that of mock acetylated TDG, whereas binding to G:T mispaired DNA was substantially reduced. Therefore, these data indicate that acetylation of the amino-terminal region selectively abrogates the G:T processing functions of TDG. In contrast, the DNA binding and mispair processing activities of phosphorylated TDG were found to be indistinguishable from mock phosphorylated protein (Fig. 3.7C and D). To confirm that we achieved efficient phosphorylation of TDG in this experiment, we carried out an acetylation reaction with this material and observed a 3-fold reduction in incorporation of radiolabelled AcCoA (data not shown). Therefore, although PKCα mediated phosphorylation does not appear to directly alter the processing functions of TDG it may prevent inhibition of G:T processing by preventing acetylation by CBP/p300.

Figure 3.7. Divergent effects of acetylation and phosphorylation on DNA mispair processing. (A) Acetylation of TDG selectively abrogates G:T processing. Purified acetylated TDG (acTDG) (25 ng) was prepared as described in Figure 3.5 and base excision assays were carried out using asymmetrically radiolabelled duplex oligonucleotides (10 ng) bearing either G:U or G:T mispairs. Reaction products were treated with alkali to cleave the abasic sites and analyzed by denaturing PAGE and autoradiography. (B) Acetylated TDG does not stably bind oligonucleotides bearing a G:T mispair. Aliquots of base excision reactions described above were subjected to electrophoretic mobility shift assays to determine binding to G:U or G:T mispaired oligonucleotides. (C) Phosphorylation of TDG does not detectably alter G:T/U processing activity. Phosphorylated (pTDG) and mock phosphorylated TDG (12 ng) were tested for ability to excise mispaired uracil and thymine. (D) DNA-binding analysis of phosphorylated and mock phosphorylated TDG. Aliquots of the base excision reactions were subjected to electrophoretic mobility shift analysis. Figures S.3.4 and S.3.5 show images of EMSA gels that include unbound DNA probe.

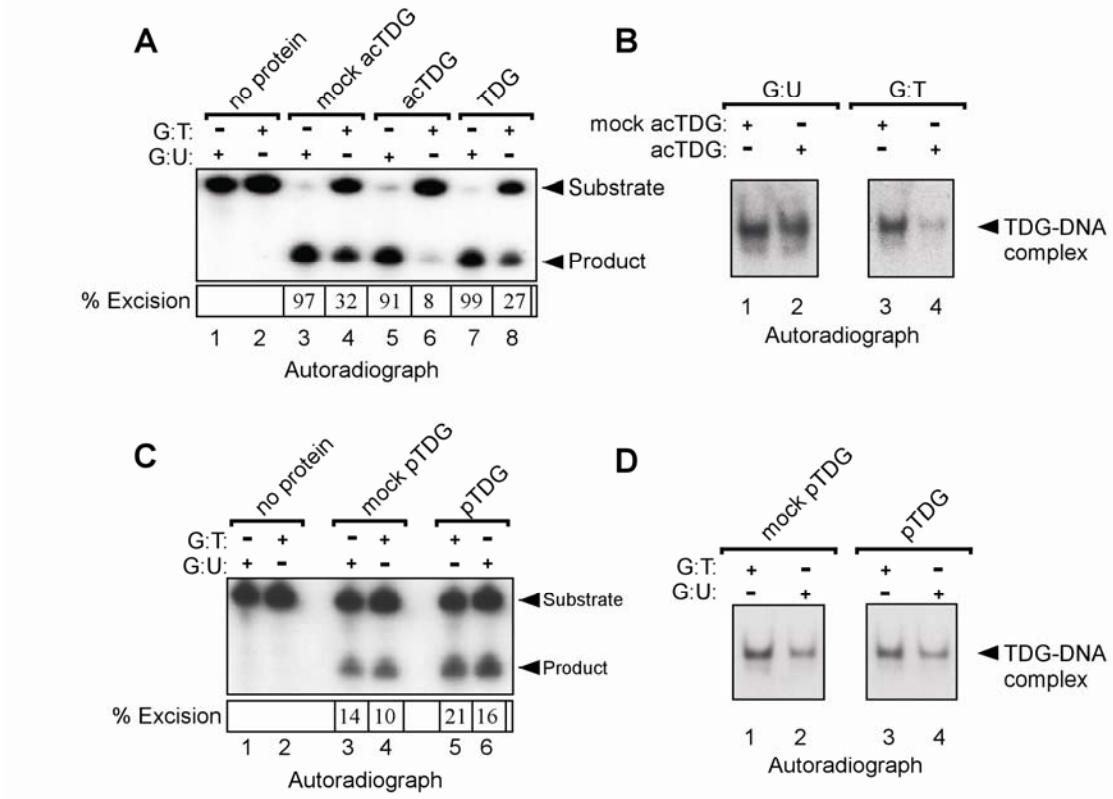
A
no protein
mock acTDG
acTDG
TDG
G:T:
G:U:
Substrate
Product
% Excision
97
32
91
8
99
27
1 2 3 4 5 6 7 8
Autoradiograph
B
G:U
G:T
mock acTDG:
acTDG:
TDG-DNA complex
1 2 3 4
Autoradiograph
C
no protein
mock pTDG
pTDG
G:T:
G:U:
Substrate
Product
% Excision
14
10
21
16
1 2 3 4 5 6
Autoradiograph
D
mock pTDG
pTDG
G:T:
G:U:
TDG-DNA complex
1 2 3 4
Autoradiograph

Figure 3.7

3.4 Discussion

We have elucidated a unique interplay between acetylation and phosphorylation in regulating the DNA repair functions of TDG. We show that these posttranslational modifications occur on adjacent residues in the amino-terminus and are mutually exclusive. Remarkably, acetylation by CBP/p300 selectively abrogates G:T processing while phosphorylation by PKCα may preserve this function *in vivo* by preventing CBP-mediated acetylation. Our findings suggest that the opposing regulatory roles of CBP/p300 and PKC may have profound effects on the functions of TDG in CpG maintenance and epigenetic regulation.

We investigated a regulatory role for PKC in TDG-mediated base excision on the basis of the proximity of putative PKCα/β/γ phosphorylation sites and acetyl-acceptor lysines (K94, K95 and K98). These residues are located within a sequence motif conserved in mouse, rat and human TDG (^{93}SKKSGKS^{99}). Our studies indicate that in mouse fibroblasts PKCα translocates to the nucleus in response to PMA stimulation and phosphorylates residues in the amino-terminal DNA binding domain of TDG. Accordingly, we have shown that in NIH 3T3 cells, stably-expressed TDG coimmunoprecipitates with endogenous PKCα. Furthermore, 2D-PAGE analysis and metabolic labeling indicate that TDG is phosphorylated in a PMA-dependent manner *in vivo* and this effect was abrogated by a PKCα/β-specific inhibitor. Using recombinant bacterially expressed proteins we have shown that PKCα interacts directly with TDG and this association requires the amino-terminal region of TDG (residues 1-122). The phosphorylation sites were mapped *in vitro* using peptides bearing alanine substitutions of potential phosphoacceptor residues. We identified two serine residues (S96 and S99) that when mutated in the context of full-length TDG, substantially reduced phosphorylation by PKCα. Consistent with this, *in vivo* PMA-dependent phosphorylation of transiently

expressed TDG was substantially reduced when the phosphoacceptor serines were substituted with aspartate. Although we have focused on PKCα in our studies, these phosphoacceptor serines may be phosphorylated by other PKC subtypes. We have used PMA to induce nuclear translocation of PKCα; however, it is well known that in fibroblasts this is a normal physiological response to growth factor (e.g. PDGF, EGF) stimulation (37). Therefore, phosphorylation of TDG may be regulated by mitogenic signals. Interestingly, in undifferentiated P19 EC cells, PKCα is mostly nuclear and colocalizes with TDG, consistent with a role in the regulation of nuclear processes.

Considering the proximity of phosphoacceptor serines and acetyl-acceptor lysines and the different charge characteristics of these residues upon covalent modification, it should perhaps not be surprising that acetylation effectively prevents phosphorylation and vice versa. We found that acetylation of recombinant TDG decreases subsequent phosphorylation by 10-fold while phosphorylation decreases acetylation by at least 3-fold. Furthermore, substitution of phosphoacceptor serines with aspartate, which mimics phosphorylation, reduced acetylation *in vitro* while replacement of positively charged lysines with alanine reduced phosphorylation. Consistent with *in vitro* studies, PMA-induced phosphorylation *in vivo* was abrogated by removal of acetyl-acceptor lysines and reduced by CBP overexpression. Surprisingly, the phosphorylation mimic (S96-99D) was acetylated *in vivo* at similar levels to wild-type TDG. This is most likely attributable to acetylation of TDG *in vivo* at other lysine residues by other acetylases. The mutually exclusive nature of these adjacent modifications is reminiscent of the cross-talk observed on the amino-terminal KS dipeptide of histone H3; in this case, phosphorylation of S10 blocks both K9 acetylation and methylation while K9 dimethylation antagonizes S10 phosphorylation (8, 10, 28, 44). Similar cross-talk as been suggested for non-histone proteins (27, 55).

In exploring the functional significance of these covalent modifications, we first established whether they could occur when TDG is bound to DNA as conformational changes in this context have been reported (17). We found that DNA-bound TDG was very resistant to acetylation while CBP autoacetylation was not altered. In contrast, phosphorylation by PKCα was much less affected by the presence of DNA. These findings suggest that *in vivo* PKCα may phosphorylate DNA-bound TDG while acetylation by CBP/p300 requires uncoupling from DNA. The observation that CBP/p300 mediated acetylation abrogates binding to duplex DNA and G:T processing may be relevant to both the CpG maintenance functions of TDG as well as its recently postulated role in gene-specific CpG demethylation (34). Acetylated TDG retains the ability to process G:U mispairs *in vitro*, consistent with previous reports indicating that this function does not require the amino-terminal region (36, 48). However, the reduced ability of acetylated TDG to bind DNA may interfere with the genome scanning functions attributed to DNA glycosylases, which could severely hinder detection of DNA mispairs and other DNA lesions *in vivo*. G:U and G:T mispairs at CpG dinucleotides are generated by hydrolytic deamination of cytosine and methyl cytosine, respectively. In order to restore cytosine methylation following repair of G:T mispairs, a mechanism is required to discern between mispairs arising from either the methylated or unmethylated cytosine. CBP/p300-mediated acetylation may provide a mechanism to discriminate between the two deamination products. Recent studies employing chromatin immunoprecipitation (ChIP) assays, have shown that TDG transiently occupies the promoters of several estrogen responsive genes (2, 22) and it has been postulated that TDG and other BER enzymes are essential for the cyclical CpG demethylation patterns observed on these genes during transcription. As the DNA binding activity and G:T processing functions of TDG are dramatically reduced by CBP-mediated acetylation, this may serve as a powerful mechanism to regulate CpG demethylation and/or the release of TDG from

promoters. The finding that CBP/p300 mediated acetylation of Polβ reduces end trimming activity and impairs participation in BER (18) suggests that CBP/p300 may generally act as negative regulator of BER. This is also in agreement with the role of acetylation in reducing the nuclease activity of another BER enzyme, flap endonuclease 1 (Fen1) (19). A recent study provided evidence for the direct excision of 5-methylcytosine by MBD4 and this activity is stimulated by PKC phosphorylation (23). Therefore, PKC signaling likely plays a central role in regulating CpG demethylation by MBD4 and TDG.

CBP/p300 as well as other protein acetylases respond to DNA damage and other cellular stresses by acetylating cellular proteins such as the tumor suppressor p53 (31, 45, 49). Covalent modification of key cellular regulatory proteins is an integral signaling mechanism in DNA damage response that leads to cycle arrest, apoptosis and cellular senescence. Acetylation of TDG may serve to block DNA repair as part of an apoptotic response to cellular stresses such as excessive DNA damage. In this context, it is interesting to note that both phosphorylation by PKC and acetylation by p300 have been shown to inactivate Polβ, while in this study we demonstrate that phosphorylation of TDG by PKCα may preserve G:T processing by preventing acetylation by CBP/p300. Altogether these findings suggest that the crosstalk between different signaling pathways could provide exquisite regulation of the different steps of BER in response to physiologic signals or stresses. Although in this study we have focused on the classic functions of TDG in processing G:T/U mispairs, it also processes other damaged bases and may be important in cellular responses to oxidative stress. Along these lines, since PKC isoenzymes are activated by reactive oxygen species (14, 25), TDG may be a crucial downstream target that would also be subject to opposing regulation by CBP/p300.

The tight binding of TDG to abasic sites produced by base excision prevents enzyme turnover and limits processing efficiency (48). It has been postulated that sumoylation of

mammalian TDG serves to promote release of the enzyme from abasic sites (17). Structural analysis of an amino- and carboxy-terminal deleted human TDG conjugated to SUMO revealed the presence of a protruding helix that interferes with DNA binding (1). However, analysis of sumoylated full-length human TDG by limited proteolysis suggested that sumoylation promotes conformational changes involving interaction of the carboxy- and amino-terminal regions (48). We have shown previously that mouse TDG contains two separate conserved amino-terminal and carboxy-terminal SUMO-binding motifs that interact intramolecularly with the conjugated SUMO and may account for the sumoylation-induced conformational changes (36). Interestingly, both sumoylation and acetylation by CBP/p300 abrogate DNA binding and processing of G:T mispairs (see Fig. 3.8). However, in contrast to sumoylation which can occur on DNA, we show that CBP-mediated acetylation requires the uncoupling of TDG from DNA. Furthermore, acetylated TDG retains the ability to form stable complexes with abasic sites as evidenced by the stable binding observed following the processing of G:U mispairs. It is plausible that acetylation promotes limited conformational changes within the amino-terminus in contrast to the more extensive changes that are associated with sumoylation. Interestingly, substitution of lysines 94, 95 and 98 with alanines did not mimic the effects of acetylation on DNA binding and this mutant was resistant to displacement by APE when bound to abasic sites (Fig. S.3.3). These findings suggest that these lysine residues play critical roles in DNA binding and mispair processing and the effects of acetylation are not strictly due to loss of positive charges.

Figure 3.8. Cross-talk between TDG posttranslational modifications. Previous studies have shown that sumoylation of human and mouse TDG induces a dramatic increase in G:U processing activity by promoting enzyme turnover (19,20). In contrast, sumoylation (19,20) or acetylation by CBP (this study) abrogate DNA binding and G:T processing (17, 36). TDG sumoylation also drastically reduces interactions with CBP/p300, thereby preventing efficient acetylation (20). The present studies reveal that phosphorylation of serine residues adjacent to acetyl-acceptor lysines by PKCα prevents acetylation by CBP and may preserve G:T processing *in vivo*.

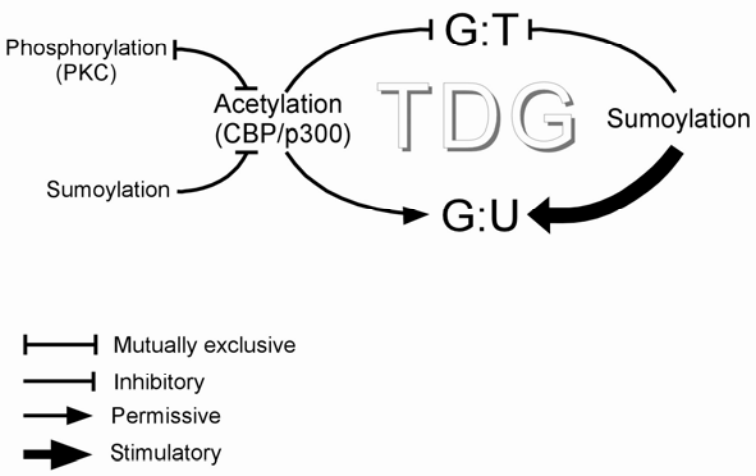
Phosphorylation
(PKC)
Acetylation
(CBP/p300)
Sumoylation
G:T
TDG
Sumoylation
G:U
Mutually exclusive
Inhibitory
Permissive
Stimulatory

Figure 3.8

We have provided biochemical evidence for the interplay of CBP/p300 and PKCα in modulating the DNA repair functions of TDG. Our studies provide insights into the complex roles of posttranslational modifications in regulating genome maintenance and gene expression pathways. The fact that both CBP/p300 and PKC signaling pathways are deregulated in oncogenesis (13, 16, 21) suggests that TDG may be a downstream target that may be functionally compromised and contribute to the genomic instability associated with cancer. Interestingly, TDG has recently been shown to efficiently excise 5-fluorouracil from DNA and plays a role in cellular responses to this commonly used chemotherapeutic agent (9, 26). Our studies indicate it may be possible to alter the DNA damage processing functions of TDG *in vivo* by targeting the signaling pathways that mediate acetylation and phosphorylation of this enzyme. Future studies will establish the utility of our findings in this context and whether TDG is suitable target for cancer therapy.

3.5 Bibliography

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3.6 Supplemental information

Figure S.3.1. Clustal W alignments of TDG orthologs. Accession numbers: Mouse [Mus musculus], AAH10315; Rat [Rattus norvegicus], NP_446181; Human [Homo sapiens], NP_003202; Chicken [Gallus gallus], NP_990081; Xenopus [Xenopus laevis], NP_001084290 NP_001084291; Zebrafish [Danio rerio], NP_001018587 XP_688633; Drosophila [Drosophila melanogaster], AAD33588. Note that Drosophila TDG is 1095 amino acids long and only part of the sequence is shown. Alignments were performed on the European Bioinformatics Institute website (http://www.ebi.ac.uk/).

CONSENSUS SYMBOLS:

An alignment will display by default the following symbols denoting the degree of conservation observed in each column: "*" means that the residues or nucleotides in that column are identical in all sequences in the alignment. ":" means that conserved substitutions have been observed, according to the color table above. "." means that semi-conserved substitutions are observed.

AVFPMILW	RED	Small (small+ hydrophobic (incl.aromatic -Y))
DE	BLUE	Acidic
RK	MAGENTA	Basic
STYHCNGQ	GREEN	Hydroxyl + Amine + Basic - Q
Others	Gray	

```
CLUSTAL 2.0.12 multiple sequence alignment

TDG_Mouse           -MDAEAARSYS--LEQVQALYSFPFQQMMAEVPNMAVTTGQQVPAVAPNMATVTEQQV--
55

TDG_Rat             ----------------------------MMAEAP--------------NMADVAGQQM--
16

TDG_Human           -MEAENAGSYS--LQQAQAFYTFPFQQLMAEAP--------------NMAVVNEQQMPE
42

TDG_Chicken         -MEAEELGRYYAYLQQAQAFYTFPFHQMMTAPP--------------TMEAMTEQPTLE
44

TDG_Xenopus         -MEAQDPSSYY---QPAQPYYPFSYHQMMNVPS--------------NMDLGNEQQTLH
41

TDG_Zebrafish       -MDERLYGSLPHAPSEYLQQWVQSAQQHLQTLQAQYP----------HMANGSAGFMME
48

TDG_Drosophila      MGEELHMHSPSHRHLDAVTTGPGRYGILVSNDTPECLSR---------EMYRHSQQSTTV
51

                                                 :                  *


TDG_Mouse           ------------------------------PADAPVQ--EPAPEAPKR-RKRKPRAAEPQ
82

TDG_Rat             ------------------------------PAEAPAQ--DPVPEAPKR-RKRKTRAAEAQ
43

TDG_Human           ----------------------------EVPAPAPAQ--EPVQEAPKG-RKRKPRTTEPK
71
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TDG_Chicken         ----------------------------GIPEPNLAQ--EPPKEVKKGGRKRKAKATEPK
74

TDG_Xenopus         PLTGVPAHELQAFSGMAANEPQVLHTLTGVPAQEPVNGEMPIPEMIPNPAEAEPTTGKRK
101

TDG_Zebrafish       GQREDAG----------------MQQMPVHPEDAAQLQPAAAQTAPAKGKRARQTNKEPK
92

TDG_Drosophila      ----------------------------LEQTDSSSCGINFKPMPKKRGRKKKLVAVNAD
83

TDG_Mouse           EPVEPKKPATSKKSGKSTKS------KEKQEKITDAFK-VKRKVDRFNGVSEAELLTKTL
135

TDG_Rat             DPVEPKKPAASKKSGKSTKS------KEKQEKITDTFK-VKRKVDRFNGVSEAELLTKTL
96

TDG_Human           QPVEPKKPVESKKSGKSAKS------KEKQEKITDTFK-VKRKVDRFNGVSEAELLTKTL
124

TDG_Chicken         Q---PKKPAAKKE--KATKS------KGKQEKITDTFK-VKRKVDRFNGVSEAELLTKTL
122

TDG_Xenopus         RGKAPSEPKPKKPAAKSAKAPK----SGKQEKITDAFK-VKRKVNRFNGVSEAELLTKTL
156

TDG_Zebrafish       PKGEPKPRAKPGPKPKKAKEDKEAPPAEGQEKIDETFKKVKRKVDRFKGMSEEEVMKRTL
152

TDG_Drosophila      TSQMTTPVDQQKVSAGRADCE-----DGGGDQAAKPKE--RKKHDRFNGMSEEEVIKRTI
136

                        ..           :.           ::  .. :  ::* :**:*:** *::.:*:
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TDG_Mouse           PDILTFNLDIVIIGINPGLMAAYKGHHYPGPGNHFWKCLFMSGLSEVQLNHMDDHTLPGK
195

TDG_Rat             PDILTFNLDIVIIGINPGLMAAYKGHHYPGPGNHFWKCLFMSGLSEVQLSHMDDHTLPGK
156

TDG_Human           PDILTFNLDIVIIGINPGLMAAYKGHHYPGPGNHFWKCLFMSGLSEVQLNHMDDHTLPGK
184

TDG_Chicken         PDILTFDLDIVIIGINPGLMAAYKGHHYPGPGNHFWKCLFMSGLSNEQLNHMDDHTLPHK
182

TDG_Xenopus         PDILTFNLDIVIIGINPGLMAAYKGHHYPGPGNHFWKCLFLSGLSDKQLNHLDDHSLPEK
216

TDG_Zebrafish       PDILIPNLDYVIIGINPGLMAAYIGRWFPGPGNHFWKCLFLSGFTEKLLNHMDDQSLPEK
212

TDG_Drosophila      PDHLCDNLDIVIVGINPGLFAAYKGHHYAGPGNHFWKCLYLAGLTQEQMSADEDHKLIKQ
196

                    ** *  :** **:******:*** *: :.**********:::*:::  :.  :*:.*  :

TDG_Mouse           YGIGFTNMVERTTPGSKDLSSKEFREGGRILVQKLQKYQPRIAVFNGKCIYEIFSKEVFG
255

TDG_Rat             YGIGFTNMVERTTPGSKDLSSKEFREGGRILVQKLQKYQPRIAVFNGKCIYEIFSKEVFG
216

TDG_Human           YGIGFTNMVERTTPGSKDLSSKEFREGGRILVQKLQKYQPRIAVFNGKCIYEIFSKEVFG
244

TDG_Chicken         YGIGFTNMVERTTPGSKDLSSKEFREGGRILMQKLQKYKPRIAAFNGKCIYEIFSREVFG
242

TDG_Xenopus         YGIGFTNMVERTTPGSKDLSSKEFREGGRILLEKLQKYKPRIAVFNGKCIYEIFSKEIFG
276
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```
TDG_Zebrafish       YGIGFTNMVARATPGSKDLSSKELREGGKILVEKIKQFKPLIAVFNGKCIYEMFCRELFG
272

TDG_Drosophila      G-IGFTNMVARATKGSADLTRKEIKEGSRILLEKLQRFRPKVAVFNGKLIFEVFS-----
250

                      ******* *:* ** **: **::**.:**::*::::* :*.**** *:*:*.


TDG_Mouse           VKVKNLEFGLQPHKIPDTETLCYVMPSSSARCAQFPRAQDKVHYYIKLKDLRDQLKGIER
315

TDG_Rat             VKVKNLEFGLQPHKIPDTETLCYVMPSSSARCAQFPRAQDKVHYYIKLKDLRDQLKGIER
276

TDG_Human           VKVKNLEFGLQPHKIPDTETLCYVMPSSSARCAQFPRAQDKVHYYIKLKDLRDQLKGIER
304

TDG_Chicken         IRVKNLEFGLQPHKVPETETLCYVMPSSSARCAQFPRAQDKVHYYIKLKDLRDQLKGIAP
302

TDG_Xenopus         VKAKKIDFGIQPHRIPETDTICYLMPSSSARCAQFPRAQDKVHHYIKLKELRNQLRGVET
336

TDG_Zebrafish       KKPKTLEFGLQPHKIPDSDTALYLMPSSSARCAQFPRAQDKVHFYIKLRELRDQLKGVIK
332

TDG_Drosophila      -GKKEFHFGRQPDRVDGTDTFIWVMPSSSARCAQLPRAADKVPFYAALKKFRDFLNGQIP
309

                       * :.** **.::  ::*  ::**********:*** *** .*  *:.:*: *.*

TDG_Mouse           NTDVQEVQYTFDLQLAQEDAKKMAVKEEKYDPGYEAAYGGAYGE----NPCNGEPCGIAS
371
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TDG_Rat             STDVQEVQYTFDLQLAQEDAKRTAVKEEKYDPGYEAAYGGACGE----NPCNGEPCGFAS
332

TDG_Human           NMDVQEVQYTFDLQLAQEDAKKMAVKEEKYDPGYEAAYGGAYGE----NPCSSEPCGFSS
360

TDG_Chicken         NTEVQEVQYTFDLQLAQEDAKKMAVKEEKYDPGYEAAYGGAYCDR---APYESEQCNFSS
359

TDG_Xenopus         NREIQEVQYTFDLQLAQEDAKRQAIKEEKYDPGYNSALGEQFNEQT--TSGESGMCNFST
394

TDG_Zebrafish       QKEVEEVNYTFDLGLAKEDAKRIAVKEEQYDPGYEAAFGGAYGEAAPEGGQSNGICNFSA
392

TDG_Drosophila      HIDESECVFT-DQRIRLCSAQQQVDIVGKINKTHQPPLGDHPSSLTVVSNCSGPIAGDAE
368

                      : .*  :* *  :   .*:: .    : :  ::.. *    .       ..  .. :


TDG_Mouse           NGLTAHSAEPRGE-----------ATPGDVPNGQ--WMAQSFAEQIPSFNN-CGTREQEE
417

TDG_Rat             NGLTANSAELGGE-----------SAPSDVPNGQ--WMAQSFAEQIPSFNN-CGTGEQEA
378

TDG_Human           NGLIES-VELRGE-----------SAFSGIPNGQ--WMTQSFTDQIPSFSNHCGTQEQEE
406

TDG_Chicken         NGTAPSNPQYCEG-----------SSFGEVPNGQ--WMTQSFADQIPEFSA--GMTQERE
404

TDG_Xenopus         DATVPSNAEFNG---------------QAQNGQ--WIPQPIAEQMSTYNH--SGDQQQG
434
```

```
TDG_Zebrafish       SEENANTAEKAPTK---------PSDVGQVQDGQ--WMTQSFADQIPDIGS--SSQAQNW
439

TDG_Drosophila      CGIVAEESDQVQSEKMIPQMDPTVPSSSNATDGKSFSYTAENTPLLPVSNHNPSINENNY
428

                           :                       :*:    .   :  :.  .   .   :.

TDG_Mouse           ESHA------------------------------------------------------
421

TDG_Rat             GSHAGSHA--------------------------------------------------
386

TDG_Human           ESHA------------------------------------------------------
410

TDG_Chicken         GSSA------------------------------------------------------
408

TDG_Xenopus         GSNA------------------------------------------------------
438

TDG_Zebrafish       GV--------------------------------------------------------
441

TDG_Drosophila      LSVMGSQQPLSQQPLEKKKRGRPKKIKGQDIIDHSVGGKASIAGQHIPSHDFNNILNLSV
488
```

Figure S.3.2. Coimmunoprecipitation of transiently overexpressed TDG and PKCα. The indicated expression vectors (7.5 mg per 10 cm culture plate) were transfected in NIH 3T3 cells and lysates were immunoprecipitated using a FLAG-affinity matrix. Captured protein complexes were separated by SDS-PAGE and analyzed by immunoblotting.

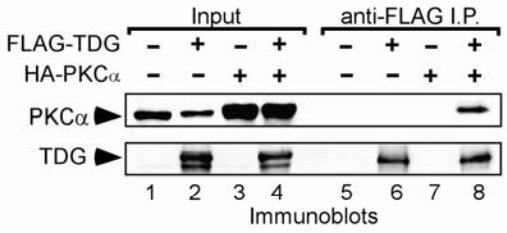
Input
anti-FLAG I.P.
FLAG-TDG
HA-PKCα
PKCα
TDG
1 2 3 4 5 6 7 8
Immunoblots

Figure S.3.2

Figure S.3.3. Alanine substitution of acetyl acceptor lysines enhances DNA binding. TDG(K94-95-98A) bound to a G:T mispair-bearing oligonucleotide is refractory to displacement by GST-APE. TDG or TDG(K95-95-98A) (100 ng each) were pre-incubated with a biotinylated oligonucleotide bearing a G:T mispair and then 1 or 2 μg of GST-APE was added to the reaction and incubated for 30 minutes. The DNA-protein complexes were captured using streptavidin coated paramagnetic beads. Bound TDG was detected by immunoblotting (upper panel). Signal volume is shown in the lower panel.

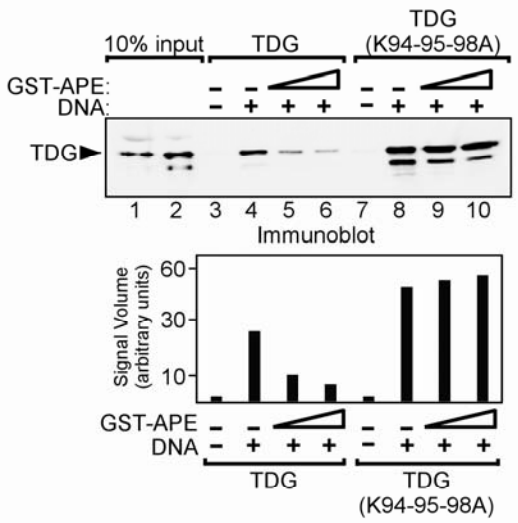
10% input
TDG
TDG
(K94-95-98A)
GST-APE:
DNA:
TDG
1 2 3 4 5 6 7 8 9 10
Immunoblot
Signal Volume
(arbitrary units)
60
30
10
GST-APE
DNA
TDG
TDG
(K94-95-98A)

Figure S.3.3

Figure S.3.4. DNA-binding analysis of acetylated and mock acetylated TDG (acTDG and mock acTDG). Aliquots of base excision reactions (Fig 3.7A) were analyzed using EMSA.

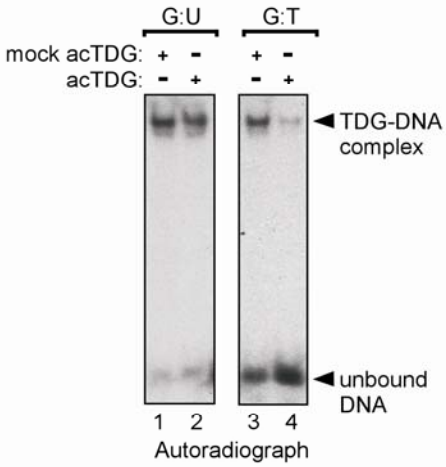
G:U
G:T
mock acTDG: + - + -
acTDG: - + - +
TDG-DNA complex
unbound DNA
1 2 3 4
Autoradiograph

Figure S.3.4

Figure S.3.5. DNA-binding analysis of phosphorylated and mock phosphorylated TDG (pTDG and mock pTDG). Aliquots of base excision reactions (Fig 3.7C) were analyzed using EMSA.

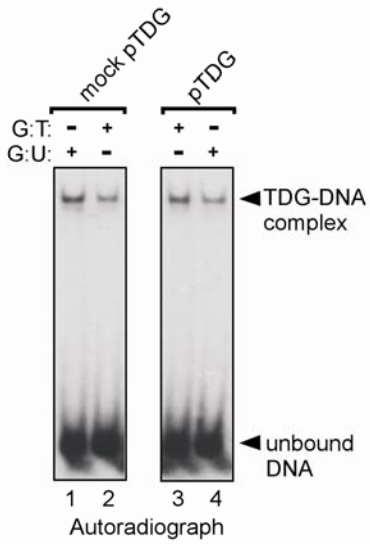
mock pTDG
pTDG
G:T: - + + -
G:U: + - - +
TDG-DNA complex
unbound DNA
1 2 3 4
Autoradiograph

Figure S.3.5

Chapter 4: Subnuclear localization of TDG is regulated by posttranslational modifications.

4.1 Introduction

Methylation of cytosine within CpG dinucleotides is an important modification in vertebrates used to regulate transcription. While an effective mechanism for repression of transcriptional activity, cytosine and 5-methylcytosine are prone to spontaneous hydrolytic deamination, generating uracil and thymine mispairs respectively. Left unrepaired, these mispairs will result in C to T transition mutations upon replication. These mispairs are major contributors to mutations in tumor genomes which alter CpG content and CpG methylation patterns (55-57). For example, examination of the IARC p53 mutation database indicates that C to T mutations at CpGs may be found in the p53 gene in almost half of all human tumor samples analyzed (53). These mutations are also major contributor to genome instability on an evolutionary time scale as evidenced by a loss of methylated CpG sequences over time resulting in approximately $1/5^{th}$ the expected frequency of CpG within the genome (7, 33, 34).

Performing a critical role in safeguarding CpG dinucleotides from degradation, DNA glycosylases TDG and MBD4 are able to excise mispaired uracil and thymine bases within CpG sequences, thereby initiating the base excision repair (BER) pathway. Repair of the product abasic site is completed by APE, Polβ and DNA ligase β, which comprise the remainder of the BER pathway (26).

TDG possess an unusually large hydrophobic catalytic pocket (4) which allows processing of various bases damaged as a result of alkylation (22), halogenation (47), and other environmental toxins (72). In NIH 3T3 mouse fibroblasts, MBD4 associates primarily with heterochromatin, whereas TDG preferentially associates with

transcriptionally active euchromatic regions (66). However, overexpression of Dnmt3a in these cells promotes TDG localization to heterochromatic regions (35). A small fraction of TDG has also been shown to localize to heterochromatic regions in P19 mouse embryonic carcinoma cells in a Dnmt3b-dependent manner (8). TDG interacts with a number of sequence-specific transcription factors including c-Jun (14), thyroid transcription factor 1(TTF-1) (44), retinoic acid receptor (RAR), retinoid X receptor (RXR) (68), estrogen receptor α (ERα), and several other nuclear receptors (13), as well as transcriptional coactivators SRC1 (38), and cyclic AMP response element binding protein (CREB) binding protein (CBP)/p300 (66), suggesting that TDG and BER may play a role alongside these important coregulators of transcription in facilitating gene expression.

The links between transcription and BER were further strengthened in recent studies by Metivier at al. demonstrating that TDG and BER proteins APE, Polβ and DNA ligase, participate in the cyclical demethylation of the transcriptionally active, estrogen responsive *pS2/TFF1* and *Wisp-2* promoters. When TDG, along with DNA methyltransferases Dnmt3a and Dnmt3b bind these methylated promoters, the methyltransferase can promote deamination of methylated cytosines, producing G:T mispairs. The mispair is recognized and corrected by TDG and BER, resulting in replacement of the methylated cytosine with an unmethylated base. The methyltransferases in turn, remethylate the previously methylated cytosines, silencing the promoter until the next signal for gene expression is received (43).

Deregulation of gene methylation patterns, including hypermethylation of tumor suppressor genes (eg. *p16, RASSF1A* and *RUNX3*) (48) or hypomethylation of oncogenes (eg. *WNT5A, CRIP1 and S100P*) (70), has been associated with promoting carcinogenesis (27, 31, 67). The TDG gene has also been shown to be hypermethylated and partially

silenced in multiple myeloma cell lines suggesting that decreased cellular levels of this enzyme contributes to genome instability and cellular transformation (52).

TDG functionally associates with the transcriptional coactivators CREB binding protein and its homologue p300 (CBP/p300). These essential cellular proteins are acetyltransferases and function as transcriptional coactivators (20, 30). CBP/p300 and TDG have been shown to form stable complexes on DNA which are competent for excision of mispaired bases. Additionally, TDG synergistically co-activates CBP-mediated transcription and is also a substrate for CBP/p300-mediated acetylation on amino terminal lysine residues 70, 94, 95, and 98 (66). Recently, we have shown that acetylation at these sites selectively abrogates TDG G:T processing activity while G:U processing is unaffected (45). Furthermore, we were able to infer from studies of an alanine substitution mutant (K94-95-98A) of TDG that the effect of acetylation was likely mediated by conformational changes in the amino terminus of TDG and not through disruption of direct TDG-DNA interactions.

As shown in the previous chapters, TDG is phosphorylated on serines 96 and 99 by PKCα (45) and is also modified by covalent attachment of small ubiquitin-like modifier (SUMO) (sumoylation) on lysine 341 (46). We have shown that sumoylation of TDG induces allosteric changes in TDG which are mediated by amino and carboxy terminal SUMO binding motifs (SBM1 and SBM2, respectively). These changes prevent sumoylated TDG from stably binding DNA, resulting in a loss of G:T processing, but not G:U processing function. Additionally, sumoylation of TDG prevents interactions with CBP and prevents CBP-mediated acetylation. PKCα-mediated phosphorylation of TDG was also shown to prevent CBP-mediated acetylation and reciprocally, acetylation prevented phosphorylation, making these modifications mutually exclusive on TDG.

This complex interplay between PTMs may cooperate in vivo to regulate TDG function and subcellular localization.

Considering the evidence that the incidence of C to T mutations at CpG is dramatically increased in ageing tissues (5, 12, 15, 26, 49), we investigated whether changes in TDG subcellular localization and sumoylation occur in senescent cells. Cellular senescence defines a normal biological response to DNA damage, and other stresses, which results in cell-cycle arrest. This mechanism can block cellular transformation by preventing the proliferations of cells that have incurred excessive DNA damage (49). We found that in normal diploid mouse and human fibroblast, replication- and drug-induced senescence was associated with redistribution of TDG from the nucleus to the cytoplasm. Additionally, there was also a dramatic increase in the fraction of high-molecular weight TDG in senescent cells consistent with increased sumoylation. Similar changes in TDG were also elicited by oxidative stress induced by hydrogen peroxide treatment. Our studies also revealed that in MCF-7 breast carcinoma cells a point mutant of TDG which could not be sumoylated was excluded from nucleoli while wild-type TDG could be driven into these non-membrane-bound organelles upon overexpression of SUMO. Finally we examined the effect PKC activation, using the agonist PMA, on the subcellular localization of TDG. We found that PMA treatment decreased colocalization of TDG with sites of active transcription and promoted entry into heterochromatic regions from which TDG is otherwise largely excluded. Collectively, these findings suggest that post-translational modification may play an important role in regulating TDG function in response to cellular stress.

4.2 Materials and Methods

Plasmids

pCMX-based mammalian expression vectors for CFP-TDG and TDG(K341R) have been previously described (46, 66). The pCMX-based mammalian expression vector for YFP-SUMO was constructed by Marc Tini.

Antibodies

TDG-specific rabbit polyclonal antibody has been previously described (46). Human PKCα-specific polyclonal antibodies were obtained from Santa Cruz Biotechnology (sc-208, or sc-208-G). Monoclonal (H14) antibody to phosphorylated RNA polymerase II was obtained from Abcam (ab24759).

Cell culture and transfections

NIH 3T3 fibroblasts were maintained in Dulbecco's minimal essential medium (DMEM) containing penicillin/streptomycin and supplemented with 10% fetal calf serum. IMR-90 cells were maintained in DMEM containing penicillin/streptomycin and supplemented with 2.5% fetal calf serum and 7.5% fetal bovine serum. Mouse embryonic fibroblast (MEF) and MCF-7 cells were maintained in DMEM containing penicillin/streptomycin and supplemented with 10% fetal bovine serum. For subcellular localization experiments, cells were grown under serum-free conditions for 4 hours and then treated with either 100 nM PMA or vehicle (DMSO) for 15 minutes in serum containing media. For overexpression studies, 6-well plates were seeded with MCF-7 cells on coverslips and grown to approximately 80% confluency. Cells were then transfected with the indicated

YFP-SUMO, and CFP-TDG fusion expression vectors using the Polyfect transfection reagent (Qiagen).

Preparation of whole cell extracts for immunoblotting

Whole cell extracts for immunoblotting were prepared from 100 mm plates of the indicated cells. Cells were grown to approximately 80% confluency and treated as indicated. Cells were then harvested by scraping, pelleted by centrifugation at 300xG for 2 minutes. Cell pellets were then washed with PBS before being resuspended in 500 µl of Laemmli buffer containing 100 units Benzonase (Roche) and incubated at 95° Celsius for 5 minutes.

Immunoprecipitations

Whole cell extracts for immunoprecipitation were prepared from 100 mm dishes of NIH3T3 cells stably expressing FLAG-TDG and control cells transduced with the empty expression vector (pLNCX). Cells grown to 80% confluency were harvested by scraping, then pelleted by centrifugation and resuspended in 500 µl of Lysis buffer (50 mM Tris HCl pH 7.9, 300 mM NaCl, 1 mM EDTA, 1mM EGTA 10% glycerol, 0.5% Triton X-100, 1 mM DTT, 50 mM sodium fluoride, 200 µM sodium orthovanadate and proteinase inhibitors - 20 µg/mL Pepstatin A, 10 µg/mL Aprotinin, 1 µg/ml Leupeptin, 0.5 mM PMSF) and incubated on ice for 30 minutes. Subsequently, the cell lysate was diluted with 500 µl of dilution buffer (50 mM Tris-HCl pH 7.9, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1 mM dithiothreitol (DTT), 50 mM sodium fluoride, 200 µM sodium orthovanadate and proteinase inhibitors) and insoluble material removed by centrifugation. Whole cell extracts were precleared twice with 50 µl (50% v/v) rabbit IgG-agarose (Sigma-Aldrich) for 30 minutes at 4^{o} C and immunoprecipitated with 50 µl

(50% v/v) anti-FLAG affinity resin (M2 agarose, Sigma-Aldrich). Immunoprecipitated proteins were separated by SDS-PAGE and detected by immunoblotting.

Immunostaining and microscopy

NIH 3T3 cells were fixed for 15 minutes with 4% formaldehyde in phosphate buffered saline (PBS) followed by a 10 minute incubation with 0.1 M glycine in PBS. Cells were then permeabilized with 0.5% Triton X-100 in PBS for 10 minutes. Immunostaining was performed with the appropriate primary antibodies and fluorophore-conjugated Donkey secondary antibodies (CY3, FITC) (Jackson ImmunoResearch Laboratories) diluted with 1% BSA/PBS solution. Epifluorescence imaging was performed on an Axiovert 200M inverted microscope equipped with an Apotome (Carl Zeiss) using appropriate fluorophore-specific filter sets. Z-series images (63X magnification) were acquired at 0.5 μm intervals and processed with Axiovision software and Adobe Photoshop. Fluorescence intensity plots were obtained by performing a line scan bisecting the cell using Axiovision software.

4.3 Results

TDG is responsible for repairing an array of toxic base lesions which contribute to mutations which have been found to accumulate in aging tissues. We therefore decided to examine whether alterations in TDG regulation may occur in ageing cells. IMR-90 diploid fibroblasts are derived from a 16-week female fetus and are capable of approximately 50-60 population doublings before entering senescence. The cellular distribution of TDG in low (LP) and high passage (HP) fibroblasts was examined by indirect immunofluorescence using an antibody that recognizes both mouse and human TDG (46). We found that in high-passage number IMR-90 cells TDG may be found in a punctate pattern in the cytoplasm and nucleus (Figure 4.1A). We next examined LP cells treated with the histone deacetylase inhibitor (HDACi) sodium butyrate which has been shown to induce alterations in cells similar to senescence (64). In these cells we found that TDG underwent a similar redistribution to that seen in IMR-90 cells naturally entering senescence (Figure 4.1B). To corroborate these findings, we performed the same analysis in sodium butyrate-treated mouse embryonic fibroblasts (MEFs) and found that TDG underwent a similar redistribution in these cells (Figure 4.1C). These observations suggested that TDG may undergo redistribution in aging cells and furthermore, that a similar pattern of relocalization may be stimulated by treatment with histone deacetylase inhibitors.

Figure 4.1. Redistribution of TDG to cytoplasm in senescent and sodium butyrate treated cells. (A) TDG undergoes redistribution from the nucleus to cytoplasm in high-passage IMR-90 cells. IMR-90 cells were passaged until they were no longer dividing, then fixed in 4% formaldehyde and TDG was detected by indirect immunofluorescence with a TDG-specific antibody. Cells were imaged using a Carl Zeiss Axiovert 200M microscope equipped with Apotome at 60x magnification. Using appropriate filters, 0.5 μm sections were taken. High passage number (HP) and low passage (LP) number cells are indicated. (B) Treatment of IMR-90 cells with sodium butyrate causes redistribution of TDG from the nucleus to cytoplasm. LP IMR-90 cells were treated with 10 mM sodium butyrate for 48 hours, and TDG localization was detected as in Figure 4.1. (C) Treatment of mouse embryonic fibroblasts (MEFs) with 10 mM sodium butyrate causes redistribution of TDG from the nucleus to cytoplasm. MEFs were treated with 10 mM sodium butyrate and analyzed as described above.

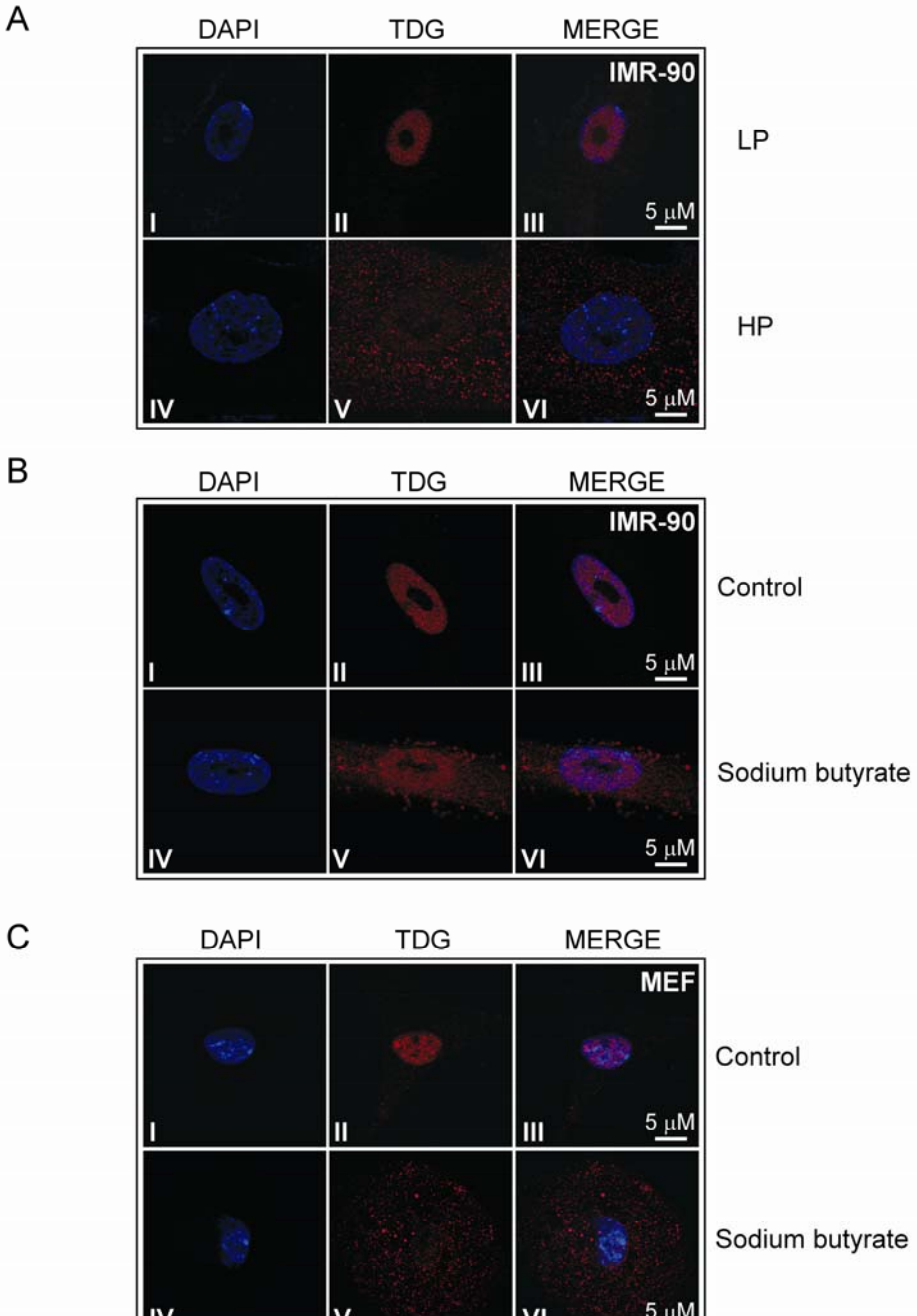
A
DAPI
TDG
MERGE
IMR-90
LP
HP
I
II
III
IV
V
VI
5 μM
B
DAPI
TDG
MERGE
IMR-90
Control
Sodium butyrate
I
II
III
IV
V
VI
5 μM
C
DAPI
TDG
MERGE
MEF
Control
Sodium butyrate
I
II
III
IV
V
VI
5 μM

Figure 4.1

In order to assess the sumoylation status of TDG after HDACi treatment we fractionated cellular lysates by SDS-PAGE and detected TDG by immunoblotting. Sumoylated TDG is approximately 10 kD greater in mass than unmodified TDG although the modified and unmodified proteins resolve at an apparent size of approximately 60 and 80 kD respectively when resolved by SDS-PAGE. By comparing the relative intensities of the immunoreactive TDG bands we were able to approximate the proportion of TDG which was sumoylated in the lysate. Interestingly, we noted that in lysates prepared from untreated IMR-90 cells TDG migrated similarly (~ 80 kD) to sumoylated TDG in lysates prepared from NIH 3T3 cells (Figure 4.2A). When we examined IMR-90 lysates treated with 1 μM TSA or 10 mM butyrate for 48 hours, we found that TDG from HDACi-treated cells migrated at approximately 90 kD, while control lysates showed bands at both 80 and 90 kD. These observations are consistent with the modification of TDG by conjugation of either SUMO-1 or SUMO-2/3 (3) and SUMO-2/3 may then be sumoylated by SUMO-1 (40). This suggested that redistribution of TDG in HDACi-treated cells may be related to further sumoylation of TDG.

Figure 4.2. Cellular redistribution of TDG may be associated with changes in posttranslational modification. (A) Cellular lysates from immortalized NIH 3T3 mouse fibroblasts and human IMR-90 fibroblasts were prepared in Laemmli buffer and immunoblotted for TDG. The expected relationship between a presumptive sumoylated (S-TDG) and TDG normally seen in NIH 3T3 cells is indicated. (B) IMR-90 cells were treated with either trichostatin A (TSA) (1 μM) or sodium butyrate (10 mM) for 48 hours and lysates were prepared and analyzed as in (A). The putative sumoylated TDG band is indicated.

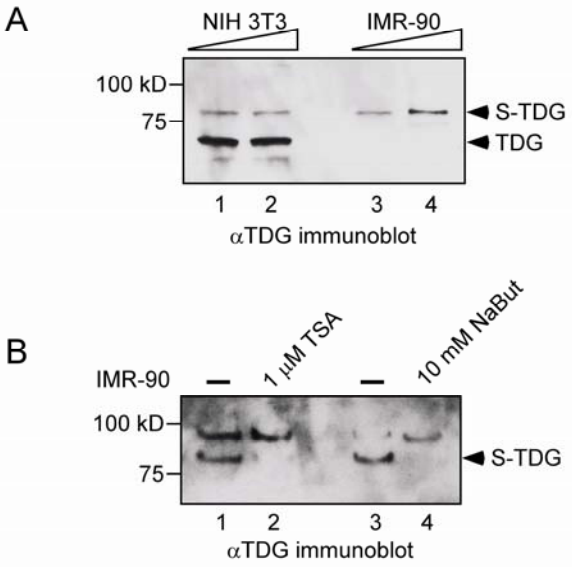
A
NIH 3T3
IMR-90
100 kD
75
S-TDG
TDG
1 2 3 4
αTDG immunoblot
B
IMR-90
1 μM TSA
10 mM NaBut
100 kD
75
S-TDG
1 2 3 4
αTDG immunoblot

Figure 4.2

We have shown that crosstalk between acetylation and phosphorylation and between acetylation and sumoylation in the regulation of TDG functions (45, 46). In order to determine whether phosphorylation or acetylation of TDG could alter sumoylation in response to cellular stress, we serum-starved NIH 3T3 cells stably transfected with FLAG-TDG expression vector (NIH 3T3-TDG) or empty vector (NIH 3T3-FLAG) for four hours then treated with phorbol ester PMA, which promotes phosphorylation of TDG (45), the biologically inactive phorbol ester 4αPMA, sodium butyrate, or various concentrations of hydrogen peroxide (H_2O_2). The latter is a natural signaling molecule causes oxidative damage to cells, and has also been shown to activate PKCα (32). Additionally, H_2O_2 has been shown to alter sumoylation through regulation of SUMO conjugating enzymes, causing a decrease in global sumoylation at low concentrations, and increase at high concentrations (9). We prepared whole cell lysates from treated cells and immunoprecipitated FLAG-TDG using FLAG-specific M2 agarose. After fractionation of immunoprecipitates by SDS-PAGE, we detected TDG by immunoblotting with a TDG-specific antibody. This analysis showed that although phorbol esters do not alter sumoylation as detected by this method (Figure 4.3, lanes 4 and 5), peroxide (compare lane 2 to lanes 6-9) and HDACi treatment (compare lane 2 to lane 3) strongly promotes a shift in TDG mobility consistent with its sumoylation (46).

Figure 4.3. Oxidative stress and sodium butyrate treatment alter post-translational modification of TDG. NIH 3T3 cells stably expressing FLAG-TDG were starved in serum-free DMEM media then treated with the indicated stimuli for one hour (overnight for sodium butyrate (NaBut) treatment). FLAG-TDG was then captured by immunoprecipitation of lysates prepared from these cells using FLAG-specific M2 agarose. Immunoprecipitates were then fractionated by SDS-PAGE and TDG was detected by immunoblotting with a TDG-specific antibody.

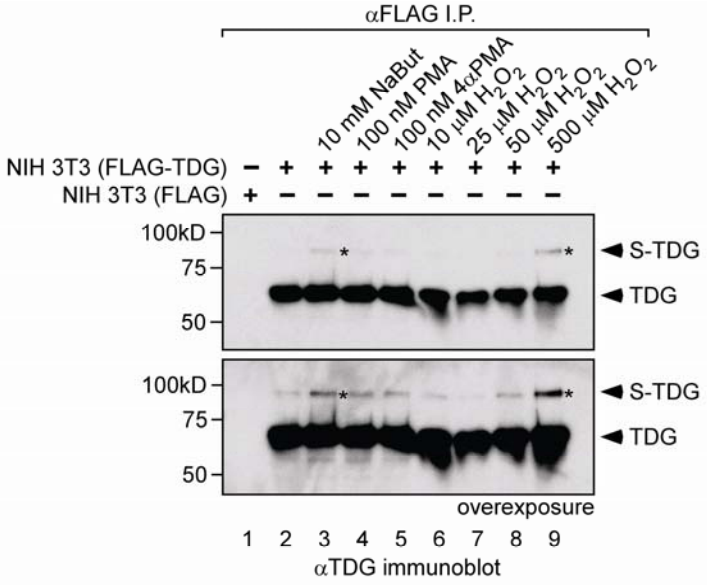
αFLAG I.P.
10 mM NaBut
100 nM PMA
100 nM 4αPMA
10 μM H2O2
25 μM H2O2
50 μM H2O2
500 μM H2O2
NIH 3T3 (FLAG-TDG) − + + + + + + + +
NIH 3T3 (FLAG) + − − − − − − − −
100kD
75
50
S-TDG
TDG
100kD
75
50
S-TDG
TDG
overexposure
1 2 3 4 5 6 7 8 9
αTDG immunoblot

Figure 4.3

These studies suggested that sumoylation of TDG occurs in response to oxidative stress while previous studies also indicate that TDG sumoylation prevents localization to the PODs (46). Therefore, we were interested in further investigating the role of sumoylation in regulating the cellular localization of TDG. Analysis of cellular lysates by immunoblotting with a TDG-specific antibody indicates that the percentage of TDG sumoylated varies between 5 and 50% depending on which cell type is examined [(Tini, unpublished)(45, 46)]. Specific detection of S-TDG in cells is problematic as there is no immunological method for distinguishing S-TDG from TDG. In order to determine the subcellular localization of S-TDG we carried out a comparative analysis of the cellular distribution of cyan fluorescent protein (CFP) fusion proteins of wild-type TDG and the sumoylation-deficient mutant TDG(K341R) cotransfected with yellow fluorescent protein (YFP)-SUMO. This approach allowed us to identify differences in the colocalization of YFP-SUMO and CFP-TDG(K341R) compared to CFP-TDG. The subcellular regions where CFP-TDG(K341R) does not appear to colocalize with YFP-SUMO may represent cellular compartments where localization is dependent on sumoylation of TDG. This approach did not allow us to specifically identify areas where only unmodified TDG localized or where both unmodified and S-TDG colocalized. Using this approach we found that YFP-SUMO and CFP-TDG colocalized extensively within the nucleus, including in nucleolar regions, from which TDG is normally excluded (46). CFP-TDG(K341R) however, did not colocalize with SUMO in the nucleoli, and rather accumulated around these non-membrane bound structures (Figure 4.4, compare panels IV and VIII). When we coexpressed FLAG-promyelocytic leukemia protein (PML) with YFP-SUMO we observed accumulation of SUMO within the PML oncogenic domains (PODs) (42, 46). Interestingly, in these cells neither exogenous TDG or TDG(K341R) localized to the nucleoli, suggesting that PML can regulate the nucleolar localization of TDG. We did observe accumulation of YFP-TDG and -TDG(K341R) in the PODs, suggesting that SUMO interactions were important for regulation of TDG localization to

these subnuclear compartments (Figure 4.4, compare panels XII and XVI). Interestingly, YFP-TDG(K341R) localized more readily to the PODs compared with YFP-TDG, suggesting that sumoylation of TDG negatively regulates POD targeting. Previously, TDG(K341R) was shown to accumulate within the PODs in the absence of SUMO overexpression (46). Additionally, sumoylated TDG was shown to be deficient in intermolecular SUMO-binding ability. Together with observations that selective desumoylation of PML within the PODs results in a loss of accumulation of TDG to the PODs (46), these observations suggest that sumoylation of TDG in concert with intracellular trafficking of SUMO may contribute to regulation of TDG subcellular localization which is dependent on TDG SUMO binding activity.

Figure 4.4. Sumoylation of TDG may promote nucleolar localization. Expression vectors for the indicated proteins were cotransfected into MCF-7 cells using Lipofectamine 2000 transfection reagent. After allowing 24 hours for expression, cells were fixed in 4% formaldehyde/PBS and fluorescent fusion proteins were detected by epifluorescence microscopy using an Axiovert microscope (Zeiss) and the appropriate filters. Representative 0.5 μM sections are shown. Arrows indicate nucleoli, or promyelocytic leukemia (PML) oncogenic domains (PODs) where appropriate.

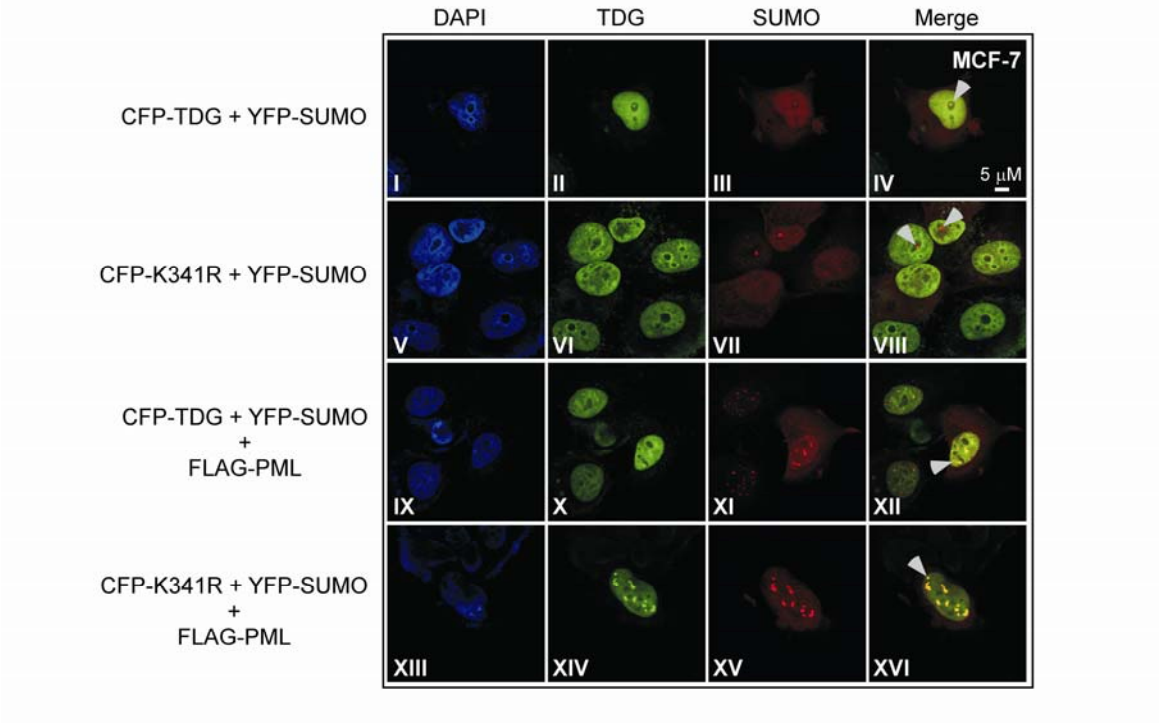
DAPI
TDG
SUMO
Merge
MCF-7
CFP-TDG + YFP-SUMO
CFP-K341R + YFP-SUMO
CFP-TDG + YFP-SUMO
+
FLAG-PML
CFP-K341R + YFP-SUMO
+
FLAG-PML
5 μM
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
XIII
XIV
XV
XVI

Figure 4.4

We were intrigued by the observed cytoplasmic redistribution of TDG in senescent fibroblasts and hypothesized about the possible consequences this may have on genome maintenance and transcriptional regulation. To further explore the association of TDG with active transcription we investigated the extent of TDG colocalization with nuclear transcription sites. In order to minimize the effects of the observed alterations in TDG regulation related to passage number in standard MEF cells or from the IMR-90 cells lines we utilized P19 mouse embryonic carcinoma stem cells for this purpose. We immunostained P19 cells with antibodies specific for TDG to detect endogenous TDG and phosphorylated RNA polymerase II (p-polII) to detect sites of active transcription (54). When we examined the localization of endogenous TDG and p-polII we found a dramatic colocalization of the two proteins consistent with a role for TDG in active transcription and the coupling of BER to transcription (Figure 4.5, panel V). Interestingly, treatment with PMA reduced the extent to which TDG and p-polII colocalized (Figure 4.5, compare panels V and X), suggesting that phosphorylation events control either directly or indirectly the cellular functions of TDG.

Figure 4.5. Colocalization of TDG with nuclear transcription sites in P19 embryonic carcinoma cells: Influence of PKC activation. TDG associates with sites of active transcription, but dissociates upon treatment with phorbol ester PMA. P19 embryonic carcinoma cells were treated with 100 nM PMA for 20 minutes before fixation with 4% formaldehyde. Endogenous TDG and phosphorylated RNA polymerase II (p-polII) were detected in by immunostaining with specific antibodies and 0.5 μm sections were obtained as described above. Arrows indicate heterochromatic regions.

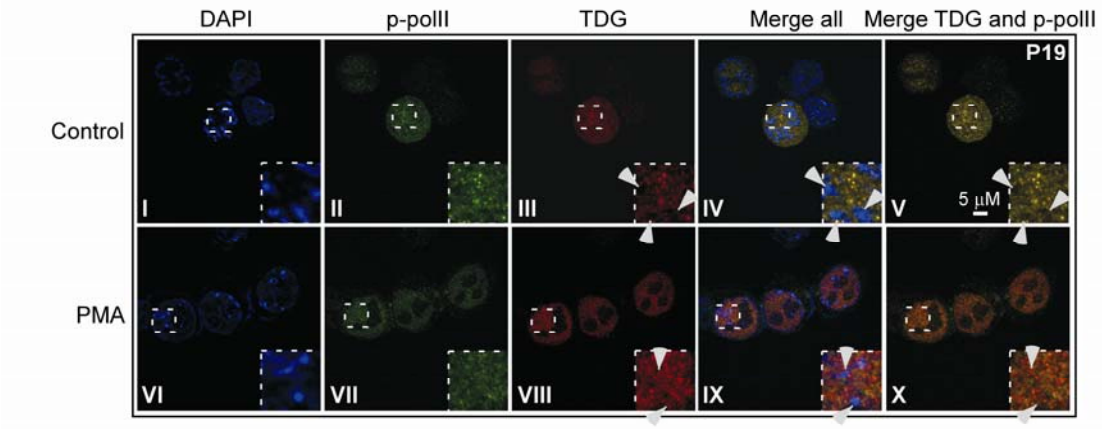
DAPI
p-polII
TDG
Merge all
Merge TDG and p-polII
Control
PMA
P19
5 µM
I
II
III
IV
V
VI
VII
VIII
IX
X

Figure 4.5

We also immunostained endogenous TDG and PKCα in P19 cells treated with PMA. In untreated P19 cells, the majority of PKCα was detected throughout the nucleus excluding the nucleoli, and no gross changes in PKCα localization were detected after PMA treatment. However, a fraction of TDG relocalized to heterochromatic regions and showed an increased localization with PKCα (Fig 4.6, compare panels V and X. Arrows indicate heterochromatic regions.). This was not accompanied by a drastic loss of localization of TDG with euchromatic areas of the nucleus. These results suggest that PKC activation promotes localization of with transcriptionally inactive heterochromatic regions.

Figure 4.6. Nuclear colocalization of TDG and PKCα is altered by phorbol ester treatment. IMR-90 cells were treated and analyzed as in Fig. 4.5 using antibodies toward endogenous TDG and PKCα. Arrows indicate heterochromatic regions.

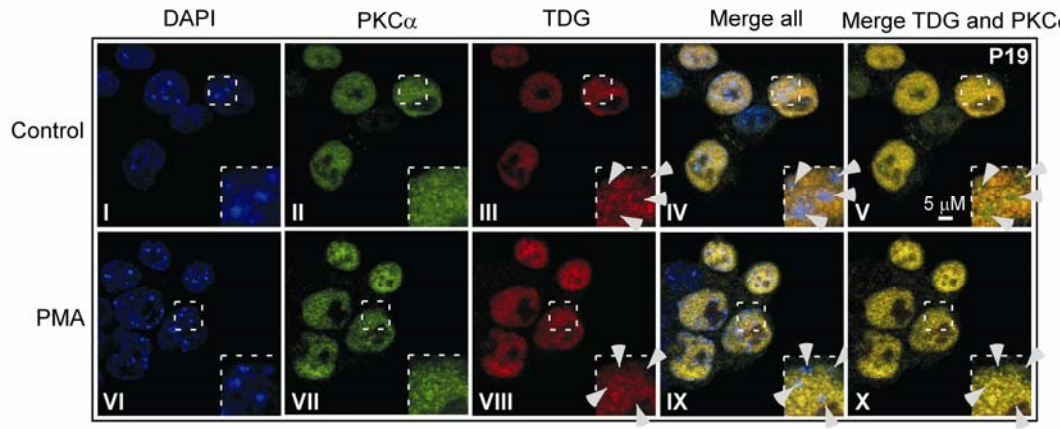
DAPI
PKCα
TDG
Merge all
Merge TDG and PKCα
Control
PMA
P19
5 μM
I
II
III
IV
V
VI
VII
VIII
IX
X

Figure 4.6

4.4 Discussion

We have shown that in senescent mouse and human fibroblasts TDG is found in both the nuclear and cytoplasmic compartments in contrast to the strictly nuclear localization observed in proliferating fibroblasts. The cytoplasmic localization coincides with altered posttranslational modification consistent with increased sumoylation or ubiquitination. Interestingly, exposing asynchronously cycling fibroblasts to oxidative stress also induced sumoylation of TDG. When we examined the localization of sumoylated TDG in breast carcinoma cells however, we found that sumoylation was associated with entry into nucleoli. We also found that TDG associates with sites of active transcription and that activation of PKC, previously shown to phosphorylate TDG, resulted in decreased association with sites of active transcription and increased association with heterochromatin. Our findings suggest that TDG PTMs are important regulators of TDG subcellular localization and that the effects PTMs on TDG may be cell-type and/or cell-cycle dependent. Importantly, we have shown that external agents may be used to alter TDG PTM in cells.

Cells may enter senescence in response to extended proliferation, telomere loss, oncogene activation, or oxidative damage (39). It has been proposed that cells in culture undergo senescence due to exposure to super-physiological concentrations of oxygen as opposed to the lower oxygen concentration found *in vivo*, suggesting that senescence is likely caused by oxidative stress. Furthermore, some evidence suggests that cells grown in low oxygen conditions may grow indefinitely (11). The most obvious sign of senescence is growth arrest caused by failure to progress from G1 to S phase (59) also termed G0. Analysis of aging mouse tissues has revealed that they are deficient in base excision repair (BER) (21) and accumulate oxidative DNA damage (2, 17, 24, 28, 29, 61) suggesting that these cells sustain genomic damage over time which may contribute to

evasion of protective cell cycle arrest mechanisms and carcinogenesis. For example, telomerase has been found to be activated in over 90 percent of human tumors and cancer cells (10, 23). Extensive evidence for misregulation of p53 and p21 or Rb mediated cell cycle arrest pathways has also been demonstrated in transformed cells (1, 16, 19, 25, 60, 65). BER is the predominant DNA repair pathway for repair of oxidative lesions in cells (37, 58) and so we chose to examine regulation of the essential protein (M. Tini, unpublished) and BER enzyme TDG, in senescent IMR-90 diploid cells. We found that TDG underwent a dramatic relocalization from nucleus to cytoplasm in these cells. Interestingly, there is a concomitant alteration in TDG distribution from being smoothly distributed within the nucleus, to forming distinct foci throughout the cell. Similarly, misregulation of BER protein subcellular localization to both nuclei and mitochondria has been observed with oxoguanine DNA glycosylase (OGG1) and AP endonuclease (APE) (62).

To exclude that extended culture had resulted in selection of aberrant cells, we also induced senescence by treating IMR-90 and mouse embryonic fibroblast (MEF) cells with histone deacetylase inhibitor (HDACi) sodium butyrate which induces a senescent phenotype (51). We found that this treatment induced dramatic redistribution of TDG from nucleus to cytoplasm similar to that seen in high passage number IMR-90 cells. This raised the intriguing possibility that TDG localization was regulated by post-translational modification (PTM) in cells and that this regulation may be altered as cells age. The BER protein APE, also a substrate for CBP-mediated acetylation, has been shown to be highly-acetylated and in aging cells (62) and this modification enhances APE binding to negative calcium response elements in the parathyroid hormone promoter (6). Although extended treatment with sodium butyrate induces numerous changes which result in a state similar to senescence (71), if an increased percentage of TDG is acetylated in aged cells this may predispose them to accumulating G:C to A:T transition

mutations since acetylated TDG does not excise thymine from G:T mispairs (45). This is supported by evidence showing an accumulation of these mutations in aging tissues (17) although a comparative measure of the G:T-mispair processing capacity of young and aged tissues has yet to be done. Furthermore, evidence indicates that senescence may act as a tumor suppressor, yet paradoxically correlative evidence suggests that because senescent cells are still susceptible to DNA damage and rely on maintenance of an elaborate senescence program mediated by the p53 and Rb pathways, which have been shown to be commonly misregulated in tumors, senescence may also contribute to carcinogenesis through reestablishment of cell division after transforming DNA damage (11). While the chance of incorporating DNA lesions such as mispairs may be offset by a lack of cell division, spontaneous DNA damage continues even after an organism has ended its natural life as a result of the inherent instability of the molecule (36).

Immunoblotting analysis of TDG in IMR-90 cells treated with sodium butyrate or trichostatin A (TSA) by immunoblotting identified a shift in mobility consistent with sumoylation of TDG, suggesting that acetylation promotes sumoylation. This could possibly be occurring in the cytoplasm, where we observe an increase in TDG localization. While sumoylation of TDG reduces acetylation of the protein *in vitro* (46), the effect of acetylation on sumoylation has not yet been investigated. Sumoylation likely prevents acetylation through steric hindrance of the acetylase (46), but such a mechanism does not preclude sumoylation of acetylated TDG. Previous studies indicate that phosphorylation and acetylation are mutually exclusive (45) yet treatment with PMA did not reduce sumoylation. It is possible that phorbol ester treatment may also promote sumoylation of TDG since both modifications reduce the charge of residues within the hydrophilic TDG amino terminus. Metabolic labeling experiments indicate that sumoylated TDG is indeed phosphorylated (45), although it is not clear which modification occurs first, nor whether there is an interplay between the modifications, or

what the nature of such an interplay may be. TDG was originally identified as a phosphoprotein based upon observations of differential mobility of TDG species in whole cell lysates fractionated by SDS-PAGE. These differences in mobility could be eliminated by pretreatment of lysates with calf thymus intestinal phosphatase (68). In this report, the authors observed TDG species around 60 kD and just over 84 kD. TDG, despite being a 45 kD peptide, normally resolves at about 60 kD when fractionated by SDS-PAGE. Interestingly, sumoylated TDG normally migrates at approximately the same position as the phosphatase-sensitive band in these experiments (68). If this were the case, it may mean that phosphorylation of TDG stabilizes TDG sumoylation while phosphatase treatment acts to destabilize TDG sumoylation.

Hydrogen peroxide has been shown to affect multiple signaling pathways and is itself a signaling molecule (18). Acting through CBP, peroxide treatment has been shown to cause acetylation of the FOXO4 transcription factor, reducing its transcriptional activity (69). PKCα is a classical PKC isozyme that normally requires calcium and diacylglycerol for activation. Some evidence suggests that PKCα, and perhaps other PKC isoforms, may be activated in the absence of these cofactors by hydrogen peroxide (32). Hydrogen peroxide stress has also been shown to modulate sumoylation of proteins negatively at low concentrations and positively at high concentrations (9). When we treated NIH 3T3 cells stably transduced with FLAG-TDG, with concentrations of hydrogen peroxide ranging from 10-500 μM we found a large increase in the proportion of S-TDG from concentrations beginning as low as 50 μM. The exact concentrations of hydrogen peroxide required to effect such changes is likely dependent on the culture conditions and cell type as previous studies performed in HeLa cells show that simply doubling the concentration of peroxide over a shorter treatment period may result in an overall decrease in sumoylation of cellular proteins, which is mediated by modulating the interactions between SUMO conjugating enzymes Uba2 and Ubc9 (9).

Considering the evidence that TDG sumoylation may be regulated in response to oxidative stress, we wanted to specifically determine the subnuclear localization of S-TDG. We found that when coexpressed with YFP-SUMO-1, CFP-TDG accumulates in nucleolar regions whereas CFP-TDG(K341R) was excluded, and instead accumulated around these non-membrane bound structures. In both cases, YFP-SUMO localized to the nucleoli. Coexpression of PML, a major component of the heavily sumoylated PML oncogenic domains (PODs), caused YFP-SUMO to accumulate in the PODs instead of nucleoli. Interestingly, both YFP-TDG and YFP-TDG(K341R) preferentially localized to the PODs in these cells. The lack of YFP-TDG(K341R) localization to nucleoli suggests that sumoylation is a dynamic process which can regulate TDG subcellular localization. Since TDG(K341R) is not defective in SUMO-binding, the differences in nucleolar localization are attributable to lack of sumoylation and not noncovalent SUMO interactions. Therefore, sumoylation appears to be a requisite step for entrance into these regions. SUMO proteases exist in the nucleolus and it is possible that sumoylated TDG crosses into the nucleoli where it is promptly desumoylated (63, 73). We have previously shown that SBMs in TDG are necessary for POD translocation and that TDG sumoylation negatively regulates this process (46). The observation that localization of YFP-SUMO to the PODs upon overexpression of PML causes a corresponding accumulation of TDG and TDG(K341R) to these structures indicates that SUMO binding is a critical determinant of the localization of TDG within the nucleus. Our current findings suggest that transient sumoylation may permit access into certain subcellular compartments such as the nucleoli and PODs. Interestingly, inhibition of proteasomal degradation by treatment with the peptide inhibitor MG132 in MCF-7, HeLa, and IB-4 cells causes a redistribution of PML and SUMO, as well as another POD constituent sp100, from the PODs to the nucleoli. Subsequent removal of MG132 allowed reconstitution of the PODs (41). This indicates that POD components, including SUMO, traffic to nucleoli in a process related to proteasomal degradation. These observations suggest that TDG

trafficking with SUMO to nucleoli may promote degradation of TDG by the proteasome, and that sumoylation may play an important role in permitting progression of this pathway in degrading TDG.

Senescence and oxidative stress have been associated with dramatic changes in chromatin structure and transcriptional programs (50, 74). These changes to the transcriptional program are associated with euchromatinization of some previously heterochromatic regions. There is an overall increase in heterochromatic regions in senescent nuclei compared to early passage cells (50), and this chromatin remodeling may act as a mechanism for silencing a portion of the numerous genes downregulated to create the senescence gene signature (74). TDG has been shown to associate preferentially with transcriptionally active euchromatic regions and act as a coactivator for multiple transcription factors in actively dividing cells and we were therefore interested in quantifying the extent to which TDG may be involved with transcriptional activity in cells. When we examined this at a gross level by immunostaining P19 cells with antibodies specific for endogenous TDG and transcriptionally active phosphorylated RNA polII (p-polII), we observed extensive colocalization of these proteins throughout the nucleus. Interestingly, treatment of cells with PMA caused reduced p-polII-TDG localization but increased TDG localization to heterochromatic foci which also contained PKCα. Examination of the effect this had on TDG-PKCα colocalization demonstrated an increase in colocalization similar to that seen between nuclear TDG and PKCα in NIH 3T3 fibroblasts (45). It is possible that phosphorylation of TDG in cycling cells serves to target TDG to heterochromatin without drastically decreasing the levels of TDG acting in euchromatic regions, while redistribution of TDG in senescent cells may serve to reduce the amount of TDG within the nucleus, thereby reducing the amount of TDG-mediated transcription occurring in these quiescent cells.

These observations suggest that the subcellular localization of TDG is altered in senescent fibroblasts, concurrent with alterations in TDG PTM. Additionally, we have shown for the first time that alterations in TDG PTM may induced by oxidative damage to proteins and DNA that promote cellular senescence or growth arrest. Interestingly, we have also demonstrated that TDG may be extensively associated with sites of active transcription in actively cycling cells, suggesting that BER mediated by TDG may play a more general role in transcriptional regulation than previously understood, and that modification of TDG and relocalization of the protein may contribute to the senescent transcriptional program.

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Chapter 5: Conclusion

TDG plays a role in maintaining genome stability through BER and also participates in transcriptional regulation. Consequently, TDG is predicted to play a role in diverse aspects of cellular function. The TDG protein possesses a central, highly conserved core region which is sufficient for excision of uracil from G:U mispairs within a CpG context. Less conserved are amino- and carboxy-terminal regions. Of these, the lysine and serine-rich amino terminus is of particular interest because it mediates numerous protein-protein interactions as well as tight DNA interactions necessary for excision of thymine from G:T mispairs within CpGs. Numerous TDG PTM have been characterized, and a majority are directed toward the amino terminus. We therefore hypothesized that PTM of the amino terminus may act to regulate TDG function. Accordingly, the aim of this thesis was to examine how TDG PTM may alter TDG subcellular localization, protein-protein interactions, and enzymatic function. The findings presented in this thesis and their implications are discussed in the following sections.

5.1 Summary

In Chapter 2 we examined the role of covalent attachment of SUMO (sumoylation) and non-covalent SUMO binding in regulating TDG subnuclear localization, protein-protein interactions, DNA binding, and enzymatic function. Initially, we undertook an examination of TDG subnuclear localization which indicated that TDG may localize to PODs by binding to SUMO conjugated to PML. Subsequent experiments revealed a novel amino-terminal SUMO binding motif ^{145}IVII^{148} (SBM1) which was conserved in various TDG orthologs and corresponded to a SUMO binding motif consensus sequence I/V-X-I/V-I/V (35) which could be recognized in both forward and reverse orientations (36). Mutation analysis showed that SBM1 was required for TDG-SUMO interactions and also for activation of CBP-mediated transcription. Analysis of *in vitro* sumoylated

TDG (S-TDG) showed that S-TDG did not bind stably to DNA and was deficient in excision of thymine from G:T mispairs, yet capable of excising uracil from G:U mispairs. Protein-protein interaction studies showed that sumoylation of TDG also prevented binding to free SUMO and abrogated interactions with CBP, including CBP-mediated acetylation of TDG.

In Chapter 3 we took a closer look at the TDG amino terminus and discovered the existence of PKCα phosphorylation sites on amino acid residues proximal to acetylated lysines 94, 95, and 98. The close proximity of the substrate residues for acetylation (K95, 95, and 98) to these acetylated serines (S96, 99) led us to investigate a potential relationship between these modifications. Remarkably, we found that acetylation and phosphorylation were mutually exclusive on TDG. Analysis of the functional consequences of these modifications on TDG substrate interactions showed that acetylated TDG lacked the ability to excise thymine from G:T mispairs, and was unable to bind an oligo bearing the same mispair while phosphorylated TDG retained these functions. Interestingly, while it appeared that sumoylation and acetylation of TDG both led to a loss of tight DNA interactions – resulting in a loss of G:T mispair processing ability, sumoylated TDG actually displayed an enhanced ability to process G:U mispairs while we did not observe the same with acetylated TDG.

In Chapter 4 we examined whether TDG regulation is altered in ageing cells. We examined the localization of endogenous TDG in IMR-90 and MEF cells approaching senescence and found that TDG is differentially localized in aging cells. Interestingly, we also found that inhibition of histone deacetylases may contribute to this process. Exposure of cells to oxidative stress, which is thought to contribute to ageing and has been shown to activate PKC as well as CBP, in addition to modulating cellular levels of sumoylation through targeting of SUMO conjugating enzymes, led to similar changes in

TDG mobility. An analysis of the consequences of TDG sumoylation suggested that this modification, may regulate TDG localization to nucleoli – normally sparsely populated by TDG. Interestingly, we also found that PML may play a role in coordinating SUMO-based regulation of TDG localization. To conclude Chapter 4, we examined colocalization between endogenous TDG and sites of active transcription in cells by immunostaining P19 embryonic carcinoma stem cells with TDG- and phosphorylated RNA polymerase II (p-polII)-specific antibodies and found extensive colocalization between TDG and p-polII which could be decreased by treatment of cells with the PKC-stimulating agent PMA, resulting in increased TDG association with heterochromatic regions from which TDG is otherwise largely excluded.

5.2 Significance and Conclusions

Together these data show that TDG is subject to multiple PTM including acetylation, phosphorylation, and sumoylation. Additionally, TDG has been shown to be polyubiquitinated during S-phase, leading to degradation of the protein (9). Furthermore, we demonstrate that these PTM are involved in an interplay which can regulate all aspects of TDG function including susceptibility to further PTM (Figure 5.1). It is important to note that many of these findings are novel observations. Accordingly Chapters 2 and 3 have been published in the journal of Molecular and Cellular Biology, and Nucleic Acids Research respectively. Although a complete understanding of how TDG participates in pathways mediating cellular processes is still unclear, these findings serve to show that TDG PTM can be a powerful regulator of TDG function.

Figure 5.1. Cross-talk between TDG posttranslational modifications. Previous studies have shown that sumoylation of human and mouse TDG induces a dramatic increase in G:U processing activity by promoting enzyme turnover (10, 28). In contrast, sumoylation (10, 28) or acetylation by CBP (this study) abrogate DNA binding and G:T processing (16, 41). TDG sumoylation also drastically reduces interactions with CBP/p300, thereby preventing efficient acetylation (28). Phosphorylation of serine residues adjacent to acetyl-acceptor lysines by PKCα prevents acetylation by CBP and may preserve G:T processing *in vivo* (27). This regulation may occur over rapid or extended time periods, in response to external stimuli and pharmacological agents, resulting in altered TDG function, localization, and interaction with DNA substrates or protein partners.

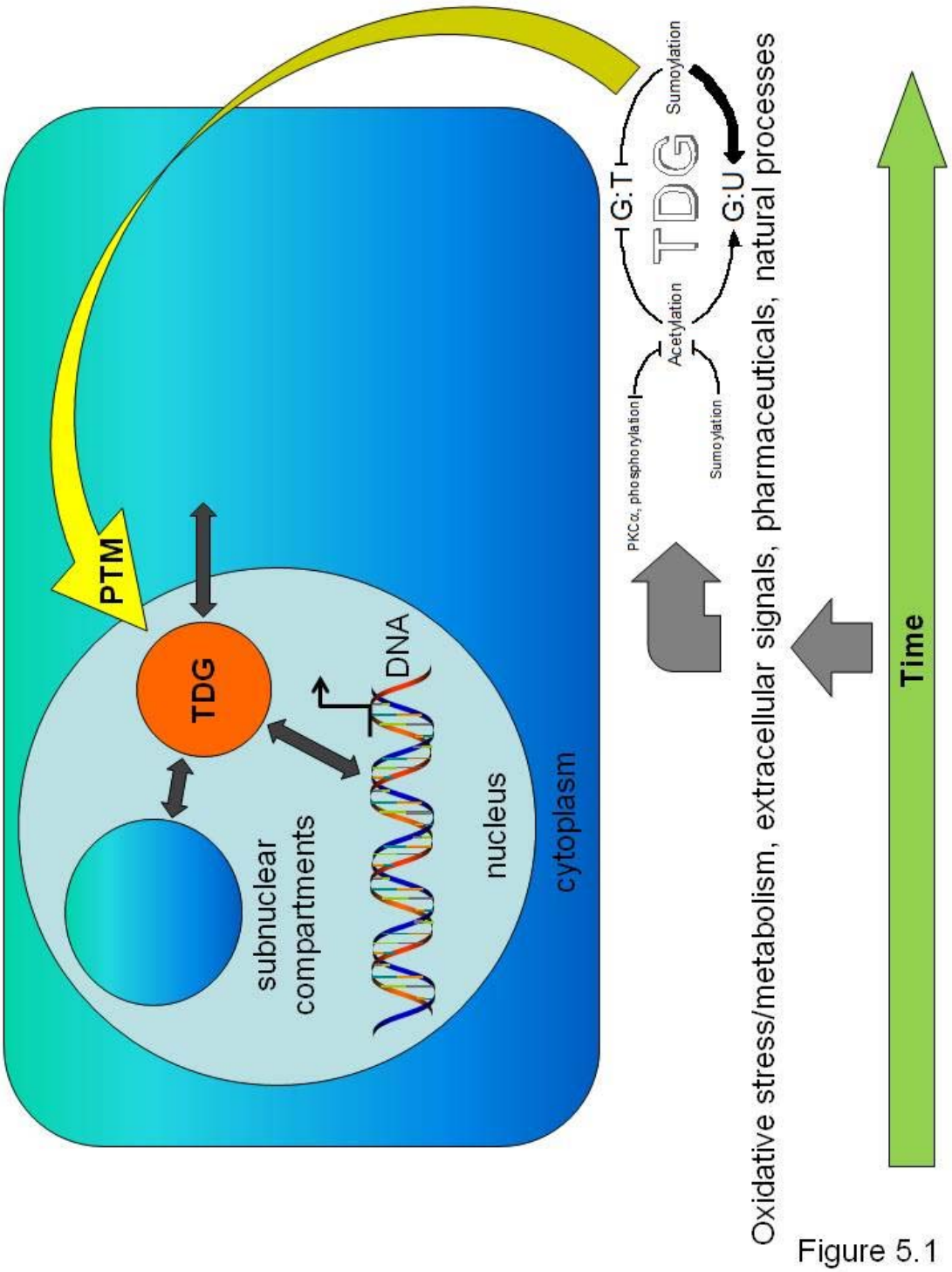
PTM
TDG
DNA
subnuclear compartments
nucleus
cytoplasm
PKCα, phosphorylation
Acetylation
Sumoylation
TDG
G:T
G:U
Sumoylation
Oxidative stress/metabolism, extracellular signals, pharmaceuticals, natural processes
Time

Figure 5.1

We began our investigations into TDG PTM by examining the roles of sumoylation and SUMO binding in regulation of TDG. In early studies examining TDG sumoylation, it was proposed that sumoylation of TDG occurred primarily on DNA and was a mechanism for facilitating conformational changes in TDG which promote its disassociation from abasic sites (10, 37). Here we present a number of observations which suggest that sumoylation may occur in other contexts as well. Our first indication that sumoylation may not be exclusively associated with DNA repair activities came from observations that glycosylase deficient point mutant TDG(N151A) was sumoylated as readily as wild-type TDG. Despite being unable to excise mispaired bases, TDG(N151A) was able to bind abasic sites, although it seemed unlikely that this could account for the degree of sumoylation observed. Furthermore, we showed in Chapter 3 that APE could effectively displace TDG from abasic sites in the absence of sumoylation *in vitro* and endogenous levels of APE should suffice to do the same *in vivo* (27). Other observations outlined in Chapter 4 pointed to a number of potential roles for sumoylation in TDG function. For example, we showed that co-expression of YFP-TDG and CFP-SUMO resulted in nucleolar localization for TDG, but sumoylation-minus mutant TDG(K341R) was excluded from these non-membrane-bound subnuclear compartments, indicating that sumoylation may play a role in facilitating entry into these areas. Interestingly, when we examined TDG regulation in senescent cells we noted an apparent increase in sumoylated TDG in addition to a dramatic relocalization of the protein, suggesting that sumoylation may facilitate passage into the cytoplasm or alternatively that TDG may be subject to increased sumoylation within the cytosol. With respect to the senescence program, it is unclear whether sumoylation of TDG is necessary for establishing senescence or if it is a product of the process.

Once sumoylated, TDG displays altered biochemical properties. In Chapter 2 we propose that this is due to changes in TDG conformation attributable to intramolecular SUMO-

binding mediated by the dual SUMO binding motifs SBM1 and SBM2. Consequently, S-TDG no longer excises thymine from G:T mispairs, or binds free SUMO. Additionally, sumoylation of TDG reduces interactions with CBP as well as CBP-mediated acetylation of TDG. We were also able to replicate experiments performed with human S-TDG using mouse S-TDG to show that sumoylated TDG is more efficient than unmodified TDG in excision of uracil from CpGs due to increased turnover on the product abasic site (28, 37). This seemingly implies that S-TDG may also be more efficient at processing mispairs *in vivo*, however the base-flipping mechanism of glycosylase function requires that TDG systematically scan the genome, flipping out each base until an aberrant base is detected and excised. It seems unlikely that S-TDG, with its reduced DNA-binding ability would serve such a function efficiently. Interestingly, we also found an increase in TDG sumoylation in cells exposed to oxidative stress. This observation may be interpreted in two ways; if sumoylation of TDG is simply a switch to produce a more efficient glycosylase, then it would be predicted that increased S-TDG would promote cell survival. If not, then it would promote genome instability and apoptosis or senescence.

We have also presented evidence indicating that the SUMO binding activity of TDG plays an important role in regulating TDG function. For example, we show that TDG can bind to SUMO conjugated to p53. This observation opens the door to an entirely new set of interacting proteins for TDG as it could potentially interact with any number of sumoylated proteins and is an important consideration for future studies. For example, after purification of a putative TDG cellular complex it may be tempting to dismiss an interaction mediated through a sumoylated protein as artefact if it cannot be verified *in vitro* with unmodified proteins. Interestingly we also found that mutants of TDG deficient in SUMO binding were unable to activate CBP-mediated transcription on a reporter gene (28). Although the nature of the requirement for SUMO binding activity in

this context is unclear, it suggests sumoylation of TDG may also reduce activation of CBP-mediated transcription by preventing SUMO binding. Such a prediction is supported by our observation that sumoylation of TDG reduces direct interactions with CBP. These findings may have a broader effect on transcriptional regulation in addition to affecting pathways directly regulated by TDG. This is because CBP/p300 transcription is partially regulated through competition between transcription factors for a limited number of CBP/p300 molecules in the nucleus (3, 18, 32). In Chapter 4 we show that senescence is accompanied by a concurrent increase in TDG sumoylation and dramatic subcellular redistribution which excludes approximately half of the cellular compliment of TDG from the nucleus. This may serve to reduce TDG-associated activation CBP while allowing other factors access to this important mediator of transcription. Hundreds of genes are differentially regulated in senescence and such a redistribution of transcription-associated factors would contribute to effecting these changes (43).

Acetylation of TDG by CBP/p300 was first observed by Tini et al who identified the acetylated lysine residues and showed that acetylation of TDG promotes dissociation of TDG-CBP complexes and reduces interactions with APE *in vitro* (39). In Chapter 3 we show that acetylation of TDG is inhibited by DNA interactions and that acetylation of TDG reduces stable DNA binding and processing of thymine from G:T mispairs (27). The latter observation is of particular interest because it provides a mechanism for regulating pathways that utilize thymine glycosylase activity such as BER and cyclical demethylation of methylcytosine on transcriptionally-active promoters. With respect to transcription, analysis of a glycosylase deficient mutant of TDG (mouse TDG N151A) has indicated that this activity is dispensable for activation of some transcriptional pathways as it was able to activate CBP-mediated transcription on a reporter gene (39). Similarly, glycosylase activity was found to be dispensable for ER-mediated transcription in reporter gene assays (5). However, glycosylase activity was shown to be required for

participation in cyclical demethylation of the transcriptionally active estrogen responsive *pS2* and *Wisp-2* gene promoters (25). This suggests that acetylation of TDG could selectively inhibit glycosylase-dependent transcription while leaving glycosylase-independent pathways active and potentially provide an additional level of transcriptional control to the cell in response to nuclear receptor ligands such as estrogen. Use of PTM to regulate TDG participation in transcriptional processes would be consistent with observations that interactions between CBP and some transcription factors may be regulated by upstream PTM (17). For example, phosphorylation of cyclic-AMP response element binding protein (CREB) at serine 133 by protein kinase C (PKC) facilitates interaction with CBP allowing transcriptional activation (15).

Another possibility includes use of TDG acetylation as a mechanism for discriminating between damaged methylated or unmethylated cytosine residues (26). Because the product of cytosine deamination and 5-methylcytosine deamination are uracil and thymine respectively acetylated TDG would permit determination of the undamaged state of the lesion because it discriminates between the products of cytosine deamination whereas unmodified TDG processes both lesions. In fact, Metivier et al employed a similar principle to distinguish between the products of cytosine or 5-methylcytosine deamination in an *in vitro* assay designed to demonstrate that Dnmt3 deaminates 5-methylcytosine. They compared base excision by TDG to that of UNG, a glycosylase capable of excising uracil but not thymine mispaired with guanine, on a defined template bearing either methylated or unmethylated cytosine residues and were able to show that abasic sites were created on methylated templates only when pre-treated with Dnmt3 catalytic fragments followed by treatment with TDG, indicating that 5-methylcytosine had been deaminated and the resulting thymine residue excised (25). *In vivo*, acetylated TDG could take the place of UDG to facilitate discrimination between formerly methylated or non-methylated cytosines. In this scenario one would expect that TDG

would associate with cellular machinery which is competent for methylation of cytosine and it was been shown that TDG associates with cytosine methyltransferases Dnmt3a and 3b (2, 20, 25). While it remains to be determined whether acetylation of TDG alters this interaction our investigations into the properties of acetylated TDG, described in Chapter 3, show that acetylation of TDG induces conformational changes in the protein which alter DNA interactions and these conformational changes may also promote differential association with protein complexes *in vivo* (27).

In Chapters 3 and 4 we firmly establish phosphorylation as an important PTM in regulation of TDG. Interestingly, we showed in Chapter 4 that treatment of P19 cells with PKC agonist PMA increased TDG localization to heterochromatic foci. Previously, separate studies showed that TDG localizes to heterochromatic regions in complex with both Dnmt3a and 3b. TDG was shown to recruit Dnmt3a to DNA *in vitro* and colocalize with Dnmt3a in heterochromatic foci in untreated NIH 3T3 cells (20). Dnmt3b was shown to be necessary for association with heterochromatic minor and major periocentromeric repeats in untreated P19 cells (2). In Chapter 3 we show that phosphorylation of TDG reduces acetylation of the protein, preventing loss of G:T processing activity (27). This suggests that phosphorylation would be favorable for TDG participation in establishing patterns of cyclical cytosine demethylation on transcriptionally active promoters along with Dnmt3a and 3b because it would prevent acetyltransferases from stalling transcription by neutralizing TDG before excision of the mutagenic mispaired thymine residue. There is much still to be learned about the consequences of TDG phosphorylation and its role in regulating TDG function beyond inhibiting acetylation of TDG.

During analysis of TDG in lysates prepared from P19 embryonic stem cells and mouse embryonic fibroblasts by SDS-PAGE fractionation and immunoblotting with a TDG-

specific antibody we observed a novel shift in TDG mobility consistent with near complete sumoylation of the protein. Interestingly, when we analyzed lysates prepared from these cells after entry into senescence, we noted a further discrete alteration in TDG mobility similar to that which might be seen upon conjugation of another SUMO peptide to TDG (Chapter 4). Although dual sumoylation of TDG *in vivo* has not yet been described and the TDG peptide contains only one SUMO conjugation consensus site, TDG has been shown to be sumoylated by SUMO-3 in vitro (1), and SUMO-3 may be sumoylated by SUMO-1 which might explain the two discrete alterations in mobility (24). While only a few proteins are known to be modified by both SUMO-1 and SUMO-2/3, sumoylation of TDG with either SUMO-1 or -2/3 has been shown to result in similar biochemical properties for the sumoylated protein (1). SUMO-1 and -2/3 do however, localize differently within cells (44) and we observed concomitant modification and redistribution of TDG in senescent cells suggesting that the second sumoylation event may be a signal for relocalization (Chapter 4). Alternatively, TDG has been shown to be polyubiquitinated (9) and it is possible that the observed alterations in mobility may be a result of monoubiquitination [see Hardeland et al. (2007) figure 5b lane 4 right panel (9)]. Further study is required to elucidate this novel mechanism of TDG regulation.

The findings reported in this thesis, within the context of previous studies done to investigate TDG function, have highlighted the amino terminus as a hotspot for PTM and protein-protein interactions in addition to being a critical mediator of TDG enzymatic function. Sequence analysis shows that the amino terminus is more evolutionarily divergent than the central core of the protein and it appears that function conferred by this divergent region correlate with mechanisms of genome regulation being utilized within the organism in question. For example, mammalian TDG is much more efficient at excising thymine from G:T mispairs than its *Drosophila* ortholog which surveys a genome possessing almost no DNA methylation (23) and excises thymine mispairs at a

physiologically irrelevant rate (8). Accordingly, discovery of a role for TDG thymine glycosylase activity in the process of cyclical demethylation of cytosine indicates that the amino terminus plays an important role in facilitating the complex regulatory mechanisms of higher organisms. To date most insights into the structure-function relationship of the TDG amino terminus have been gained through indirect evidence based upon partial proteolysis and functional assays performed with truncated proteins (10, 28, 37). Only one study describes an NMR-based examination of the TDG amino terminus and the authors show that the amino terminus assumes different conformations when examined in the presence or absence of the entire TDG peptide (33), suggesting that it is not feasible to study this region outside the context of the full molecule. Furthermore, our investigations show that the amino terminus is neither a modular DNA binding domain (28), nor sufficient for interactions with PKCα outside of the context of full-length TDG (27). Therefore, the findings outlined within this thesis represent important insights into the regulation of TDG and should provide a strong basis for future studies into TDG function in cellular and animal models, potentially leading toward use of TDG as a target for future therapeutics.

5.3 Research Impact and Future Directions

Cancer is a leading cause of death in affluent countries and the number of cases and deaths due to cancer are expected to double to over 26 million new cases and 17 million deaths per year by 2030 (38). The etiology of this disease is complex and many factors have been found to contribute to carcinogenesis. This poses a hurdle for chemotherapeutics as tumors have proven to be heterogeneous and cells within millimeters of each other may react to therapy differently. Compounding this problem, the tumor microenvironment has also been shown to foster carcinogenesis and tumor survival (6). Despite these therapeutic hurdles, there are common traits or hallmarks of

cancer cells which permit their identification and selective targeting by chemotherapeutics. For example, cancer cells commonly exhibit a tendency toward genomic instability and it is thought that as few as two mutations may destabilize the delicate balance of homeostasis and facilitate carcinogenesis. Once transformed, malignant cells undergo a number of genomic rearrangements which shuffle the genome, contributing to tumor heterogeneity and permitting evasion of normal processes that allow apoptosis and necrosis. For example, sampling of human tumors shows that one of the most often damaged genes is that of the p53 DNA damage response protein (30). Dysfunction of p53 and other cell cycle arrest genes is significant because they permit cell cycling to continue without first repairing damaged DNA, further promoting an accumulation of damage (31). This drastic loss of genomic stability displayed in all cancers means that the malignant cells become increasingly reliant on remaining DNA repair pathways to prevent complete genomic disintegration. This has led to the widespread use of chemotherapeutics which promote DNA damage to induce apoptosis in cancer cells.

The cancer therapeutic 5-fluorouracil 5-FU is widely used in combination therapy with other agents to treat a number of human cancers (21). *In vivo*, 5-FU produces metabolites such as fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP), and fluorouridine triphosphate (FUTP) (21). FUMP may be incorporated into RNA, disrupting RNA metabolism at multiple levels (13) while FdUMP may be directly incorporated into DNA during synthesis, creating A:5-FU or G:5-FU mispairs. FdUMP also causes nucleotide pool imbalances by inhibiting thymidylate synthase (11), which produces dTMP from dUMP, thereby increasing production of dUTP at the expense of dTTP. The increasing pools of U and 5-FU are incorporated into DNA and their active removal causes fragmentation of the genome as futile cycling of BER creates abasic sites which are susceptible to becoming double-stranded breaks (14). It has been shown that

TDG excises 5-FU with high efficiency from G:5-FU and A:5-FU DNA mispairs as well as from single stranded DNA *in vitro* (8). TDG was also shown to mediate 5-FU toxicity in cells (19).

In light of the important role TDG plays in effecting the cytotoxicity of this widely used chemotherapeutic, it may be useful to supplement 5-FU treatment with compounds which alter regulation of TDG PTM to favor apoptosis or cell cycle arrest. Studies are currently underway to gauge the effectiveness of agents generally targeting acetylation or phosphorylation to treat cancers by use of HDACi or kinase inhibitors alone or in combination have shown promise (4, 7, 29, 42). Previous findings indicating that TDG is a major mediator of 5-FU cytotoxicity (19) as well as the findings outlined within this thesis suggest that combining 5-FU treatment with pharmacological agents to selectively alter TDG PTM and promote 5-FU glycosylase activity may increase effectiveness of 5-FU-based therapy.

There has recently been a burst of important findings about TDG function however large gaps still exist about how these functions may be regulated and ultimately manipulated *in vivo*. Significantly, we have described here the existence of novel phosphorylation sites on TDG, and the only known examples of stimuli which alter TDG sumoylation and association with chromatin. Additionally, we have shown for the first time that TDG may be localized to the cytoplasm in senescent cells and in response to HDACi treatment. In order to most effectively employ TDG PTM-targeting therapeutics to compliment current cancer therapy strategies, more must be known about the mechanism by which TDG functions and the biological processes which would be affected by such interventions.

A largely neglected aspect of TDG function is its association with RNA. In previous studies TDG was purified as part of a putative demethylase complex from chick embryos and this complex included an RNA component which was assigned no specific role in the

demethylation process. TDG was also found to be present in a complex with Dnmt3a/b containing an RNA component. Although it has been suggested that the CpG-rich composition of these RNAs may promote targeting of the complex to CpG islands, the nature of any TDG-RNA interaction remains unclear and it has not yet been shown that the glycosylase interacts directly with RNA. Interestingly, TDG residues ^{94}KKSGK^{98} resemble an RNA binding motif identified in adenosine deaminases acting on RNA (ADAR) 1 and 2 KKXXK (40). Substitution of lysine residues within the KKXXK motif to EAXXA has been shown to abrogate RNA binding in ADAR1 and 2 (40) and may be a useful tool to further investigate putative TDG-RNA interactions within these complexes. Intriguingly, these conserved residues are also substrates for CBP/p300-mediated acetylation which we have shown in chapter 3 to also be regulated by PKC phosphorylation.

Future investigations should be directed toward completing our understanding of how TDG PTM regulate TDG function and subsequent PTM. For example, we have shown that sumoylation of TDG reduces acetylation of the protein through allosteric changes in the TDG peptide (28), but evidence presented in Chapter 4 indicate that acetylation may promote TDG sumoylation. Based upon the findings of Chapter 4, and close examination of previously published data which is discussed above (9), further investigation into the possibility that TDG is monoubiquitinated is also required. Monoubiquitination is a widely used PTM regulating diverse cellular processes including gene expression and may play a role in regulating TDG function as well (12). Finding answers to these questions is possible using the methods described in this thesis although it would be useful to develop antibodies or mass spectrometry methods which are able to detect TDG PTM in samples isolated from cells. Mass spectrometry-based methods would allow the detection of modified residues, and can be used to further verify crosstalk between modifications. For example, mass spectrometry of samples isolated from differentially

treated cells could be used to verify the mutual exclusivity of acetylation and phosphorylation on the amino terminus *in vivo* (27).

Modification-specific antibodies could be used to purify modification-specific TDG complexes from cells and to examine the subcellular localization of modified TDG. It will be important to capture these complexes and analyze their components in order to gain insight into the role TDG PTM play in regulating cellular functions. Since it is not possible to raise antibodies toward sumoylated TDG, we may use TDG(K341R) to identify proteins interacting with sumoylated TDG by comparing proteins in complex with TDG to those in complex with TDG(K341R) and identifying any not interacting with the sumoylation-minus mutant protein. These may be interacting specifically with sumoylated TDG. However, due to the small amount of S-TDG in most cells, this strategy may not be effective. We have already shown that sumoylation of TDG reduces interactions with CBP *in vitro* and that the SUMO binding activity of TDG is necessary for activation of CBP-mediated transcription. Therefore, we are confident that isolation and comparison of various TDG complexes would identify differences in the protein complexes formed by TDG and TDG SBM mutants or between TDG and sumoylated TDG *in vivo*.

Considering the increasing body of evidence for the transcriptional cofactor role of TDG, it is essential to identify target genes to gain further insights into its biological functions. As described in this thesis, both SUMO-binding and DNA glycosylase activities of TDG have been shown to be important for transcriptional regulation (25, 28). Determining the overall compliment of genes activated or repressed by targeting TDG may be achived using techniques such as chromatin immunoprecipitation - sequencing (ChIP-Seq) (22) to determine which promoters TDG associates with, followed by quantitative PCR (qPCR) using cells stably transfected with defined mutants of TDG in order to determine whether

loss of TDG function affects gene expression. Classification of these genes into subgroups dependent on specific TDG activities would provide a useful guide for future studies into potential therapeutics targeting TDG. These investigations are now routine and may be performed using either modification-specific TDG antibodies or stable cell lines transduced with TDG modification mimics for analysis by ChIP-seq and qPCR (22, 34).

5.4 Perspective

As discussed in this thesis, TDG is a critical cellular protein involved in multiple physiological processes including maintenance of genomic integrity and transcriptional regulation, including removal of epigenetic marks. Abberant regulation of any of these processes has been shown to contribute to disease and carcinogenesis. Here we have shown that TDG is subject to multiple PTM which can dramatically alter TDG function *in vitro* and *in vivo*. Furthermore, we describe a unique interplay between PTM of TDG in which modified TDG displays differential susceptibility to further PTM. Interestingly, we also show that TDG is differentially regulated in senescent cells and importantly, we identify pharmacological agents which can alter TDG PTM in cells. A useful way to look at our findings is that we have identified biochemical switches controlling TDG and that future studies will attempt to identify how these switches can be pharmacologically regulated to potentially enhance the efficacy of cancer chemotherapy.

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PUBLICATIONS

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ABSTRACTS (POSTER PRESENTATIONS)

Mohan RD, Dong Y, Litchfield D, Torchia J, Tini M. Opposing roles of adjacent phosphorylation and acetylation in DNA mispair processing by thymine DNA glycosylase. *Stevenson Memorial Lecture and Research Day*. (2008) ***Award winner – best poster**

Mohan RD, Dong Y, Torchia J, Tini M. Regulation of DNA damage processing by covalent modification of thymine DNA glycosylase. *1st Annual Canadian Human Genetics Conference*. (2008)

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75th Annual Cold Spring Harbor Symposium – Nuclear organization. Cold Spring Harbor, New York. (2010)

Stevenson Memorial Lecture and Research Day. London, Ontario. (2008)

1st Annual Canadian Human Genetics Conference. St-Sauveur, Quebec. (2008)

National Cancer Institute of Canada (NCIC) 60th anniversary Meeting. Toronto, Ontario. (2007)

INVITED ORAL PRESENTATIONS

Mohan RD. Regulation of DNA Damage Processing by Covalent Modification of Thymine DNA Glycosylase. The Stowers Institute for Medical Research. (2009).

Mohan RD, Thillainadesan G, Croker A. Awardee experiences in the CaRTT program, and recommendations for future directions. CIHR Strategic Training Program in Cancer Research and Technology Transfer (CaRTT) Annual Retreat. (2009).

Mohan RD, Dong Y, Litchfield D, Torchia J, Tini M. Regulation of DNA Damage Processing by Covalent Modification of Thymine DNA Glycosylase. Oncology Research Day. (2009).

TEACHING

Teaching Assistant (TA). Pharmacology 463a, Principles of Toxicology. (2006)

LEADERSHIP AND SCHOLARLY ACTIVITIES

Program Advisory Committee: Canadian Institutes of Health Research (CIHR) Strategic Training Program in Cancer Research and Technology Transfer (CaRTT).

Scientific Advisor and Mentor: Post-Doctoral Fellow: Amir Mhawi; M.SC. students: Jason Gagliardi (M.Sc., Microbiology (2006), Anita Rao (M.Sc., Physiology (2008)); 4th year students: Kieran Ritchie, Monika Demidas, Laura Metcalf, Tim Richards, John Shin, Nathan Schachter, Josh Malekzadeh, Sanjeeban Mishra, Kaitlyn Shaw, Lynn Nguyen; Technician: Debbie (Yuhua) Dong. 09/2004 – Present

Data Club Presenter and Participant: The Cell Biology and Gene Regulation group at the Siebens Drake Medical Research Institute. Presentations cover my body of work and afterward I answer questions regarding the data and its implications. 10/2003 – Present

Science Fair Judge: London Ontario Regional Science Fair. 2005-2009

Shinerama Leader: Faculty of Science. The Shinerama charity supports cystic fibrosis research. 09/2001

Group Leader: Let's Talk About Science. 09/1999 – 05/2000

President: Delaware Hall Resident's Council. 03/1999 – 03/2000