THE ROLE OF KRAB-ZFP ASSOCIATED PROTEIN 1 (KAP1) IN THE CONTROL OF PROMYELOCYTIC LEUKAEMIA NUCLEAR BODY (PML NB) NUMBER

by

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ABSTRACT

Promyelocytic leukemia nuclear bodies (PML NBs) are dynamic subnuclear protein compartments that are thought to play roles in many diverse cellular functions. The major component of PML NBs is the PML protein, and PML gene expression is frequently lost in human cancers. PML NBs also play a role in the response to DNA damage. We and others have shown that PML NBs behave as DNA-damage sensors by increasing in number following the induction of DNA breaks. However, the molecular mechanism behind this phenomenon has not been elucidated. Previous studies have established that the ataxia telangiectasia (AT) mutated (ATM) and AT and Rad3-related (ATR) kinase pathways can regulate PML NB number post-DNA damage. After ATM activation, DNA repair is initiated as well as decondensation of chromatin by phosphorylation of KRAB-ZFP associated protein 1 (Kap1). PML NBs make contacts with the surrounding chromatin and can increase in number in response to perturbations in chromatin structure. Therefore, changes in PML NB number in response to DNA damage may arise due to ATM-mediated Kap1-dependent decondensation of chromatin. We have assessed PML NB number in normal and Kap1-deficient cells before and after DNA damage. Loss of Kap1 causes PML NB number to increase without DNA damage, directly implicating Kap1 in the control of PML NB number due to changes in chromatin structure. Phosphorylation of Kap1 also plays a role in regulating PML NB number. A Kap1-independent process regulates PML NB number following DNA damage, as PML NB number further increase post-DNA damage. Understanding the mechanism(s) behind this phenomenon is an important step towards individualized cancer therapy by validating the PML NB as a biomarker for tumour response to radiation and chemotherapy.

LIST OF ABBREVIATIONS USED

53BP1	53-binding protein 1
a.u.	Arbitrary units
Akt	AK mouse transformed
ALT	Alternative lengthening of telomeres
alpha-MEME	alpha-Minimum essential media eagle
AML1	Acute myeloid leukaemia 1
APBs	ALT-associated PML NBs
APL	Acute promyelocytic leukaemia
As_2O_3	Arsenic trioxide
ASF1a	Anti-silencing 1a
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia and Rad3-related
ATRIP	ATR-interacting protein
ATRX	Alpha thalassemia/mental retardation syndrome X-linked
Bak	Bcl2-antagonist/killer 1
Bax	Bcl2-associated X protein
Bcl-2	B-cell CLL/lymphoma 2
BD	Binding domain
BLM	Bloom syndrome
bp	Base pairs
BRCA1	Breast cancer 1, early onset

BRCA2	Breast cancer 2, early onset
BRCT	BRCA1 C-terminal domain
BrdU	Bromodeoxyuridine
Bromo	Bromodomain
C term	C terminus
$CaCl_2$	Calcium chloride
CBP	CREB-binding protein
Cdc	Cell division cycle
Cdk	Cyclin-dependent kinase
Chk1	Checkpoint kinase 1
Chk2	Checkpoint kinase 2
CK2	Casein kinase 2
CML	Chronic myeloid leukaemia
CREB	Cyclic adenosine monophosphate response element binding
CO_2	Carbon dioxide
DAPI	4,6'diamidino-2-phenylindole
Daxx	Death-domain-associated protein
Dbf4	Dumbell forming 4
DFC	Dense fibrillar component
DMEM	Dublecco's modified eagle medium
DMP-30	2,4,6-tris(dimethylaminomethyl)phenol
DNA	Deoxyribonucleic acid
DNAPK	DNA protein kinase

DNAPK-cs	DNA protein kinase-catalytic subunits
Dnase-I	Deoxyribonuclease I
DSB	Double-strand break
DTT	Dithiothreitol
E2F	E2 transcription factor
EDTA	Ethylenediaminetetraacetic acid
EMS	Electron Microscopy Sciences
EtBr	Ethidium bromide
FANCD2	Fanconi's anemia complementation group 2
Fas	Apoptosis stimulating fragment
FBS	Fetal bovine serum
FC	Fibrillar Centre
FISH	Fluorescent in situ hybridization
G1-phase	Growth phase #1
G2-phase	Growth phase #2
Gadd45α	Growth arrest and DNA-damage inducible 45 α
GFP	Green fluorescent protein
GSK3β	Glycogen synthase kinase 3 β
h	Hours
H1	Histone 1
H2A	Histone 2A
H2A.X	Histone 2A.X
H2B	Histone 2B

H3	Histone 3
H3K27	Histone 3 lysine residue 27
H3K36	Histone 3 lysine residue 36
H3K4	Histone 3 lysine residue 4
H3K79	Histone 3 lysine residue 79
H3K9	Histone 3 lysine residue 9
H3me3K9	Histone 3 tri-methylated on lysine residue 9
H4	Histone 4
H4K20	Histone 4 lysine residue 20
HA	Hemagglutinin
HAT	Histone acetyltransferase
HAUSP	Herpesvirus-associated ubiquitin-specific protease
HCl	Hydrochloric acid
HDAC	Histone deacetylase
HDAC7	Histone deacetylase 7
HDM2	Human double-minute homolog
HIPK2	Homeodomain-interacting protein kinase 2
HIRA	Histone cell-cycle regulation defective A
HMET	Histone methyltransferase
HP1	Heterochromatin protein 1
HR	Homologous recombination
HRc	High resolution camera
HSV-1	Herpes simplex virus-1

Hyg	Hygromycin
ICD	Interochromosomal domain
ICP0	Infected Cell Polypeptide 0
INO80	INOsitol requiring 80
IR	Ionizing radiation
Isg20	Interferon-stimulated exonuclease gene 20
KAP1	KRAB-associated protein 1
kB	Kilobases
KCl	Potassium Chloride
kDa	Kilodalton
KRAB	Krüppel-associated box domain
KRIP1	KRAB-interacting protein 1
Ku	Ku thyroid autoantigen
LIG4	DNA ligase 4
LM/ESI	Light microscopy/electron spectroscopic imaging
MAPK-1	Mitogen-activated kinase-1
MAPPs	Mitotic accumulations of PML protein
Mb	Megabases
MDC1	Mediator of DNA-damage checkpoint 1
Mdm2	Murine double-minute 2
mg	Milligrams
MHCI	Major histocompatibility class I
min	Minutes

mL	Millilitres
mM	Millimolar
MMS4	Methyl Methane Sulfonate sensitivity 4
MNase	Micrococcal nuclease
M-phase	Mitosis phase
Mre11	Meiotic recombination 11
MRN	Mre11-Rad50-NBS1
mRNA	Messenger RNA
MUS81	Methyl Methane Sulfonate and UV sensitive 81
MX1	Myxovirus resistance 1
N term	N terminus
NA	Numerical aperture
Na_3VO_4	Sodium vanadate
NaCl	Sodium chloride
NaF	Sodium fluoride
NB	Nuclear body
NBS1	Nijmegen breakage syndrome 1
ND10	Nuclear domain 10
Neo	Neomycin
NHEJ	Non-homologous end joining
NHF	Normal human fibroblast
NIH	National Institute of Health
nm	Nanometer

NMA	N-pyridoxyl-2-methylalanine-5-phosphate
NOPP140	Nucleolar and coiled-body phosphoprotein 1
NOR	Nucleolar organizer regions
NPAT	Nuclear protein of the ATM locus
NSA	Nonenyl Succinic Anhydride
NuA4	Nucleosome acetyltransferase of H4
NuRD	Nucleosome remodeling
p21	Protein 21 kDa
p27Kip1	Protein 27 kDa kinesin-related protein 1
p300	Protein 300 kDa
p53	Protein 53 kDa
PA28γ	Proteasome activator subunit γ
pAkt	Phophorylated Akt
PBS	Phosphate-buffered saline
PCNA	Proliferating-cell nuclear antigen
PFA	Paraformadehyde
PHD	Plant homeo domain
PI3K	Phophoinositide-3-kinase
PIKK	PI3K-like protein kinase
Pin1	Peptidyl-prolyl cis/trans isomerase
PIP2	Phosphatidylinositol-4,5-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PKB	Protein kinase B

PKC	Protein kinase C
PML	Promyelocytic leukaemia
PML NB	Promyelocytic leukaemia nuclear body
PODS	PML oncogenic domains
PP2A	Protein phosphatase 2A
pRb	Retinoblastoma protein
PrK	Proteinase K
PTEN	Phosphatase and tensin homologue deleted in chromosome 10
Puro	Puromycin
PxVxL	Proline-any amino acid-valine-any amino acid-leucine
Rad	RADiation sensitive
RARα	Retinoic acid receptor α
RBCC	RING-finger B box coiled-coil domain
rDNA	Ribosomal DNA
RecQ	Recombinase Q
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
RNF	Ring-finger nuclear factor
RNP	Ribonucleoprotein
RPA	Replication protein A
rpm	Rotations per minute
rRNA	Ribosomal RNA
RSC	Chromatin structure remodeling complex

RT	Room temperature
S824A	Serine residue at position 824 replaced with alanine
S824D	Serine residue at position 824 replaced with aspartic acid
SAHF	Senescence-associated heterochromatin foci
SARA	Smad anchor for receptor activation
SCE	Sister-chromatid exchange
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
Ser	Serine
SETDB1	SET-domain bifurcated 1
shRNA	Short hairpin RNA
Smad2/3	SMA and mothers against decapentaplegic 2/3
SMC1	Structural maintenance of chromosome 1
SMN	Survival of motor neurons
snoRNA	Small nucleolar RNA
snRNA	Small nuclear RNA
snRNP	Small nuclear RNP
snoRNP	Small nucleolar RNP
Sp100	Speckled 100 kDa
S-phase	Synthesis phase
SQ/TQ	Serine-Glutamine or Threonine-Glutamine
SSBs	Single-strand breaks
ssDNA	Single-stranded DNA

Su(Var)3-7	Suppressors of variegation 3-7
Su(Var)3-9	Suppressors of variegation 3-9
SUMO-1	SMT3 suppressor of mif two 3 homolog 1
SWI-SNF	SWItch/Sucrose NonFermentable
SWR1	Sick With Rat8 1
TERT	Telomerase reverse transcriptase
Tert-NHF	Immortalized normal human fibroblasts
TGFβ	Transforming growth factor β
Thr	Threonine
TIF1	Transcription intermediary factor 1
TIP60	Tat-interactive protein 60 kDa
TNFα	Tumour necrosis factor α
TopBP1	Topoisomerase (DNA) II binding protein 1
TopoII	Topoisomerase II
Trf	Telomere-repeat-binding protein
Tris	Tris(hydroxymethyl)aminomethane
U2OS	Osteosarcoma cells
UBC	Ubiquitin conjugating enzyme
UV	Ultraviolet
VP16	Etoposide
WRN	Werner
Wnt	Wingless (Wg) and INT
XLF	XRCC4-like factor

XRCC4	X-ray repair complementing defective repair in Chinese hamster cells 4
ZFP	Zinc-finger protein
μg	Micrograms
μL	Microlitres
μm	Micrometres
μM	Micromolar

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CHAPTER 1: INTRODUCTION

1.1 NUCLEAR STRUCTURE

Eukaryotic cells have evolved to compartmentalize cellular processes into organelles. The nucleus is one multifaceted organelle that not only contains our genetic information as DNA but also the molecular machinery to transcribe, replicate and repair our genes. In eukaryotic cells, DNA is normally packaged with histone proteins to form chromatin [1, 2]. The minimal repeat unit is the nucleosome composed of histone proteins where DNA wraps around the histone core [3]. Non-histone proteins allow chromatin to have different degrees of DNA compaction forming all 23 chromosomes that are further compartmentalized into discrete territories [4, 5]. Furthermore, the position of some genes on chromosomes and the position of the chromosome within the nucleus can regulate both gene expression as well as the frequency of chromosome translocations between genes [5, 6].

The space that separates chromosomal territories is termed the interchromosomal domain (ICD) [7]. A number of distinct subnuclear domains or bodies lie within the ICD including nucleoli, Cajal bodies and promyeolocytic leukaemia nuclear bodies, which are functionally complex [8-10]. Extensive architecture of the nucleus organizes and regulates the many processes that occur within the nucleus. Changes in nuclear and subnuclear body structure following cell stress correlate with a number of cellular pathologies, including the development of cancer [11, 12]. The functional significance of nuclear organization remains poorly understood, but advances in nuclear structure research can help in understanding the etiology behind certain diseases as well as help to diagnose and treat them.

1.1.1 CHROMATIN STRUCTURE

The basic building block of chromatin is the nucleosome, which is composed of the core histone proteins [3]. Heterodimers of histones H2A and H2B are complexed with two heterodimers of histones H3-H4 and this histone complex is enveloped by 146 bp of DNA to form a nucleosome particle [3]. *In vivo*, chromatin exists as a 10-nm fiber (i.e. 'beads-on-a-string' conformation) [3, 13] as well as higher-order nucleosome arrays involving the linker histone H1 to form a secondary structure of 30-nm fibers, referred to as the 'solenoid' conformation [14] (Figure 1). Tertiary structure of chromatin involves long-distance chromatin fiber interactions and interactions with numerous chromatin-associated proteins [15, 16] (Figure 1).

Chromatin organization is also complicated by the fact that chromatin can exist as either euchromatin or heterochromatin [17-19]. Euchromatin condenses during the cell cycle and decondenses during interphase to allow transcription, as euchromatin is usually gene-rich [17, 18, 20]. Heterochromatin is more compact as it remains condensed throughout interphase [20]. Heterochromatin is also transcriptionally silent, contains repetitive sequences [18, 19, 21] and can exist as two types. The first type, constitutive heterochromatin is always condensed and is the same in all cells in a species [18, 22]. For example, centromeric and telomeric chromatin are types of constitutive heterochromatin that maintain centromere and telomere structure throughout cell division [18, 22]. Facultative heterochromatin is more dynamic and regulated, as it can spend time in a euchromatic or heterochromatic state in different cell types or at different developmental stages, therefore regulating gene expression [22, 23]. The switch from actively transcribed genes in euchromatin to silenced genes in heterochromatin has been well studied in *Drosophila* by

position-effect variegation [24, 25]. Position-effect variegation occurs when a gene is inactivated due to its abnormal juxtaposition with heterochromatin [24, 25]. An example occurs in *Drosophila* where eye colour is variegated due to transposition of the white gene placing it next to heterochromatin, resulting in red-white mosaic coloured eyes, where as *Drosophila* with the wild-type white gene have red eyes [24, 25].

Many factors can alter the formation of euchromatin or heterochromatin. Certain modifications of N-terminal histone 'tails' correlate with gene activation (i.e. euchromatin) or gene silencing (i.e. heterochromatin) [26, 27]. These include at least 5 types of modifications, of which lysine acetylation and methylation are the most studied [26]. Whether a histone tail modification is gene activating or gene silencing depends on which residue(s) are modified. Hyperacetylation and trimethylated H3K4, H3K36 and H3K79 are typical in actively transcribed euchromatin [26, 28, 29]. Hypoacetylation and high levels of at specific methylation sites (H3K9, H3K27 and H4K20) are most common in a silent heterochromatic state [26, 28, 29].

Not only is H3K9 methylation important for heterochromatin formation, non-histone proteins are also associated with heterochromatin. The best characterized of these non-histone proteins is heterochromatin protein 1 (HP1), originally discovered in *Drosophila*, for which orthologs exist from yeast to humans [30]. Humans have three isoforms of HP1, α, β and γ [30]. The HP1 isoforms differ in localization: HP1 α and β are found in pericentric heterochromatin, whereas HP1 γ is present in euchromatin and excluded from heterochromatin [30]. Methylated H3K9 is the binding site for the N-terminal chromodomain in HP1 [31, 32]. The HP1 proteins are fundamental anchors in the formation of heterochromatin and silencing euchromatic genes [33]. HP1 can further

mediate protein-protein interactions through its C terminal chromoshadow domain for gene silencing. For example, suppressors of variegation (Su(Var) 3-7 and 3-9) in *Drosophila* exhibit similar localization to HP1 and interact with the chromoshadow domain of HP1 [31, 34, 35]. Transcription intermediary factors (TIF) α and β can also interact with the chromoshadow domain of HP1 [36].

TIF1β is an important co-repressor that interacts with HP1 through its chromoshadow domain [37-39], and this interaction is important for cell differentiation [40, 41]. TIF1β is also known as KRAB-associated protein 1 (Kap1) or KRAB-interacting protein 1 (KRIP1) [37, 38, 42-45]. The HP1-binding domain of Kap1 is 15 amino acids and contains a PxVxL motif (Figure 2A) [43, 44, 46]. Within the HP1-binding domain of Kap1, serine residue 473 can be phosphorylated by protein kinase C (PKC) preventing interaction with HP1 and allowing the expression of regulatory genes involved in the cell cycle [47]. The HP1-Kap1 interaction allows Kap1 to coordinate activities to regulate chromatin structure by interacting with Mi-2α, a component of the nucleosome-remodeling (NuRD) histone-deacetylase complex (HDAC) and SET-domain bifurcated 1 (SETDB1), a H3K9-selective histone methyltransferase (HMET) (Figure 2B) [48, 49]. Mi-2α and SETDB1 are recruited through the plant homeo domain (PHD) finger-bromodomain of Kap1 [48, 49] and requires sumoylation of the Kap1 bromodomain, which is mediated by the ubiqutin-conjugating enzyme UBC9 and the PHD-finger E3 ligase activity of Kap1 [50, 51].

Kap1 largely coordinates transcriptional repression through its N terminal domain by binding to proteins containing the Kruppel-associated box (KRAB) domain where Kap1 was originally discovered (Figure 2) [37, 38, 42, 52, 53]. KRAB domains are conserved in the N terminus of over 400 zinc-finger proteins (ZFPs) and are strong transcriptional

repression domains that bind DNA [54-56]. Interestingly, KRAB-ZFP transcription factors in conjunction with Kap1 participate in an autoregulatory loop to repress their own gene expression [57]. The RING B box coiled-coil (RBCC) domain of Kap1 interacts with KRAB domains (Figure 2A) [58, 59] and this interaction is conserved between three subfamilies of KRAB domains [53]. Furthermore, a single KRAB domain can bind three molecules of the Kap1-RBCC [60]. Therefore, Kap1 acts as an important molecular scaffold to coordinate activities of numerous proteins, including KRAB-ZFP, HP1, SETDB1 and Mi-2α that alter chromatin structure to silence gene transcription as outlined above (Figure 2B).

1.1.2 SUBNUCLEAR COMPARTMENTS

Within the ICD space of the nucleus lies a number of subnuclear compartments or bodies that play important roles within the nucleus, possess intricate structural complexities, and functionally are only partially characterized. Examples of subnuclear compartments are the nucleolus, Cajal bodies and promyelocytic leukaemia nuclear bodies (PML NBs), which is discussed below in further detail [8].

The nucleolus is the most prominent structure inside the nucleus, and mammalian cells contain between 1-5 nucleoli [61]. Around 700 proteins are associated with nucleoli [62, 63]. Only approximately 30% of these nucleolar proteins take part in ribosome biogenesis, which is widely believed to be the central role of the nucleolus [10, 62, 63]. Ribosome biogenesis requires synthesis, cleavage and modification of ribosomal RNA (rRNA), interactions with small nucleolar ribonuclear proteins (snoRNP) and the final assembly with ribosomal proteins to form and export 40S and 60S subunits [61, 64-66]. rRNA synthesis and assembly of ribosomes is also tightly linked to the formation of

functionally distinct sub-nucleolar domains important for nucleolar structure. Initial rRNA synthesis by RNA polymerase I occurs at the fibrillar centres (FC) [61, 64-66]. Nucleolar structure largely relies on rRNA genes and actively transcribed rDNA are termed nucleolar-organizer regions (NORs) [61, 64, 67]. NORs are tandem head-to-tail repeats of rRNA genes where RNA polymerase I initiates rRNA synthesis at the end of mitosis and clustering NORs in early G1 directs assembly of the nucleolus [65, 67, 68]. Conversely, during mitosis nucleoli disassemble as RNA polymerase I dissociates and rRNA synthesis is arrested [65, 67, 68]. Nascent rRNA is then processed at the dense fibrillar component (DFC), which contains fibrillarin and surrounds the FCs [61, 64-66]. Finally, before export to the cytoplasm, ribosomes mature in the granular centres containing nucleophosmin/nuclear phosphoprotein B23 [61, 64-66].

The remaining 70% of the nucleolar associated proteins are not attributed to ribosome biogenesis, and therefore it has been suggested that the nucleolus is a plurifunctional subnuclear compartment [62, 63, 69]. The nucleolus participates in other cellular processes including maturation of non-nucleolar RNAs and ribonucleoproteins (RNPs), messenger RNA (mRNA) export and decay, cell senescence, control of viral infection, cell-cycle progression, tumour suppression, DNA repair, telomerase function and cell stress response [10, 69-71]. Not only does the nucleolus have a dynamic structure, especially during mitosis, but protein composition is continually in flux; the steady-state accumulation of proteins that give rise to a structurally coherent nuclear domain [72, 73]. As such, the nucleolus can respond to a number of cell stressors (transcriptional inhibiton, hypoxia, DNA damage) in dynamic and dramatic ways by rapid changes in protein composition [71, 74-77]. Therefore, changes in biochemical composition of the nucleolus may

play a role in regulating the cell stress response, in particular in response to DNA damage [70, 71].

Another dynamic subnuclear compartment are Cajal bodies, which were initially termed 'nucleolar accessory bodies' by Ramón y Cajal, who first discovered them due to their association with nucleoli [78]. These structures are also referred to as 'coiled bodies' due to their coiled appearance by electron microscopy [79]. The Cajal body was eventually renamed in honour of its discoverer [79]. The number and size of Cajal bodies varies between cell types and during the cell cycle, but mammalian cells usually have 1-10 Cajal bodies per nucleus, ranging from 0.1-2 μm in diameter [79-82]. Cajal bodies behave similar to nucleoli during the cell cycle as they require high levels of transcriptional activity [83-85]. In the cell cycle, Cajal bodies disappear in mitosis and reappear during G1 growth phase [83-85]. The main structural component of Cajal bodies is p80 coilin, which is necessary for Cajal-body formation, but is not essential for life [86, 87]. However, p80 coilin is not the only protein that is localized to Cajal bodies. Other proteins that are found at Cajal bodies include: fibrillarin and nucleolar and coiled-body phosphoprotein 1 (NOPP140), which are nucleolar proteins; nuclear protein of the ATM locus (NPAT), a histone transcription factor; and survival of motor neurons (SMN) that is involved in spinal muscular atrophy and participates in the formation of small nuclear RNA (snRNA) [80-82].

Cajal bodies not only interact with numerous proteins but can also interact with many small nuclear RNA (snRNA) species including U1, U2, U4, U5, U6, U11 and U12 [8, 79, 88] and small nucleolar RNAs (snoRNAs) involved in splicing RNA polymerase I, II and III transcripts [79, 80, 89, 90]. This suggests that Cajal bodies play roles in RNA

processing. For example, Cajal bodies have been shown to interact with U7 snRNA, which is required for 3' trimming of histone pre-mRNA [79, 80, 91, 92]. It has been proposed that the Cajal body forms in order to assemble the machinery for RNA processing rather than to carry out RNA processing, as Cajal bodies do not contain DNA or non-snRNP splicing factors [80, 81, 86, 89, 93]. Cajal bodies are also mobile but exhibit restricted diffusion within the ICDs of nuclei in live cells through reversible chromatin contacts [85]. Cajal bodies can also respond dramatically to cellular stress such as viral infection or DNA damage [94, 95], which may be due to changes in chromatin structure or activation of DNA-damage or stress-induced kinase pathways [71]. For example, following ultraviolet (UV)-C radiation, proteasome activator subunit γ (PA28γ) is activated and associates with p80 coilin inducing redistribution of p80 coilin, and allowing disassembly of Cajal bodies [94].

The promyelocytic leukaemia nuclear body (PML NB) is another dynamic subnuclear compartment that, like nucleoli and Cajal bodies, undergoes dynamic changes during the cell cycle and under cell stress. The intricate structure, complex biochemical composition and plethora of functions associated with PML NBs is discussed in greater detail in the following section.

1.2. PROMYELOCYTIC LEUKAEMIA NUCLEAR BODIES

PML NBs, also referred to as PML oncogenic domains (PODS), Kremer bodies or nuclear domain 10 (ND10), were originally characterized using autoantibodies from patients with primary billary cirrhosis [96-98]. PML NBs were originally thought to be an important nuclear matrix protein [99]. However, after two decades of research, PML NBs

have proved to be an important subnuclear compartment with a dynamic and complex structure, whose function appears to be regulated by changes by its biochemical composition.

1.2.1 THE DYNAMIC STRUCTURE OF PML NBs

PML NBs are a largely proteinaceous subnuclear compartment of 0.3-1 μm in diameter, containing little RNA and making extensive chromatin contacts, attributes revealed by correlative light microscopy and electron spectroscopic imaging (LM/ESI) [100-102]. The main structural component of PML NB is the PML protein, which is essential for PML NB formation [103, 104]. However, other proteins, including the transcriptional repressor speckled 100-kDa protein (Sp100) and small ubiqutin-like modifier-1 (SUMO-1), are also important for PML NB structural integrity [101]. Sumoylation of PML and Sp100 are particularly important in mediating specific protein interactions that include recruitment of the death-domain-associated protein (Daxx) [104-107]. A potential mechanism for the sumoylation of PML may be though histone deacetylase 7 (HDAC7), which has been shown to be important for PML NB formation [108, 109]. HDAC7-dependent sumoylation of PML is induced by tumour necrosis factor (TNF) α allowing HDAC7 to associate with an E2 SUMO ligase, UBC9, stimulating PML sumoylation, suggesting HDAC7 may possess SUMO E3 ligase-like activity independent of its HDAC activity [108, 110]. Conversely, removal of SUMO by SUMO-specific proteases causes reorganization of PML NBs and redistributes some PML-NB associated proteins, such as Daxx [111-113].

There is an average of 10 PML NBs per cell in most cell types, which is reflected in one of the PML NB original names, ND10 [96]. However, the number of PML NBs in a cell can range from 5-30 NBs, which is cell type and cell cycle-dependent [101, 114]. The

contacts that PML NBs make with chromatin play a role in maintaining PML NB integrity and stability [102, 115]. PML NBs have been shown to double in number in early S-phase through a supramolecular fission mechanism, directly linking this phenomenon to DNA replication [102, 114]. PML NBs are pulled apart as the surrounding chromatin topology changes, and the resulting PML microbodies are indistinguishable to the parental body [102, 114]. This behaviour indicates that new PML NBs come from pre-existing PML NBs, and are not due to random accumulation of PML protein [102]. In late S-phase Daxx leaves PML NBs [116] and during mitosis PML NB number decreases as Sp100 and SUMO-1 are depleted and bodies begin to aggregate when chromatin contacts are broken [105, 117]. These mitotic accumulations of PML protein (MAPPs) contribute to the reformation of PML NBs in G1, which again accumulate Sp100 and SUMO-1 [117, 118].

Changes in PML NB number occur in response to changes in sumoylation status or during the cell cycle. However, PML NBs can also respond to cellular stress. Some cellular stresses can affect PML NB number by a SUMO-dependent mechanism, including viral protein expression, heat shock or heavy-metal treatment, all of which trigger the desumoylation of PML and other body components such as Sp100 (Figure 3) [101, 115]. Desumoylation leads to the blebbing of PML NBs into numerous small PML microstructures devoid of SUMO-1 [101, 115]. As cells recover from stress, PML microstructures fuse together and SUMO-1 levels again increase [101, 115]. Other cellular stresses can act in a SUMO-independent fashion, including staurosporine treatment, transcription inhibition, DNase-I treatment, apoptosis and DNA damage, all of which create changes in chromatin topology (Figure 3) [101, 102, 119]. The resulting supramolecular subunits that are released from PML NBs during DNA damage are similar to the

microbodies released during S-phase, which again suggests changes in chromatin structure may regulate PML NB number [114, 120].

1.2.2 THE COMPLEX BIOCHEMICAL COMPOSITION OF PML NBs

PML NBs are not only dynamic in their structure and behaviour, but what makes a PML NB is complex. PML NBs contain the PML protein, which is the main structural component of PML NBs. SUMO-1 is important for structural integrity and Sp100 and Daxx are important components of the PML NB whose roles have been previously discussed. PML NBs associate with numerous other proteins under various conditions that contribute to the promiscuity of PML NBs in terms of their functions (Figure 4). Furthermore, PML NBs have been shown to interact non-randomly with genomic loci. To make things more complicated, the PML protein itself has numerous isoforms, allowing PML isoform-specific interactions.

The PML gene is composed of nine exons, of which exons 6-9 are alternatively spliced resulting in seven isoforms of the PML protein that differ at the C terminus [121-123]. All PML isoforms contain the RBCC motif at the N terminus, which mediates protein-protein interactions and plays a role in PML NB formation [124, 125]. A nuclear localization signal (NLS) is also present in all PML isoforms with the exception of PML VII [122, 123]. Individual PML isoforms are identified by the motifs present in the C terminus. A nuclear exclusion signal is present in PML I [126] as is a binding site for acute myeloid leukaemia I (AML1) [127] and a nucleolar targeting signal [128]. PML IV has binding motifs for p53, human double-minute homolog (HDM2) and HDACs [129-131]. Furthermore, PML IV also regulates telomerase function by interacting with telomerase reverse transcriptase (TERT), the catalytic component of telomerase [132]. The various PML isoforms have

different effects on PML NB organization, including changes in PML NB number, size and shape in different cell types when PML isoforms are overexpressed [133]. These differences with respect to these PML NB attributes suggest these isoforms may also exhibit differences in function.

In addition to changes in associated proteins, PML NBs have been shown to be meeting places for different genomic loci [134]. PML NBs particularly associate with gene-rich and transcriptionally active regions of the genome [135, 136]. Immuno-FISH analyses have been used to identify the specific gene loci that interact with PML NBs. The major histocompatibility (MHC) class I cluster on chromosome 6, which is important in antigen presentation, has been shown to be associated with PML NBs [135]. However, the implications of the specific interaction are complicated, as some studies show that PML NB and MHCI association is related to some active genes in the MHC complex [137], although this has not been confirmed [138, 139]. PML NBs also associate with the p53 gene, which encodes a potent tumour suppressor protein mediating numerous signaling pathways [140]. Therefore, it is suggested that PML NBs may play a role in p53-mediated pathways. Furthermore, a number of viral genomes have been shown to target PML NBs during productive infections, perhaps due to the high transcriptional activity [104, 141-143]. How specific genomic loci associate with PML NBs is unclear. This association may be due to high transcription activity, as found for viral genomes, [134] or for recruitment of genomic loci by transcription factors to specifically regulate transcriptional activity [134, 144, 145].

Differences in PML isoforms and association of PML NBs with genomic loci reflect only a fraction of the complexity involved in the regulation of PML NB

composition and function. The biochemical composition of PML NBs is largely due to the variety of proteins that associate or localize to PML NBs under numerous conditions. Over 75 proteins have been described as having an association with PML NBs [144, 146, 147] (Nuclear Protein Database http://npd.hgu.mrc.ac.uk) and exchange of a variety of these components is dynamic [148]. It is difficult to attribute a specific function to PML NBs due to the plethora of proteins that interact with them and their involvement in different cellular processes (Figure 4). Therefore, like the nucleolus, PML NBs are plurifunctional compartments. How PML NBs play roles in so many cellular processes is unclear and a number of models have been suggested [101, 144, 149, 150]. One model suggests that PML NBs act as depots or storage sites to sequester nuclear proteins and maintain levels of certain nuclear factors (i.e. Daxx) [98, 151]. Another model proposes that rather than acting as storage sites, PML NBs serve as sites for post-translational modification of nuclear proteins. For example, accumulation of homeodomain-interacting protein kinase 2 (HIPK2), p53 and CREB-binding protein (CBP) at PML NBs regulates phophorylation (by HIPK2) and acetylation (by CBP) of p53 [152, 153]. A third model suggests that PML NBs are sites for specific nuclear functions such as DNA transcription, replication or repair [134, 144]. This view can be supported by the evidence presented earlier that PML NBs associate with genomic loci and viral genomes. However, it should be noted that these models are not mutually exclusive.

1.2.3 THE PLURIFUNCTIONAL PML NB

As discussed above, PML NBs are plurifunctional subnuclear compartments playing roles in viral infection, proteolysis, gene regulation, apoptosis, cell senescence, tumour suppression and DNA repair (Figure 4).

Many DNA viruses and viral proteins interact with PML NBs [134, 141]. Many initial studies were done using Herpes simplex virus 1 (HSV-1) [141, 154-156]. The main role of PML NBs in viral infection is to act as initial sites of transcription and replication for DNA viruses [154-156]. Furthermore, many regulatory proteins encoded by DNA viruses associate with PML NBs and can change PML NB structure and biochemical composition. For example, an early regulatory protein in HSV-1 infection called infected cell polypeptide 0 (ICP0) disrupts PML NBs by inducing proteasome-dependent degradation of the SUMO-1 forms of PML and Sp100 [154]. More specifically, an isoform of PML, PML Ib, is localized to the cytoplasm to promote cellular resistance to viral infection by sequestering ICP0 in the cytoplasm, limiting HSV-1 viral protein accumulation and replication [157].

A role for PML NBs in proteolysis is also suggested from studies involving the ICP0 viral protein [154]. The 11S proteasome subunits have been shown to localize to PML NBs and require sumoylation of PML [158]. Therefore, degradation of PML by proteasome-mediated pathways requires sumoylated PML.

The interactions of viral genomes [141, 142] and genomic loci [134] with PML NBs provide evidence for transcriptional role of PML NBs. The fact that nascent RNA is detected in the vicinity of PML NBs also supports a transcriptional role of PML NBs [100]. As PML cannot bind DNA directly, PML NBs may interact with transcription factors and regulators or participate in chromatin remodeling processes [144, 149]. With regards to the MHC locus, it has been suggested that PML NBs regulate transcription by organizing genes in higher-order chromatin loops to alter transcriptional activity [159]. PML NBs interact with a number of transcriptional co-activators, including CBP and p53 [129, 160], and

a number of co-repressors, including the retinoblastoma protein (pRb) [161]. Furthermore, PML NBs have been shown to co-localize with heterochromatin proteins, like HP1 [162], suggesting a role of PML NBs in the establishment of heterochromatin and gene silencing [163]. Therefore, it has been suggested that PML NBs play dual roles by stabilizing co-activator or co-repressor-transcription factor complexes [144].

The tumour-suppressive nature of PML and PML NBs has been of great interest as PML NBs play roles in apoptosis and cell senescence, which are two mechanisms that keep cell growth in check and prevent cancer [164, 165]. The first indication of a role for PML in cancer was the discovery of the PML gene itself. The PML gene was identified in a (15:17) translocation with the retinoic acid receptor α (RARα) gene in patients with acute promyelocytic leukemia (APL), where mature lymphocytes are not produced and there is an accumulation of promyelocytes [166, 167]. In patients with APL, the production of this oncogenic fusion protein, PML-RARα, causes disruption of PML NBs in the promyelocytes [103, 166, 167]. Treatment of APL with all-*trans*-retinoic acid allowed reformation of PML NBs triggering differentiation, which correlates with remission of these patients [168]. Arsenic trioxide (As_2O_3) has also been used as a treatment for patients with APL as it induces apoptosis of the leukaemic cells [169] as well as degradation of PML-RARα vby a SUMO-triggered ring-finger nuclear factor (RNF) 4 ubiquitin-mediated pathway [158, 170, 171].

Decreased PML expression has also been found in solid tumours of various histological origins, including breast, colon, lung and prostate tissue [172]. Further indication that PML is a tumour suppressor is that PML-/- mice survive, but form tumours when exposed to various physical and chemical carcinogens [173]. Therefore, PML

is a classical tumour suppressor, as its loss or disruption appears to enhance the development of cancer. Since the loss of the PML protein is correlated with both tumour formation and increased susceptibility to carcinogens, the regulation of PML protein levels has emerged as an important aspect of tumour suppression. PML can be lost or degraded by various mechanisms. Phosphorylation of PML on serine residue 117 by casein kinase II (CK2) regulates PML protein levels by promoting ubiquitin-mediated degradation, leading to tumourigenesis [174]. Inhibition of this phosphorylation event by CK2 enhances the tumour-suppressive properties of PML [174]. PML degradation is also regulated by the interaction of PML with the petidyl-prolyl *cis-trans* isomerase Pin1 [175]. Pin1-dependent degradation of PML is inhibited by PML sumoylation and relies on phosphorylation of PML by mitogen-activated kinase-1 (MAPK1) [176].

In addition to being a tumour suppressor itself, PML and PML NBs are also involved in other tumour-suppressive networks. PML has been linked to numerous other proteins involved in tumour suppression and aids in the coordination of proteins involved in these pathways. The phophoinositide-3-kinase (PI3K)-AK mouse transformed (Akt) signaling pathway regulates cellular processes including cell proliferation, growth, survival and motility needed for cells to initiate tumourigenesis [177]. Growth-factor tyrosine kinases activate PI3Ks, which catalyze the production of a lipid secondary messenger phosphatidylinositol-3,4,5-triphosphate (PIP3) [177, 178]. PIP3 recruits and activates a number of downstream effectors including Akt or protein kinase B (PKB), which mediate signals to promote cell growth and survival [177, 179]. Phosphatase and tensin homologue deleted in chromosome 10 (PTEN) regulate the conversion of PIP3 to inactive phosphatidylinositol-4,5-bisphosphate (PIP2), preventing the activation of

Akt [177, 180]. Therefore, PTEN acts as a tumour suppressor. Studies have shown that PML regulates Akt [181] as well as PTEN [182, 183]. PML recruits the Akt phosphatase protein phosphatase 2A (PP2A) to inactivate phosphorylated Akt (pAkt) in the nucleus where pAkt would normally promote cell survival and cancer progression [181]. PTEN is regulated by its translocation between the nucleus and the cytoplasm. PTEN is de-ubiquitylated by herpesvirus-associated ubiquitin-specific protease (HAUSP) resulting in the nuclear exclusion of PTEN, which leads to activation of Akt, cell survival and cancer progression [182, 183]. PML has been demonstrated to oppose HAUSP activity through a mechanism involving Daxx [183].

The potent tumour suppressor p53 is involved in a number of signaling pathways and PML plays a large role in coordinating p53 function. Firstly, PML directly interacts with the p53 gene [134, 140]. Secondly, PML, more specifically PML isoform IV, interacts with the DNA-binding domain of p53, localizes at PML NBs and acts as a transcriptional activator [130, 184, 185]. Thirdly, PML promotes p53 stabilization by sequestering the p53 ubiquitin-ligase murine double-minute 2 (Mdm2) to the nucleolus, preventing p53 degradation [129, 186, 187]. Furthermore, localization of HIPK2, p53 and CBP at PML NBs promotes activation of p53 by regulating the phophorylation (by HIPK2) and acetylation (by CBP) of p53 [152, 153]. These interactions between PML and p53 help facilitate transcription of p53-responsive genes, allowing cells to undergo cellular senescence [188, 189] or apoptosis [184, 190] under various conditions. Lastly, PML itself is a p53 target gene, adding an autoregulatory feature to mediating p53 function [191].

In addition to promoting apoptosis, activation of p53 also results in cellular senescence. Stabilization and activation of p53 through phosphorylation of serine residue

46 and acetylation of lysine residue 382 is induced by PML, specifically PML IV, and is important for cellular senescence [188]. PML IV is also important in mediating cellular senescence by negatively regulating telomerase by interacting with its catalytic component, TERT, resulting in shortened telomeres [132]. Furthermore, oncogenic ras induces PML to promote cellular senescence through the p53 and pRb tumour-suppressor pathways, and both p53 and pRb localize to PML NBs [189, 192]. Independently of p53 and Rb, cellular senescence is also caused by the formation of facultative chromatin into senescence-associated heterochromatin foci (SAHF), which are induced by anti-silencing factor 1a (ASF1a) and histone cell-cycle regulation defective A (HIRA) [193]. HIRA must localize to PML NBs before interacting with ASF1a and does so through glycogen synthase kinase 3 β (GSK3β) -mediated phosphorylation of HIRA by downregulating Wnt signals that normally promote cancer [194].

PML and PML NBs not only regulate p53-dependent apoptosis but also some other forms of apoptosis that are p53-independent. PML interacts with Daxx, a proapoptotic factor in Apoptosis stimulating fragment (Fas)- and transforming growth factor β (TGFβ)-mediated apoptosis [173, 190, 195, 196]. Furthermore, cytoplasmic PML has been shown to play a role in TGFβ signaling by interacting with the TGFβ signaling proteins SMA and mothers against decapentaplegic 2/3 (Smad2/3) and Smad anchor for receptor activation (SARA) to induce either growth arrest, cellular senescence or apoptosis [197]. Finally, PML can induce apoptosis in a p53-independent manner by regulating checkpoint kinase 2 (Chk2) autophosphorylation and activation, in which PML induces this pathway [198, 199].

An interesting twist in the role of PML in cancer is that the presence of PML has not only a tumour-suppressive effect in somatic cells, preventing the development of cancer, but aids in the maintenance of cancer stem cells [200, 201]. PML appears to regulate self-renewal by cancer stem cells, such as haematopoetic stem cells in APL or leukaemia-initiating cells in chonic myeloid leukaemia (CML) to promote cellular transformation [200, 201]. Despite these recent studies, PML and PML NBs are still viewed as being tumour suppressive in nature. Accumulation of mutations in tumour-suppressor genes, including PML, can lead to cancer. Functional DNA-damage signaling pathways and DNA-repair mechanisms in a cell are important to prevent mutations from accumulating. The role of DNA-damage signaling and DNA repair in genome stability and their relation to PML is discussed below.

1.3 THE ROLE OF ATM AND ATR KINASES IN GENOME STABILITY AND DNA-DAMAGE SIGNALING

Various mechanisms for sensing and repairing DNA lesions are highly conserved from yeast to mammals. Ataxia telangiectasia-mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) are transducing kinases that are important in the initial cellular responses to DNA damage [202-205]. Mutations in ATM and ATR have been associated with rare autosomal recessive disorders, ataxia telangiectasia and Seckel syndrome, respectively [206-208]. Although these kinases have similar roles in DNA-damage signaling, the diseases they cause show different symptoms. Ataxia telangiectasia is characterized by cerebellar degeneration leading to severe neuromotor dysfunction, immunodeficiency, radiosensitivity and increased susceptibility to cancer [209]. On the other hand, Seckel syndrome is exemplified by mental retardation, microcephaly, characteristic facial

features including pronounced nose and ears, dwarfism and radiosensitivity [210]. Furthermore, ATM-deficient mice [211] show severe growth defects yet are viable, whereas an embryonic lethal phenotype is present in ATR-knockout mice, suggesting an essential function for ATR [212]. These phenotype differences in loss or mutated ATM and ATR indicate clearly that ATM and ATR play distinct cellular roles.

Despite these differences, both ATM and ATR belong to the PI3K-like protein kinase (PIKK) family, which possess serine/threonine kinase activity through their PI3K domain [203, 213, 214]. ATR was originally discovered based on its sequence homology with ATM [215]. ATM and ATR share substrate specificity as they target serine or threonine residues followed by a glutamine (SQ/TQ motif) [216, 217]. ATM and ATR recognize and phosphorylate over 700 proteins with an SQ/TQ motif; many of these proteins are involved in networks that respond to DNA damage [203, 218, 219]. Many of these common ATM/ATR substrate are involved in cell-cycle checkpoint control, including checkpoint kinases Chk2 and Chk1, the tumour suppressor p53 and the mediator breast cancer 1, early onset (BRCA1) [202-204, 218]. Other proteins are involved in DNA repair and chromatin modifications. Early activation of ATM and ATR along with their mediators leads to phosphorylation of specific targets resulting in downstream effects involving activation of transcription, cell-cycle control, apoptosis and DNA repair [220]. Therefore, the ATM/ATR DNA-damage response involves activation of ATM and/or ATR, followed by the recruitment of mediator proteins allowing for signals to be transduced activating effector proteins for cell-cycle control, DNA repair or modification of chromatin.

1.3.1 ATM AND ATR ACTIVATION

ATM and ATR activation are complex processes. ATM is strongly activated by ionizing radiation (IR) and radiomimetic drugs that create double-strand breaks (DSBs) [221]. Inactive molecules of ATM are maintained as dimers or higher order multimers in which the kinase domain is blocked by this interaction [222]. The MRN complex, which is composed of meiotic recombination 11 (Mre11), Radiation sensitive (Rad) 50 and Nijmegen breakage syndrome 1 (NBS1), is one of the first sensors of DNA damage and is an accessory complex for recruitment of ATM to sites of damage (Figure 5) [223, 224]. The MRN complex regulates ATM in at least two different ways, *via* oligonucleotides produced from MRN-dependent processing of DNA breaks, which stimulate ATM activity [225], and by the direct interaction of ATM with the MRN complex through the C-terminal domain of NBS1 [226]. These MRN-ATM interactions result in an intermolecular phosphorylation event on serine residue 1981 of ATM, releasing active ATM monomers [222].

ATR is strongly activated by UV radiation, replication-fork arrest and chemicals that create single-stranded DNA (ssDNA) [227-229]. When ssDNA results from these types of damage, replication protein A (RPA) coats ssDNA forming a critical structure that recruits ATR-interacting protein (ATRIP), which in turn allows formation of the ATRIP-ATR complex [230, 231]. Following the induction of single stranded breaks (SSBs), the clamp loader Rad17 is recruited and activated, which then loads the proliferating cell nuclear antigen (PCNA)-911 (Rad9-Rad1-Hus1) complex onto DNA [232]. Topoisomerase (DNA) II binding protein 1 (TopBP1) then binds the 911 complex and ATRIP [233, 234] and

ATR becomes further activated resulting in the phosphorylation of Rad17, 911 and TopBP1, which again further stimulates ATR kinase activity (Figure 5) [205].

However, activation of ATM and ATR is not as simple as it seems. ATM and ATR have been thought to act in parallel and independently of each other, but there is also communication and cooperation between ATM and ATR in response to DNA damage. Following IR, ATM is activated first, followed by the sequential activation of ATR [235-238]. Moreover, following UV radiation, replication stress or chemicals that create single-stranded DNA (ssDNA), activation of ATR results in ATM phosphorylation and activation [239].

1.3.2: MEDIATORS OF ATM AND ATR KINASES

Mediators are proteins directly downstream of ATM and ATR kinases that play a variety of roles including cell cycle control, DNA repair and chromatin modifications (Figure 6) [205]. An important event that occurs after ATM and ATR activation is phosphorylation of a histone variant, H2A.X, which comprises approximately 10% of the total H2A [240, 241]. Both ATM and ATR phosphorylate H2A.X on serine residue 139, producing what is termed γ-H2A.X, which can spread over a 2-megabase (Mb) domain surrounding the damage [240, 242, 243]. Phosphorylated H2A.X accumulates in large protein complexes termed 'repair foci' [244]. The number of repair foci correlates linearly with the number of DNA DSB [244]. Furthermore, formation of these γ-H2A.X foci appears to preferentially occur in euchromatin, whereas heterochromatin is refractory to the phosphorylation of H2A.X [39, 245]. Furthermore, heterochromatin correlates with an impediment to ATM-dependent signaling and repair of DSBs [246]. Finally, transcription is

inhibited at γ-H2A.X foci allowing repair to occur in which the γ-H2A.X signal is lost, but only at low levels of damage [247, 248].

Other mediators are recruited following phosphorylation of H2A.X, mainly after ATM activation. Mediator of DNA-damage checkpoint 1 (MDC1) binds γ-H2A.X through its BRCA1 C-terminal (BRCT) domain [249, 250]. This interaction amplifies the γ-H2A.X signal, either by tethering ATM or by inhibiting γ-H2A.X dephosphorylation [251]. MDC1 promotes further recruitment of the MRN complex, aiding the spreading of γ-H2A.X [252]. MDC1 is phosphorylated by CK2 and this phosphorylated MDC1 interacts with the MRN complex through the N terminus of NBS1 [252, 253]. Following damage, not only does the MRN complex bind free DNA ends, but more MRN complex is recruited from the pool of bound MRN-MDC1 [252]. The γH2A.X-MDC1 interaction also acts as a platform for the recruitment of other proteins involved in the DNA-damage response [254] including p53-binding protein (53BP1) and BRCA1 [255]. In addition, ubiquitylation of histones occurs surrounding the break by the E3 ligases RNF8 and RNF168 and the E2 ubiquitin-conjugating enzyme UBC13, which is necessary for recruitment of 53BP1 and BRCA1 [255]. RNF8, RNF168 and UBC13 are recruited by the γ-H2A.X-MDC1 interaction and phosphorylation of MDC1 [255-257]. Finally, live-cell experiments have revealed the order of recruitment of these factors, namely MDC1, followed by NBS1, 53BP1 and BRCA1 [258]. Many mediators may be recruited, but only a select few will be required depending on what downstream effect the cell chooses to carry out.

1.3.3: DOWNSTREAM EFFECTORS OF ATM AND ATR KINASES

The roles of ATM and ATR kinases are best understood by activation of their downstream effectors and the resulting changes that occur. These cellular changes include modulation of the cell cycle and modification and/or remodeling of chromatin to facilitate DNA repair [220].

1.3.3.1 CELL-CYCLE CONTROL

An important event that occurs after ATM and ATR activation is cell-cycle control. Within the cell cycle there are many checkpoints that a cell must pass to continue dividing. When DNA damage is present, these checkpoints become activated to prevent accumulation of DNA damage and to allow time for DNA repair to occur [259]. All eukaryotic cells have four phases in the cell cycle, G1 (growth), S (DNA synthesis), G2 (growth) and M (mitosis), and checkpoints take place at G1/S, intra-S and G2/M (Figure 7A) [259].

For a normal cell cycle, a number of factors are required (Figure 7A). Three phosphotyrosine phosphatases, cell division cycle 25 A, -B and –C (Cdc25 A, -B and –C) are important in cell-cycle transitions [260]. Under normal conditions, these Cdc25 proteins dephosphorylate cyclin-dependent kinases (Cdks) that act on the proteins directly involved in the cell cycle [261]. Dephosphorylated Cdk2 allows phosphoylation of Cdc45, which is a protein that is required for replication initiation and is important in regulating G1/S and intra-S checkpoints [262, 263]. For S-phase progression, structural maintenance of chromosome 1 (SMC1) and the kinase activity of Cdc7-Dumbbell forming 4 (Dbf4) are also required [264, 265]. For the entry into mitosis, the Cdc2-CyclinB complex is necessary

and its activity is dependent on the upregulation of Cdc25A and downregulation of the Wee1 kinase [266].

For checkpoint activation in response to DNA damage, ATM and ATR kinases are involved. ATM and ATR respond to their respective sources of DNA damage and have their respective pathways for checkpoint activation [220]. However, there is sequential activation of ATM and then ATR following DSB, suggesting that ATM initiates a checkpoint blockage and ATR is the maintenance kinase [236], and *vice versa* with regards to UV radiation [267]. Activation of two checkpoint kinases, Chk1 and Chk2, at all checkpoints is important to transduce the initial signal from ATM or ATR [268, 269]. Activation of ATM following DSBs results in phophorylation of Chk2 on threonine residue 68 for its activation [270], whereas ATR activation following UV damage results in Chk1 phosphorylation on serine residue 345 for its activation [271]. Cdc25 proteins are excluded from the nucleus following phosphorylation by Chk1/Chk2 and interaction with 14-3-3 proteins, thus allowing Cdc25 proteins to be their degraded [272]. Inactivation of Cdc25 phosphatases promotes phosphorylation of Cdks to inhibit the DNA-replication machinery, so cell division cannot continue [272].

For the G1/S checkpoint, there is a rapid onset of a G1/S arrest as well as maintenance of a G1/S arrest if damage is prolonged (Figure 8). For the rapid initiation of a G1/S arrest, activated ATM and ATR phosphorylate Chk2 and Chk1 respectively. Cdc25A is inactivated through phosphorylation by Chk1 and Chk2, so that Cdk2 cannot be dephosphorylated [262]. The resulting phosphorylated Cdk2, complexed with cyclin E or A, accumulates and prevents the phosphorylation of Cdc45, which is important in the initiation of DNA replication [262, 263]. Maintenance of a G1/S arrest is mediated by the

potent tumour suppressor and transcription factor p53. ATM and ATR phosphorylate serine residue 15 on p53 directly promoting p53 stabilization [221, 236]. Activated Chk1 and Chk2 phosphorylate p53 on serine residue 20 to prevent Mdm2 binding and degradation of p53 [273]. Accumulation of p53 leads to activation of its target genes including p21, which binds to the Cdk2-CyclinE complex to arrest cells in G1/S [45]. In addition, p21 binds the Cdk4-CyclinD complex to prevent it from phosphorylating the retinoblastoma protein (pRb), which in its hyperphosphorylated form inhibits transcription of S phases gene by the E2 transcription factor (E2F) [274, 275].

The Cdc45 pathway is also involved in the intra-S phase checkpoint to prevent any further initiation of DNA replication (Figure 9) [220]. However, ATM and ATR have different responses for the intra-S checkpoint (Figure 9). ATM phosphorylates SMC1 with the aid of NBS1, BRCA1 and Fanconi's anemia complementation group 2 (FANCD2) to inhibit DNA replication [264]. On the other hand, ATR inhibits initiation of DNA synthesis by inhibiting the Cdc7-Dbf4 kinase [265]. Like ATM, ATR also activates BRCA1 and NBS1 [276] promoting recovery of stalled replication forks.

As in a G1/S or intra-S checkpoint, the ATR/ATM-Chk1/Chk2-Cdc25A axis is also involved in the G2/M checkpoint (Figure 10) [220]. For example, Chk1 and Chk2 can activate the Wee1 kinase [266]. Cdc25A and Wee1 then act on the Cdc2-CyclinB complex, which promotes entry into mitosis [266]. Together, downregulation of the Cdc25A phosphatase and upregulation of the Wee1 kinase mediate phosphorylation of Cdc2 to prevent entry into mitosis [277].

1.3.3.2: DNA REPAIR

ATM and ATR are largely involved in the initial events of DNA-damage signaling and aid in transducing signals for certain processes to occur. Cell cycle control as discussed above is a way for the cell to halt cell division once DNA damage has occurred. However, this response does not eliminate the damage. The DNA damage must be repaired or else the cell will be overwhelmed by DNA damage and undergo apoptosis. Therefore, controlling the cell cycle buys time for the cell to repair the damage, and ATM and ATR allow this to happen. There are numerous mechanisms of DNA repair and the repair mechanism that is used essentially is determined by the type of damage present and the phase of the cell cycle (Figure 7B) [220]. DNA repair can be divided into six categories including direct repair of altered bases, base-excision repair, mismatch repair, nucleotide excision repair, DSB repair and cross-link repair [220, 278]. For simplicity sake, DSB repair is discussed here as ATM and ATR, along with DNA protein kinase (DNAPK), are the central DSB signaling kinases [279, 280].

In mammalian cells, DSBs can be repaired either by non-homologous end-joining (Figure 11) (NHEJ) or homologous recombination (Figure 12) (HR) [220, 279]. NHEJ basically involves re-joining the ends of DSBs, which can lead to translocations if the breaks belong to different chromosomes (Figure 11) [281]. NHEJ is preferentially used in G1 of the cell cycle but can be used throughout the cell cycle as it simply resects the ends of the break [282]. Firstly, a dimer of Ku thyroid autoantigen (Ku) proteins (Ku70/Ku80) binds both ends of the DSB, which recruits the catalytic subunits of DNA protein kinase (DNAPK-cs) [283]. The X-ray repair complementing defective repair in Chinese hamster cells 4 (XRCC4)/DNA ligase IV (LIG4) complex is recruited to ligate the two processed

DNA termini [284]. Other proteins such as the nuclease Artemis and Cernunnos/XRCC-like factor (XLF) have also been implicated the processing and ligation of the DSB in NHEJ [281, 285]. NHEJ usually results in the loss of few nucleotides and is largely error prone [281].

HR is quite different from NHEJ and relies on sequence homology between the broken ends and use of the undamaged homologous chromosome to fill in the missing information (Figure 12) [286]. Therefore, HR is largely used during S phase of the cell cycle, when sister chromatids are in close proximity until they segregate in anaphase [282]. The MRN complex recognizes the ends of the DSB before starting the recombination process (Figure 12A) [287]. Strand invasion and branch migration are mediated by RPA and Rad51 (Figure 12B) [288]. Many other proteins, including BRCA1, BRCA2, Rad52, Rad54, Rad55 and Rad57, are involved in HR, but their roles are unclear [289]. A Holliday junction forms where the strands cross over, DNA polymerases synthesize new DNA, branches migrate and Holliday junctions are then resolved by methyl methane sulfonate and UV sensitive 81 (MUS81) · methyl methane sulfonate sensitivity 4 (MMS4) (Figure 12B) [290]. HR is largely error free compared to NHEJ and is conserved from budding yeast to higher eukaryotes [286].

1.3.3.3 MODIFICATION OF CHROMATIN

Cell-cycle control may buy time for a cell to repair DNA but the DNA may not always be readily accessible. The packaging of DNA into chromatin is largely refractory to DNA repair. DNA damage, such as DSBs, causes some disruption in chromatin structure, but further modifications of chromatin may be necessary for the complete repair of DNA damage. These modifications to chromatin are partly mediated by ATM/ATR and their

downstream effectors. Some of these modifications involve post-translational modification of histones such as acetylation, ubiquitylation and phosphorylation, remodeling nucleosomes and global changes in chromatin [255].

Chromatin is more accessible and can be transcribed when histones become acetylated [26, 28, 29]. Following DSBs, acetylation of histones is mediated by the mammalian Tat-interactive protein 60 kDa (TIP60) complex in which its histone acetyltransferase (HAT) subunit is homologous to the nucleosome acetyltransferase of H4 (NuA4) in yeast [291]. TIP60 HAT and NuA4 acetylate H4 lysine residues 5, 8, 16 and 16 to facilitate the 'opening up' of chromatin surrounding the site of DNA damage [292]. These HATs also acetylate γ-H2A.X promoting the ubiquitylation of γ-H2A.X, which in turn facilitates dephosphorylation and/or turnover of γ-H2A.X [293]. UV-induced histone acetylation by the p300 HAT is mediated by p53, a downstream target of ATM/ATR, resulting in increased chromatin accessibility for DNA repair [294, 295].

In addition to HATs, a number of ATP-dependent chromatin remodeling complexes are recruited by γ-H2A.X, an event downstream of ATM/ATR activation. Some of these chromatin remodeling complexes include INOsitol requiring (INO80) and Sick with rat8 (SWR1) in yeast, the mammalian homologues of which are subunits in the TIP60 complex [255, 296, 297]. The role of INO80 and SWR1 is to remove the core histones in nucleosomes surrounding the DSB by exchanging H2A.X-H2B dimers for H2A-H2B dimers to allow NHEJ or HR to occur [298, 299]. Other chromatin remodeling complexes such as SWItch/Sucrose NonFermentable (SWI-SNF) and chromatin structure remodeling complex (RSC) have been shown to participate primarily in HR [300, 301].

While HATs and chromatin remodeling complexes affect histones and nucleosomes directly, global changes in chromatin structure can occur following DNA damage and activation of ATM/ATR. Global chromatin relaxation induced by loss of H1 or inhibition of HDACs appears to amplify the DNA-damage response through greater DSB accessibility, which enhances signaling [302]. However, chromatin containing DSBs first undergoes local decondensation surrounding the lesion in an energy-dependent manner [303]. Although in yeast DSBs appear to move to coalesce at one or more DNA-repair centres [304], DNA breaks are relatively immobile in mammalian cells and DSBs prefer to undergo translocations with neighbouring chromosomes [305]. These local changes in chromatin mobility establish an accessible environment for DNA-damage signaling and repair and are independent of γ-H2A.X [303]. Although γ-H2A.X is important for stable association of DNA-damage response proteins, it may be necessary to maintain the decondensed state of chromatin [303, 306].

Heterochromatin may be a physical block to the spreading of γ-H2A.X along chromatin as well as limiting the mobility of damaged chromatin [39, 303]. Heterochromatin is more refractory to formation of γ-H2A.X compared to euchromatin [39, 245], a situation which is also reflected by a delay in ATM-dependent signaling and repair of DSBs in heterochromatic regions [246]. Knockdown of Kap1, a transcriptional co-repressor, or other heterochromatin factors like HP1 or HDACs, alleviates the necessity for ATM in DSB repair, most likely by perturbing heterochromatin structure [246]. Kap1 is phosphorylated by ATM on serine residue 824 following DSBs leading to chromatin relaxation, also caused by loss of Kap1 (Figure 13) [307]. Phosphorylation of Kap1 also leads to the expression of p53 target genes involved in cell-cycle control and apoptosis [308, 309]. ATR

also phosphorylates Kap1 and the phosphorylated Kap1 colocalizes with a number of DNA-damage response proteins including γ-H2A.X., 53BP1, TopBP1 and BRCA1 [310]. This suggests that Kap1 may participate in the majority of DNA-repair processes by allowing chromatin decondensation upon early phosphorylation of Kap1 and access to these DNA-damage response factors [310]. HP1 is also affected by the DNA-damage response as phosphorylation HP1β by CK2 promotes mobilization of HP1β following DNA damage [311]. However, recent evidence has also shown that HP1 can be recruited to several types of DNA damage to modify chromatin structure and facilitate efficient DNA repair [312]. Therefore, the role of HP1 in the DNA-damage responses and modification of chromatin remains unresolved and controversial.

1.4 THE ROLE OF PML NBs IN GENOME STABILITY AND DNA DAMAGE SIGNALING

Many processes act together to maintain genome stability, including DNA repair, cell-cycle control and apoptosis. Maintaining genome stability is an important component in preventing the accumulation of mutations in oncogenes and tumour suppressor genes that could lead to cancer. The role of PML and PML NBs in tumour-suppression and cancer has been discussed extensively. However, PML NBs also play a role in maintaining genome stability and DNA-damage signaling, which might contribute to their role in tumour suppression. Sister-chromatid exchange (SCE), a marker for genome instability, is seen at high levels in PML-deficient mice, similar to patients with Bloom's syndrome [313]. PML-deficient mice also show an impaired p53 response to DNA damage [184]. PML and PML NBs regulate p53 on a number of different levels including p53 expression [134], stabilization [129], post-translational modifications [152] and p53 target

gene transcription [184] as discussed above. This involvement directly implicates PML and PML NBs in transducing signals for a proper p53 response (i.e. cell-cycle arrest, senescence, or apoptosis). Not only does p53 localize to PML NBs, many other proteins localize to PML NBs that are involved in DNA repair and DNA-damage signaling [101]. PML NBs also increase in number following DNA damage in an ATM- and ATR-dependent manner suggesting that PML NBs act as sensors to DNA damage [119].

1.4.1 PML NBs AND DNA REPAIR

Many DNA-repair factors colocalize with PML NBs before and after DNA damage with a complex network of interactions [101]. Some of the DNA-repair factors include: ATM, ATR and Chk2 kinases; BRCA1; histone variant H2A.X; all components of the MRN complex (Mre11, Rad50, NBS1); Rad51; Rad52; all components of the Rad9-Hus1-Rad1 complex; RPA; p53; TopBP1; telomerase repeat binding proteins 1 & 2 (Trf1 & 2); and Recombinase Q (RecQ) helicases, Bloom syndrome protein (BLM) and Werner (WRN) [101]. Behaviour of these DNA-repair proteins differs with regards to PML NBs, as some proteins localize with nuclear bodies in undamaged cells, some proteins dissociate and reassociate following DNA damage, others are relocalized in a cell-cycle-dependent manner and, finally, some only associate following damage [101, 149]. A subset of these interactions is summarized below.

In undamaged cells the MRN complex [314, 315], ATR [316], BLM [317], RPA [317] and Chk2 [198] are found in PML NBs and the nucleoplasm. The association of the MRN complex with PML NBs is mediated by the interaction between NBS1 and a PML NB component, Sp100 [318]. BLM association with PML NBs is more dynamic. During the G1 and S-phases of the cell cycle, BLM localizes to PML NBs [313, 319], whereas RPA and

Rad51 associate with BLM during the S and G2 phases of the cell cycle [317]. Normal BLM function requires association with PML NBs and this interaction plays an important role in p53 activation [319].

When cell are exposed to IR, disruption of PML NBs occurs due to loss of chromatin contacts [102], allowing distribution of PML NB associated proteins to sites of damage for repair of DSB by NHEJ using the MRN complex or by HR using BLM, Rad51 and RPA [101]. ATM activates Chk2, after which some Chk2 leaves the NB and some Chk2 remains [101, 149]. Chk2 phosphorylates PML on serine residue 117 [198], the consequences of which remain unclear in terms of PML NB disassembly but which may allow formation of supramolecular assemblies by affecting PML NB interactions [101]. Approximately 1 hour following IR and peaking at 8-12 hours post IR, TopBP1 colocalizes with PML NBs, which correlates with an increase in PML and TopBP1 protein levels [320]. p53 also behaves in a similar manner by colocalizing with PML NBs after DNA damage and p53 protein levels increase in a PML-dependent manner [321]. Following 8-12 hours post IR, ATM begins to colocalize at PML NBs and colocalization of resident proteins of the PML NB, including BLM, RPA and the MRN complex, peaks mostly when cells are arrested in G2/M, either facilitating late repair or a G2/M arrest, but this remains unclear [101, 317]. With ATM and Chk2 activation, p53 is phosphorylated and stabilized to upregulate its target genes to induce a G1/S arrest or apoptosis [322]. The juxtaposition of PML NBs and repair foci containing γ-H2A.X is also seen at later time points, but the mechanism and function of this association remains unknown [119, 321].

When cells are exposed to UV radiation, PML NBs are disrupted in a similar manner as after exposure to IR. ATR, which also mediates S-phase arrest, is activated

and, along with RPA, both proteins move from PML NBs to sites of damage [316]. The RecQ helicase WRN, normally found at nucleoli, localize to PML NBs after UV radiation along with RPA and Rad51 and may play a role in regulating p53 [323]. In addition to p53, many other proteins that are necessary for p53 activation also colocalize at PML NBs following UV radiation, such as Mdm2, CBP and HIPK2 [152, 153, 186, 187]. p53 activation is coordinated by PML NBs through p53 phosphorylation by HIPK2 and p53 acetylation by CBP, as well as by interaction with Mdm2 to prevent p53 degradation [152, 153, 186, 187]. Similarly to what is seen after IR, activation and stabilization of p53 leads to increased expression of p53 target genes for a G1/S arrest or apoptosis [322].

Many of the DNA-repair and HR proteins that associate with PML NBs (BRCA1, NBS1, Rad1/Rad9/Hus1, Rad17, RPA, Rad51, Rad52) further colocalize with Trf1/2 and telomeric DNA at a subset of PML NBs to facilitate an alternative mechanism of telomere maintenance called alternative lengthening of telomeres (ALT) [324, 325]. ALT occurs in cell lines that lack telomerase and the subset of PML NBs involved in ALT are known as ALT-associated PML bodies (APBs) [324, 326]. APBs are usually found in late-S and G2 phases of the cell cycle and persist through mitosis, in which APBs are associated with telomere repeats at sites of DNA synthesis [324, 325]. How the accumulation of these factors (ie. BRCA1, Rad51, RPA, Rad17, Rad1/Rad9/Hus1, Rad51 and Rad52) along with BLM and the MRN complex at APBs allow cooperative function with respect to telomere elongation remains unclear [101]. However, NBS1 does seem to be required for telomere elongation at APBs, through the NBS1-Sp100 interaction [318, 326].

In response to DNA damage, many signaling and repair proteins localize to and from PML NBs as discussed above. However, the functional consequences of this

complex network of interactions at PML NBs have not been revealed in its entirety. It is clear that PML NBs play a key role in transducing signals to mediate the p53 response to DNA damage. What about their role in DNA repair? As PML NBs colocalize with numerous DNA-repair proteins which are necessary for the formation of repair foci [320], PML NBs may act as a functional nexus to regulate and stabilize repair foci. PML NBs do colocalize with sites of DNA repair, such as γ-H2A.X in DSB repair [321, 327] as well as BLM at sites of nucleotide-excision repair following UV radiation [317, 328]. In addition, PML NBs have been shown to recruit ssDNA after DNA damage, which could facilitate the processing of DNA damage [300].

As for a direct role for PML NBs in DNA repair, it is not entirely clear. Most DNA-repair factors colocalize with PML NBs at later time points and usually DNA damage has been repaired at this point [101, 119, 321]. However, PML NBs may play a role in later phases of repair [149, 320] or in specific repair pathways. PML NBs may be important in repair by HR, a claim supported by a number of findings. Firstly, PML-knockout mice exhibit high levels of SCE, a defect seen in cells with faulty HR machinery [313]. Secondly, Rad51, BLM and other HR proteins colocalize to PML NBs [101]. Lastly, telomere elongation is thought to occur by HR in ALT cells, in which the association of PML NBs with telomeres plays a major role [324, 329]. However, it is unclear whether PML NBs move to sites of damage or if damaged chromatin is recruited to sites adjacent to PML NBs [101]. To date there is no convincing evidence to support the movement of PML NBs to DNA damage. Therefore, PML NBs most likely integrate signaling events for DNA repair through the accumulation and/or release of DNA-repair factors upon disruption of PML NBs following DNA damage [101].

1.4.2 PML NBs AS DNA DAMAGE SENSORS

PML NBs mediate signals for DNA repair, cell-cycle control and apoptosis. Following DNA damage, PML NBs also increase in number [101, 320, 321], similar to the behaviour of PML NBs following cell stress, as discussed above. The combination of these dynamic changes in PML NB composition and number following DNA damage suggests that PML NBs act as DNA damage sensors [101, 119].

PML NBs increase in number following various types of DNA damage including UV radiation [187, 328], IR [119, 314, 320, 321], alkylating agents [330] and topoisomerase II inhibitors etoposide (VP16) and doxorubicin causing DSBs [119]. The disruption of PML NBs following DNA damage occurs in a SUMO-independent fashion being driven by the loss of chromatin contacts with the PML NBs [101, 102]. In response to low physiological levels of IR or chemotherapy agents, PML NBs do respond in a rapid, sensitive and dose-dependent fashion by increasing in number *via* a supramolecular fission mechanism from pre-existing PML NBs [119], reminiscent of the behaviour of PML NBs in S phase of the cell cycle [114].

It was previously unknown if this behaviour of PML NBs is regulated by any of its associating DNA-damage signaling or repair proteins; however, recent evidence suggests it is [119]. Despite the major importance of PML and PML NBs in regulating p53 function, the increase in PML NB after DNA damage is surprisingly independent of p53 [119]. ATM, ATR and Chk2 kinases as well as NBS1, a component of the MRN complex, have been shown to regulate this increase in PML NB number [119]. Inhibition of these proteins through chemical inhibitors or DNA-repair-deficient cell lines impedes the increase in PML NB number following DNA damage [119]. ATM, ATR, Chk2 and NBS1

are involved in parallel yet partially redundant signaling pathways involved in the detection and repair of DNA damage, as discussed above [203, 204]. Briefly, the MRN complex, containing NBS1, senses DSBs. Through a number of mediator proteins, ATM and/or ATR are activated and ATM phosphorylates and activates Chk2. Interestingly, inhibition of both ATM and ATR using caffeine has a dramatic effect in dampening PML NB response to DNA damage, suggesting these kinases act in an additive fashion to regulate PML NB number [119]. This interpretation is consistent with their redundant roles in DNA-damage signaling [202].

Dellaire and colleagues (2006) [119] provide a model for PML NB number increase in response to DSBs (Figure 14A). Following the induction of DSBs, first there is a biophysical response due to initial topological changes in chromatin structure as PML NBs are disrupted and form microbodies through a fission mechanism from preexisting NBs. A second phase of the increase in PML NB number following DSB induction is dependent on DNA-repair processes in which ATM, ATR, Chk2 and NBS1 are involved. The importance of this increase in PML NB number following DNA damage is an increase in 'functionally active' sites for repair factors to associate and be post-translationally modified at these 'docking sites', as surface area increases for these protein interactions to occur in concert with an increase in nuclear body number [101, 119]. Thus, PML NBs can act as DNA damage sensors. Elucidation of the exact mechanism(s) behind the increase in PML NB number following DNA damage is important to validate PML NBs as a potential biomarker for tumour responses to radiation and chemotherapy agents. As such PML NBs could provide a novel means to monitor the response of cancer cells treated with DNA-damaging agents.

1.4.3 HOW DOES ATM/ATR REGULATE PML NB NUMBER?

The exact role that ATM/ATR plays in the increase in PML NB number following DNA damage is unknown. The role of ATM and/or ATR in regulating PML NB behaviour following DNA damage may be two-fold, 1) through phosphorylation of PML by Chk2 [198], ATR [129] or ATM and 2) through changes in chromatin structure following DNA damage (Figure 14B). PML NBs make extensive contacts with chromatin [102, 115] and the increase in PML NB number following DNA damage is similar to PML NB behaviour following cell stress or during S-phase, when chromatin structure is altered [102, 115, 119]. Recently it has been shown that ATM regulates global decondensation of chromatin following DSBs through phosphorylation of Kap1 [307]. Although ATM has been observed to have this effect on chromatin through Kap1, ATR has also been found to phosphorylate Kap1 [310]. Loss of both ATM and ATR has the most profound effect on the delay in PML NB number increase following DSBs [119]. However, loss of ATM and/or ATR affects many pathways in DNA-damage signaling. Therefore, I hypothesized that loss of Kap1 might isolate the role of DNA-damage-dependent decondensation of chromatin in regulating PML NB number after DNA damage.

I have found a novel pathway involving Kap1 in the regulation of PML NB number. Loss of Kap1 without DNA damage causes an increase in PML NB number which correlates with chromatin relaxation, suggesting that Kap1 may regulate PML NB number prior to DNA damage. This phenomenon is not dependent on cell type and exhibits no alterations in the cell cycle profile. Furthermore, following DNA damage, there is a further increase in PML NB number that occurs *via* a Kap1-independent mechanism but is partially dependent on phosphorylation of Kap1. I suggest that Kap1

plays an important role in maintaining PML NB integrity prior to and following DNA damage.

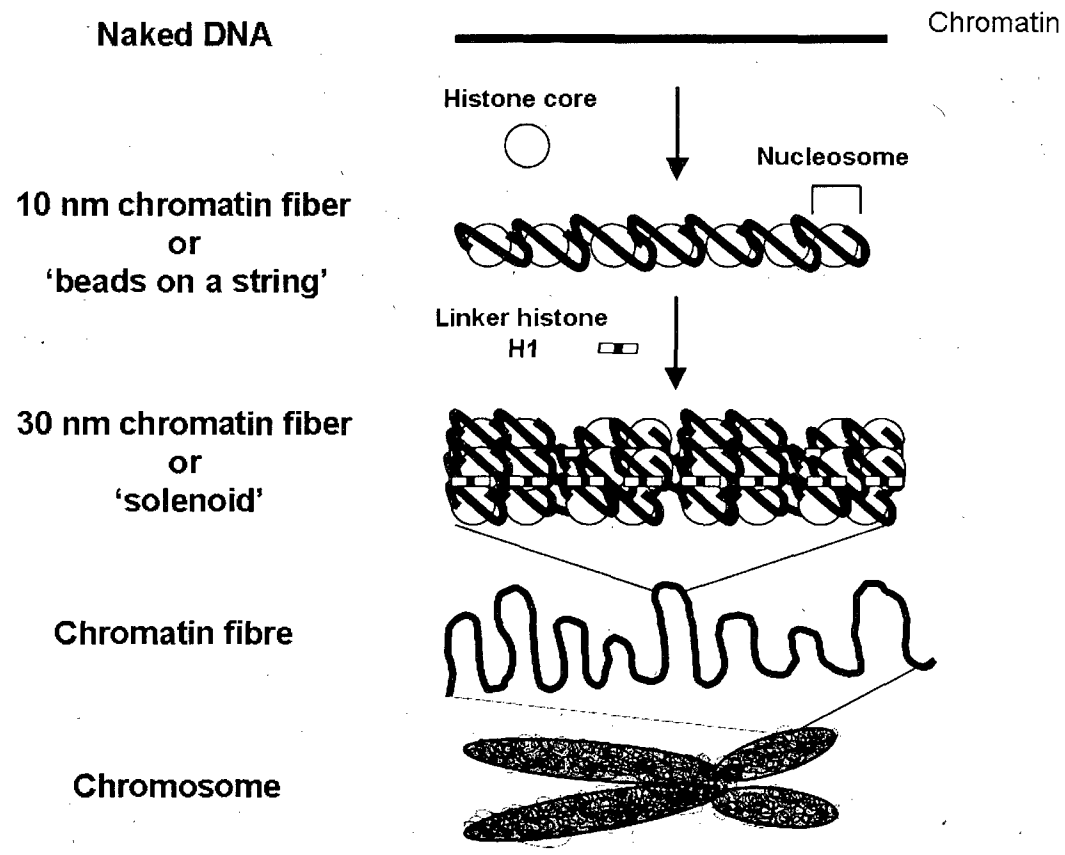


Figure 1. Chromatin structure: Naked DNA is complexed with the core histone proteins to form a 10-nm fibre or 'beads on a string'. The basic unit of the 10-nm fibre is the nucleosome, which consists of 146 bp of DNA wrapped around heterodimers of histone H2A-H2B and two heterodimers of histones H3-H4. The 10-nm fibre further compacts into a 30-nm fibre or 'solenoid' fibre. Linker histone H1 plays a role in forming the 30-nm fibres. Tertiary structure of chromatin depends on long-distance chromatin fibre interactions within the 30-nm fibres forming loops of chromatin. These compact chromatin fibres can further condensed into chromosomes seen in metaphase. Adapted from http://themedicalbiochemistrypage.org/dna.html.

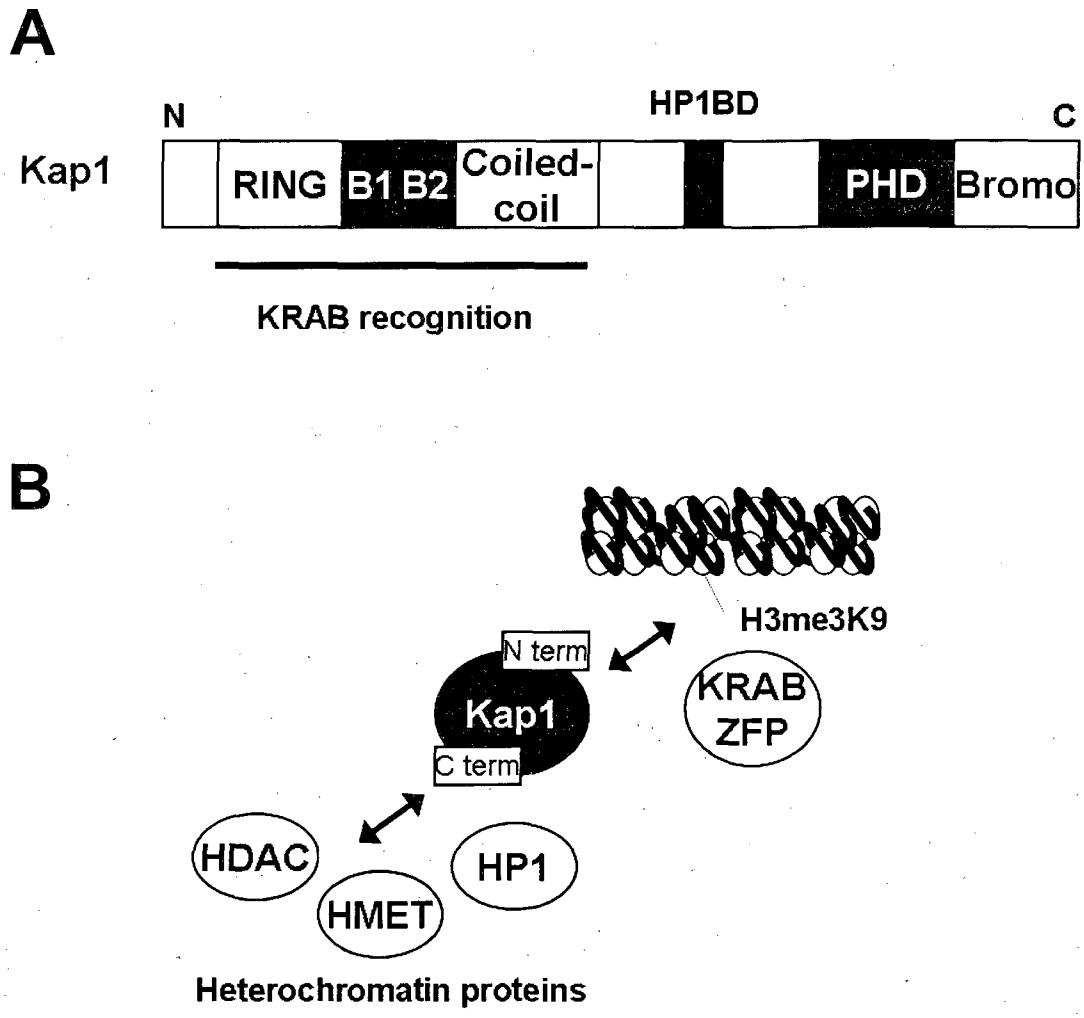


Figure 2. Kap1 structure and function. (A) Schematic illustration of the Kap1 protein and its domains: RING-finger BB-boxes coiled-coil (RBCC) domain; Heterochromatin protein 1 (HP1) binding domain (BD); Plant homeo domain (PHD) finger; Bromodomain (Bromo). The RBCC domain is the site of Kruppel-associated box (KRAB) domain binding. Adapted from Sripathy *et al.* (2006) [37]. (B) Schematic illustration of Kap1 function. Kap1 acts as a transcription intermediary factors interacting with a number of proteins to facilitate heterochromatin formation and gene silencing. Kruppel-associated box zinc-finger proteins silencing complexes (KRAB-ZFP) associate with tri-methylated histone H3 on lysine residue 9 (H3me3K9) and the N terminus of Kap1. Through its C-terminal domain, Kap1 interacts with heterochromatin proteins including HP1, histone deacetylases (HDAC) and histone methyltransferases (HMETs).

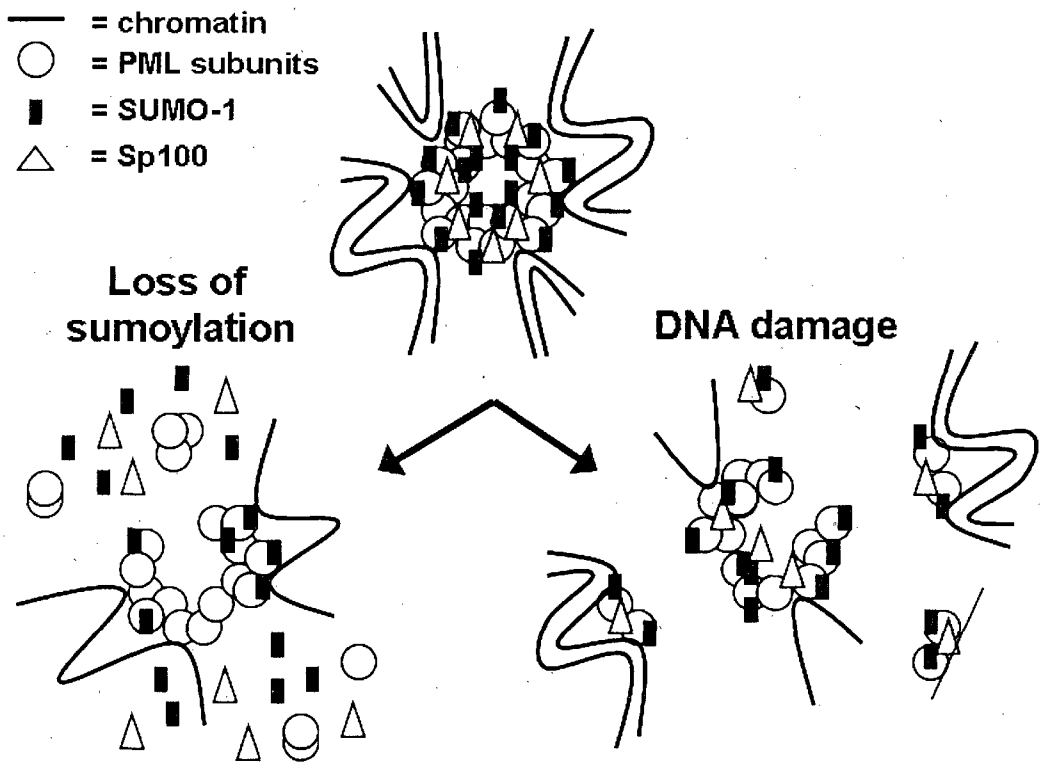


Figure 3. Mechanisms of PML NB disruption following cell stress. PML NBs can disassemble and cause an increase in PML NB number two ways: SUMO-dependent or SUMO-independent mechanisms. Many cellular stresses such as viral protein expression, heat shock or heavy-metal treatment, desumoylate PML NB components PML and Sp100. Loss of sumoylation results in the blebbing of PML NBs into numerous small PML microstructures devoid of SUMO-1. Therefore, this process is SUMO-dependent. PML NB numbers also increase in response to DNA damage caused by radiation, chemical treatment or exogenous nucleases, which cause changes in chromatin topology. PML microbodies, rather than microstructures, are released and are identical to the parent NB in Sp100 and SUMO-1 content. This PML NB response to DNA damage does not depend on SUMO-1, making it a SUMO-independent mechanism. Adapted from Dellaire and Bazett-Jones (2004) [101].

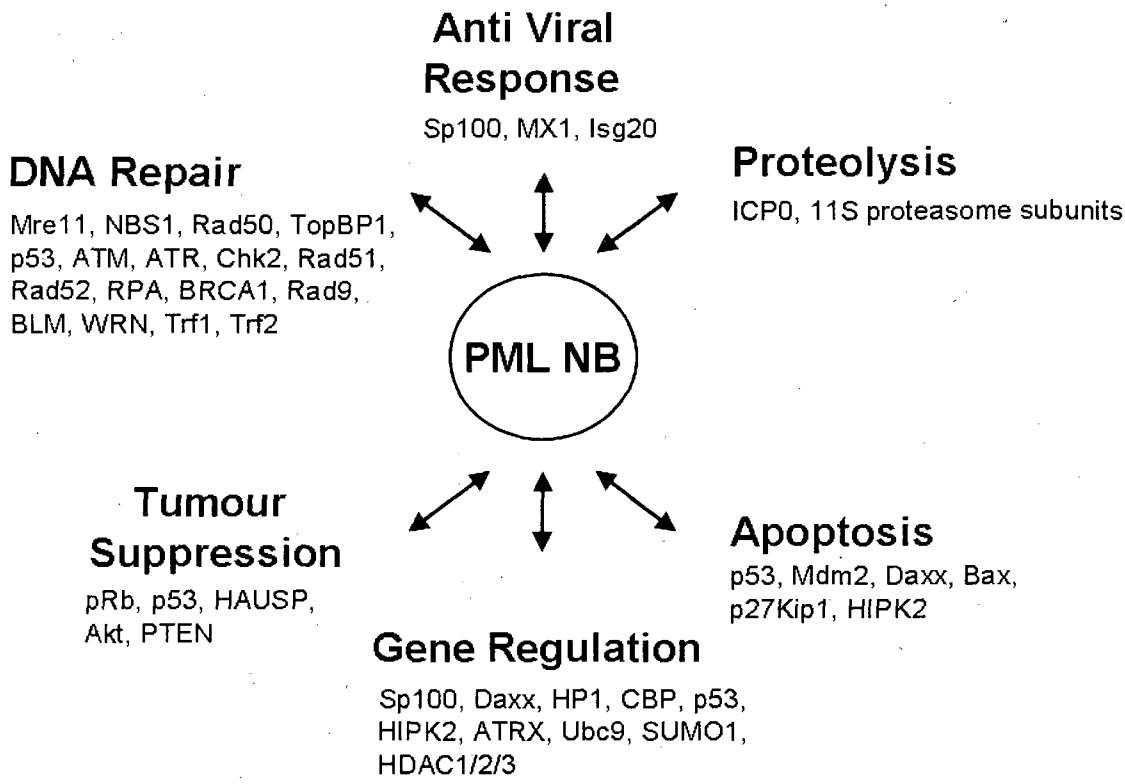


Figure 4. The plurifunctional PML NB. The diverse functions of PML NBs and some of the proteins implicated in these cellular processes are summarized in this figure. These proteins can localize at PML NBs or interact with PML directly. Furthermore, these proteins may localize to PML NBs depending on the phase of the cell cycle. A protein may either be localized in the nucleoplasm and within PML NBs, or may localize only to PML NBs upon specific stress conditions. Adapated from Dellaire and Bazett-Jones (2004) [101].

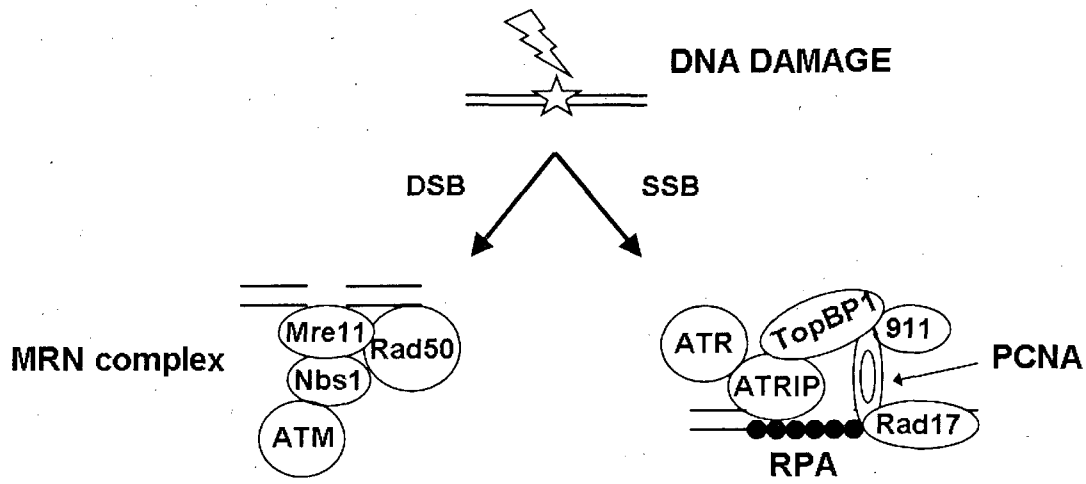
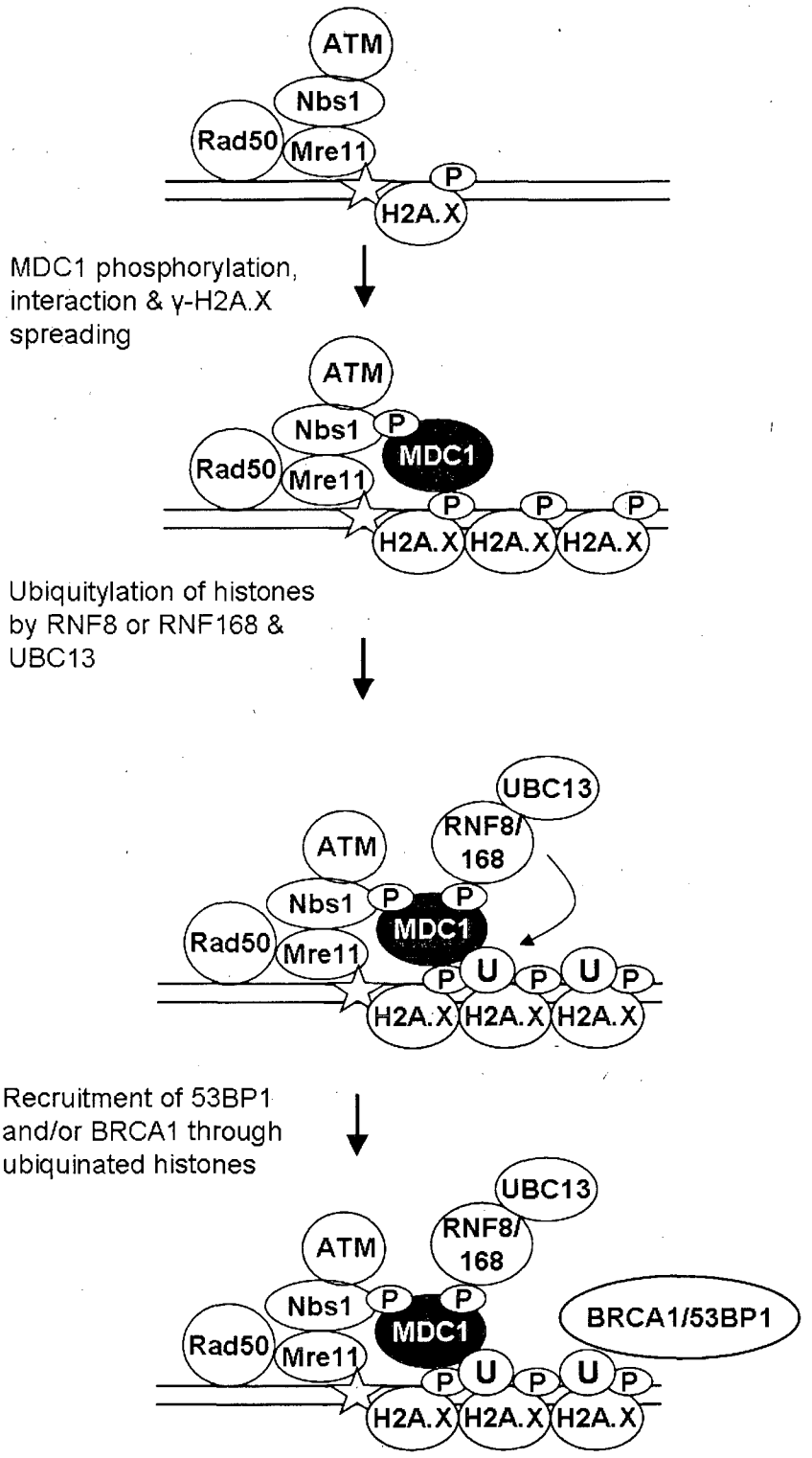


Figure 5. Activation of ATM and ATR with their respective accessory proteins. ATM is activated following the induction of double-strand breaks (DSBs) and requires the MRN complex composed of Mre11, Rad50 and NBS1 for ATM activity. ATR is activated following the induction of SSBs. RPA coats ssDNA and recuits ATRIP, which forms the ATR-ATRIP complex. The clamp loader Rad17 is recruited and loads the PCNA-911 complex onto DNA. 911 and ATRIP further recruit TopBP1 for full activation of ATR.

Figure 6. Mediators of the ATM response to DNA damage. Following activation of ATM *via* the MRN complex after the induction of DSB, histone H2A.X is phosphorylated. MDC1 is recruited and binds to phosphorylated H2A.X. MDC1 is phosphorylated by CK2 to promote spreading of H2A.X phosphorylation around the DSB through further recruitment of MRN and ATM. MDC1 mediates the recruitment of E3 ligases RNF8 and RNF168 and E2 conjugating enzyme UBC13 to regulate ubiquitylation of H2A.X and other histones to promote recruitment of BRCA1 and 53BP1. Adapted from Van Attikum and Gasser (2009) [255].


ATM
Nbs1
Rad50
Mre11
P
H2A.X
MDC1 phosphorylation, interaction & γ-H2A.X spreading
MDC1
Ubiquitylation of histones by RNF8 or RNF168 & UBC13
UBC13
RNF8/ 168
U
Recruitment of 53BP1 and/or BRCA1 through ubiquinated histones
BRCA1/53BP1

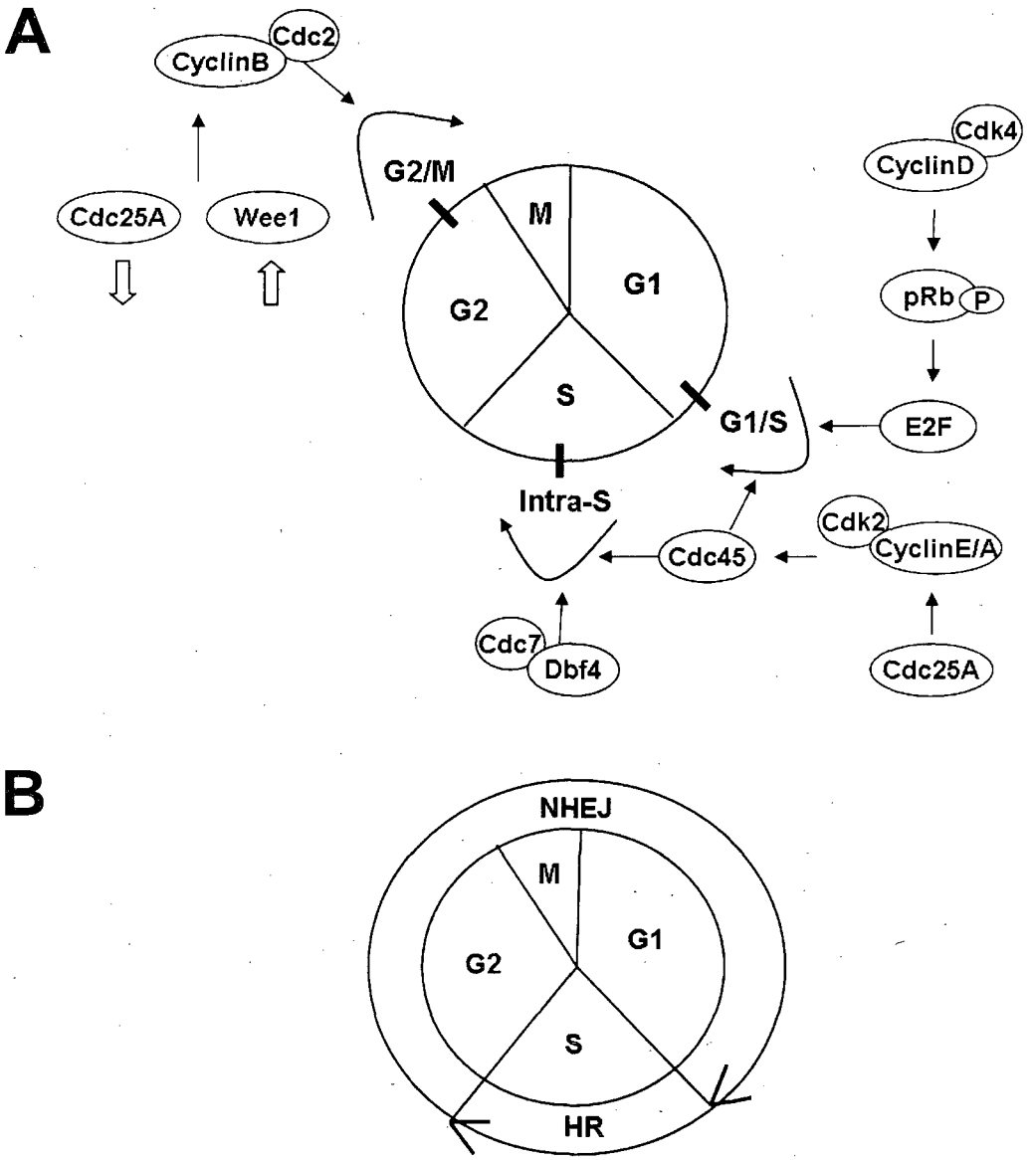


Figure 7. The cell cycle and its relationship to DNA repair. (A) Proteins that mediate the cell cycle. The eukaryotic cell cycle consists of four stages: G1 (growth), S (DNA synthesis), G2 (growth) and M (mitosis). Cell-cycle checkpoints exist at three places within the cell cycle: G1/S, intra-S and G2/M. A cell must be able to pass all checkpoints to complete the cell cycle. Many proteins mediate cell-cycle checkpoints. At G1/M and intra-S, Cdc25A must be active and readily dephosphorylate Cdk2 to allow replication initiation by Cdc45. pRb must be phosphorylated to promote the release of E2F for transcription of S-phase genes. Furthermore, Cdc7-Dbf4 is important for progression through S-phase. At G2/M, downregulation of Cdc25A and upregulation of Wee1 kinase is important to activate Cdc2-CyclinB to promote mitosis. (B) The connection between DNA repair and the cell cycle. The choice of a DNA-repair mechanism depends on the cell cycle. NHEJ can be used throughout the cell cycle. However, HR is preferred in S-phase due to close proximity of homologous chromosomes.

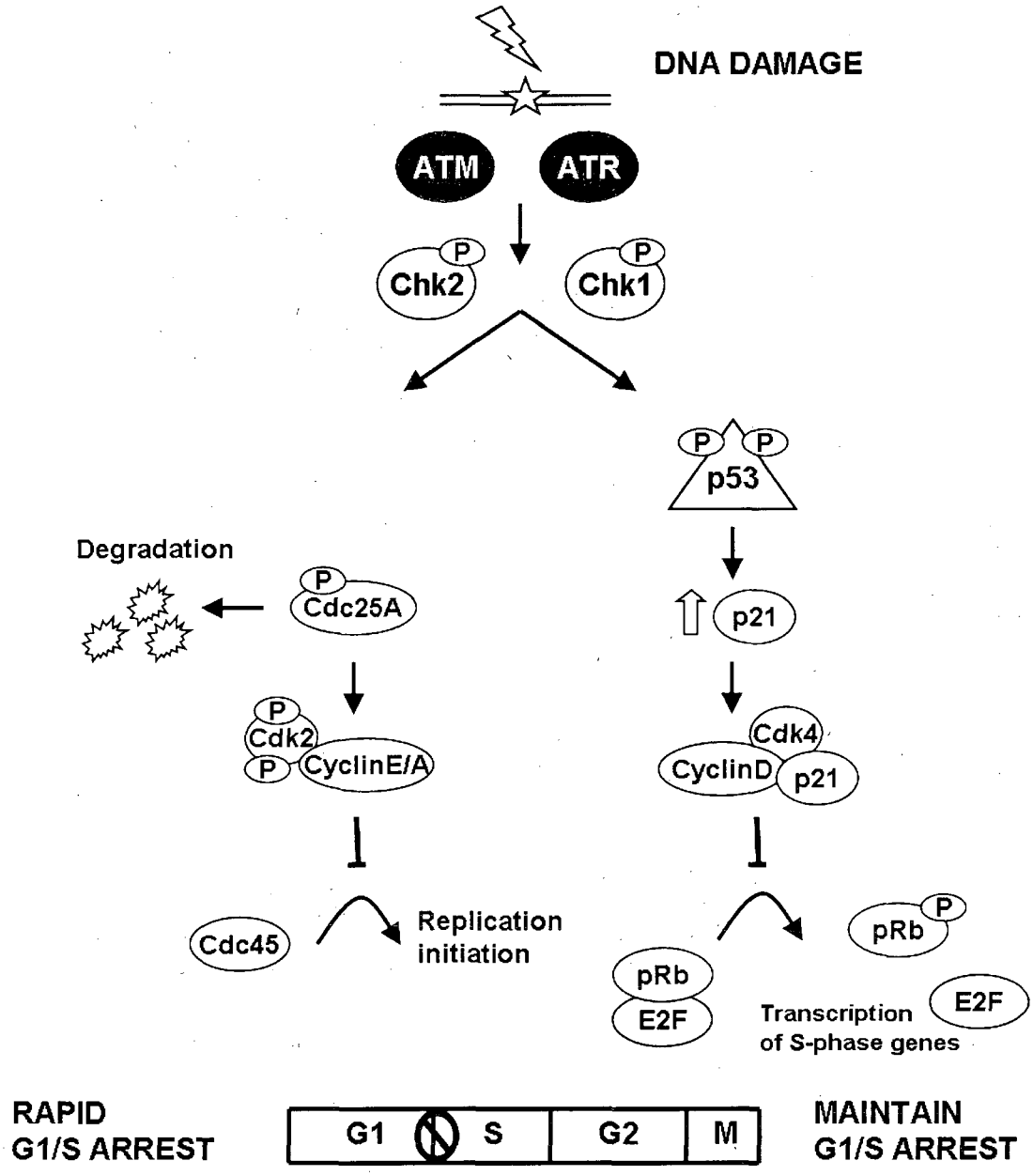


Figure 8. Activation of the G1/S cell-cycle checkpoint. ATM and ATR are activated following DNA damage, which in turn activates Chk1/Chk2. For a rapid G1/S arrest, Chk1/Chk2 phosphorylates Cdc25A, causing its inactivation and degradation. This results in the accumulation of phosphorylated Cdk2 that cannot activate Cdc45 for replication initiation. To maintain the G1/S arrest, p53 is phosphorylated by ATM/ATR and Chk1/Chk2. Phosphorylated p53 induces expression of p21, which binds to Cdk4-CyclinD, thus preventing it from phosphorylating pRb, which normally promotes release of the transcription factor E2F for expression of S-phase genes. Adapted from Sancar *et al.* (2004) [220].

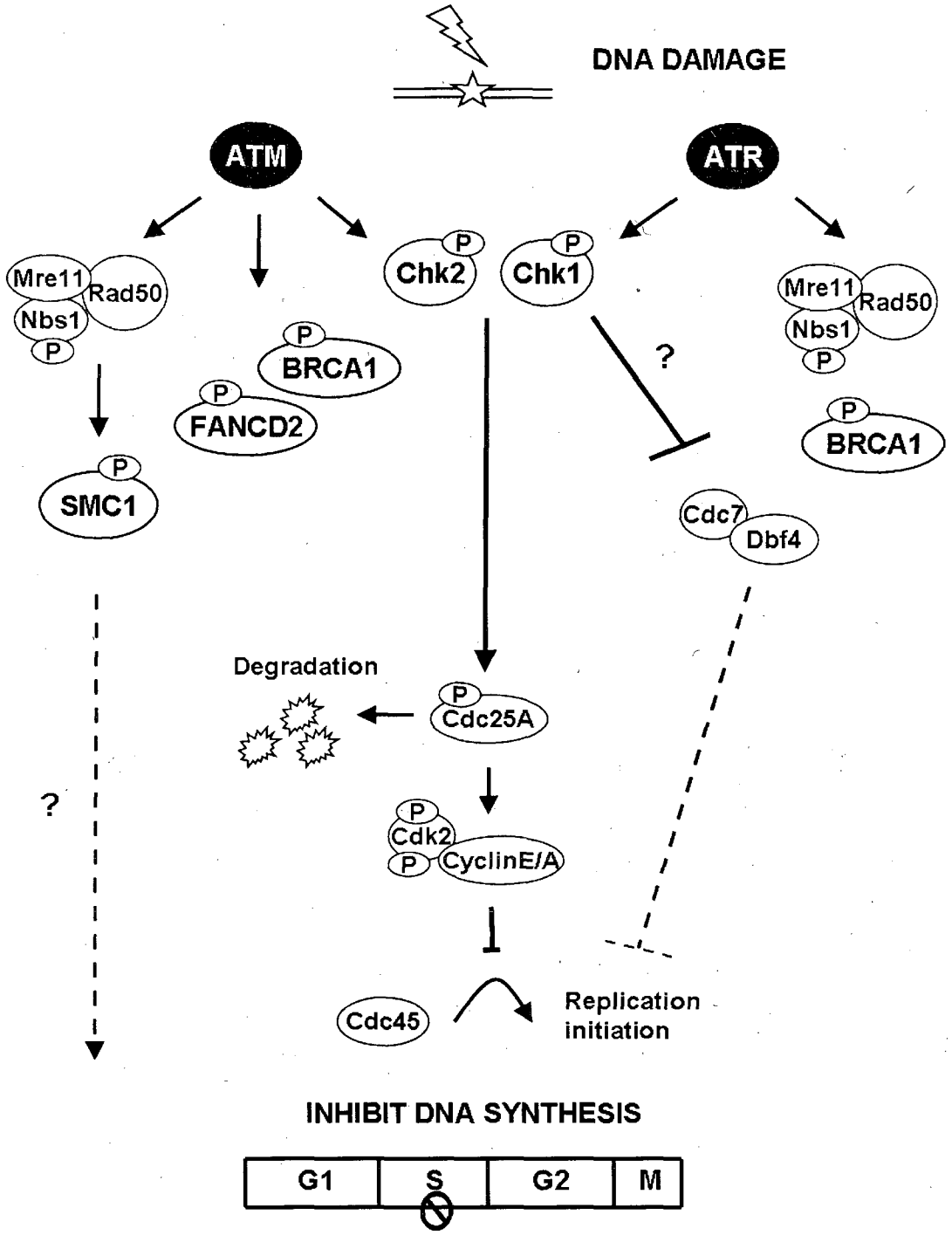


Figure 9. Activation of the intra-S cell-cycle checkpoint. ATM and ATR are activated following DNA damage, which in turn activates Chk1/Chk2. Both ATM and ATR initiate the degradation of Cdc25 and the accumulation of inactive Cdk2-CyclinE, which inhibits replication initiation by Cdc45. ATM also phosphorylates NBS1, BRCA1, FANCD2, and SMC1, which inhibits DNA replication. ATR also inhibits replication initiation through inactivation of Cdc7-Dbf4 as well as phosphorylating BRCA1 and NBS1 to prevent DNA replication. Adapted from Sancar *et al.* (2004) [220].

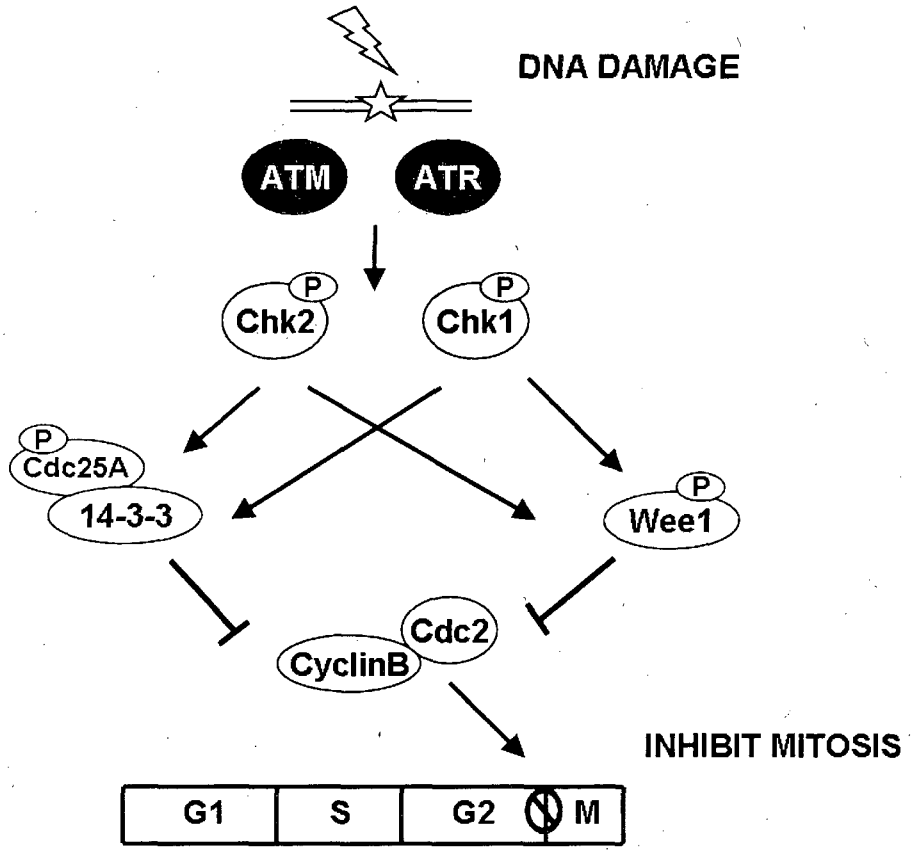


Figure 10. Activation of the G2/M cell-cycle checkpoint. ATM and ATR are activated following DNA damage, which in turn activates Chk1/Chk2. Both Chk1 and Chk2 are able to regulate phosphorylation and inactivation of Cdc25A as well as phosphorylation of the Wee1 kinase to inhibit Cdc2-CyclinB, which is necessary to promote the entry into mitosis. Adapted from Sancar *et al.* (2004) [220].

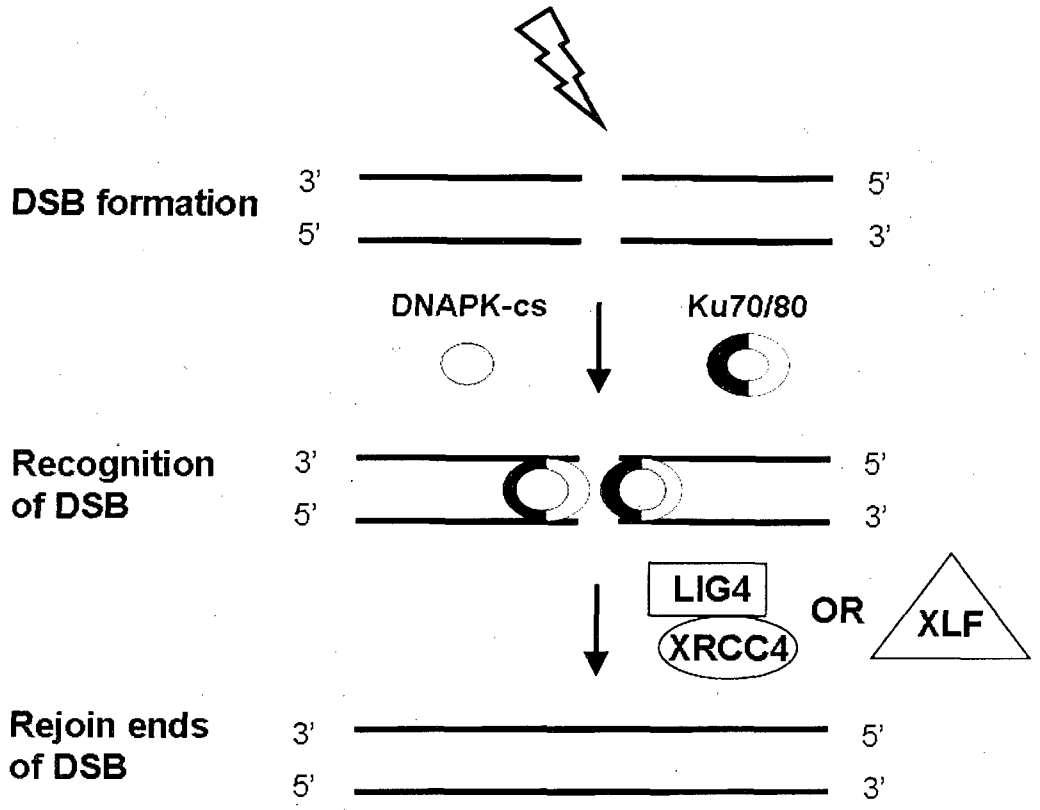
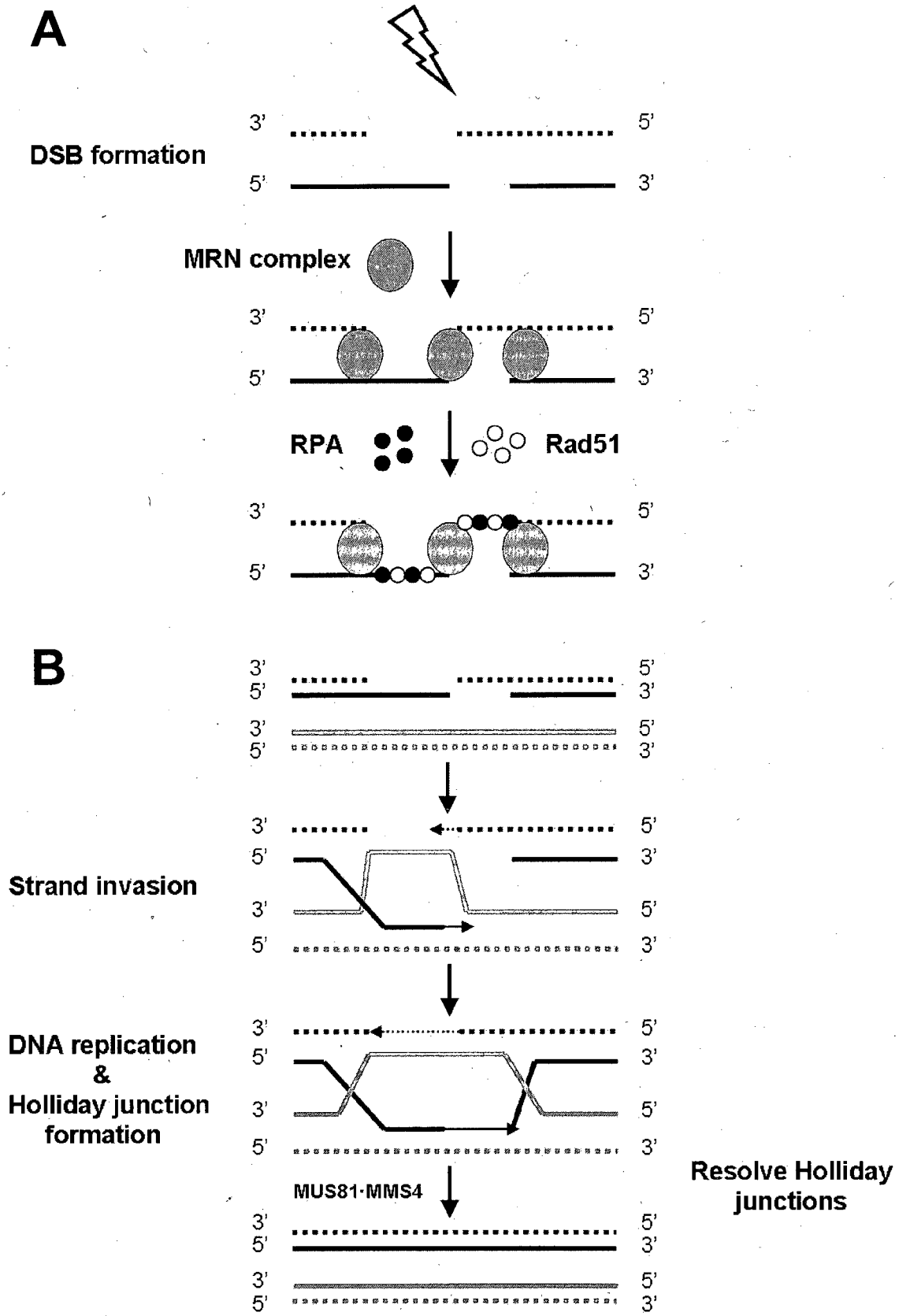


Figure 11. Non-homologous end joining (NHEJ). Following DSB formation, heterodimers Ku70-Ku80 and DNAPK catalytic subunits (DNAPK-cs) recognize the damage and bind the ends of the DSB. End processing can occur. XRCC4-Lig4 or XLF/Cernunnos mediate rejoining or ligation of the broken ends. Adapted from Sancar *et al.* (2004) [220].

Figure 12. Homologous recombination (HR). (A) Recognition of broken DNA ends. Following DSB formation, the MRN complex recognizes and binds the broken ends. RPA and RAD51 coat the ssDNA and mediate strand invasion. (B) Strand invasion and Holliday junctions. DNA replication occurs to fill in the gaps. Holliday junctions result when the two recombining strands are covalently joined by single-strand crossovers. MUS81·MMS4 resolvase cleaves the Holliday junctions, resulting in two separate duplexes. Adapted from Sancar *et al.* (2004) [220].


A
DSB formation
3'
5'
5'
3'
MRN complex
RPA
Rad51
B
Strand invasion
DNA replication
&
Holliday junction
formation
MUS81·MMS4
Resolve Holliday
junctions

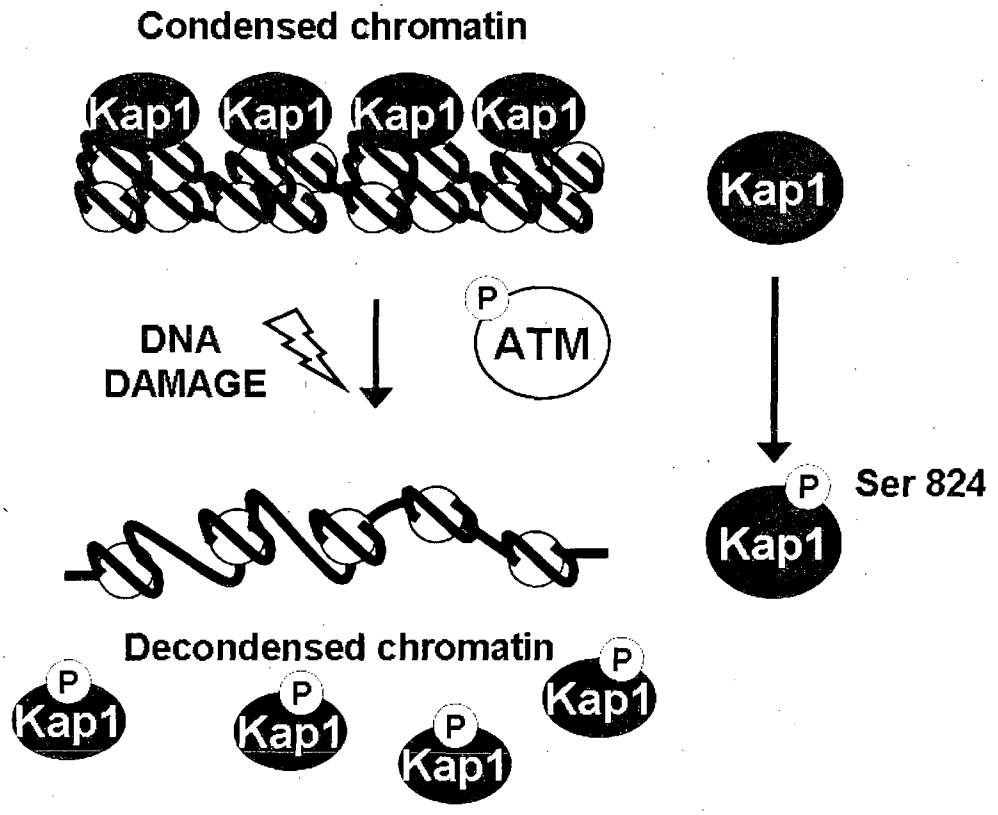
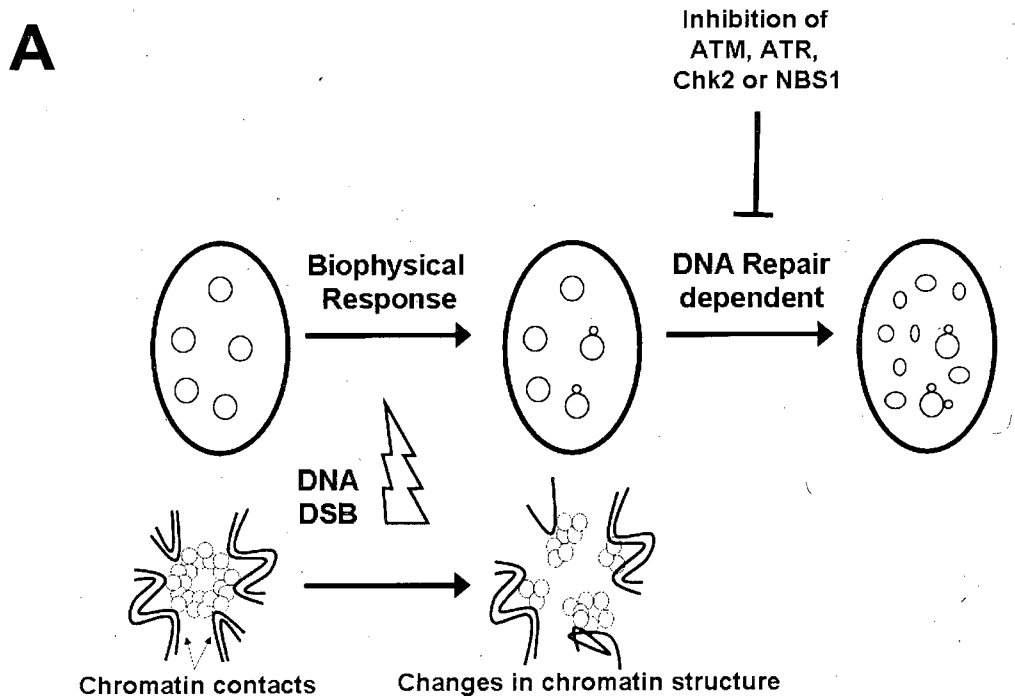
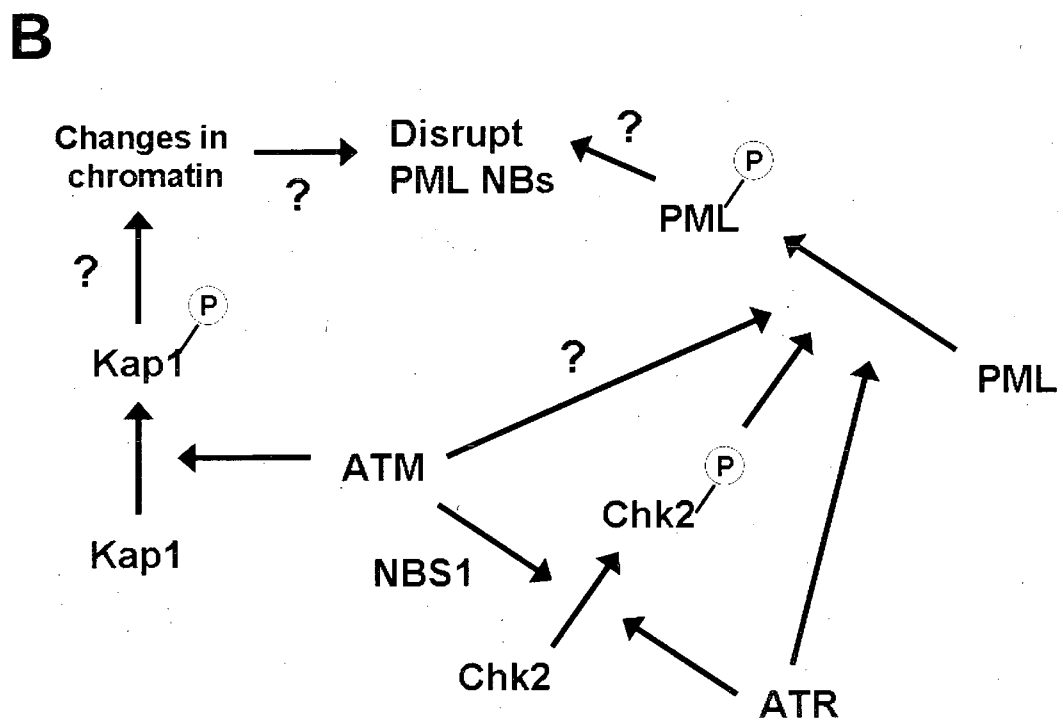


Figure 13. ATM, Kap1 and chromatin structure. Global decondensation of chromatin is mediated by ATM through phosphorylation of Kap1 on serine residue 824 following DNA damage. Kap1 maintains the structure of condensed chromatin. Once Kap1 is phosphorylated, Kap1-protein interactions are disrupted, Kap1 is no longer associated with chromatin and chromatin decondenses.

Figure 14. The biophysical and molecular components of the mechanism regulating PML NB number following the induction of DSBs. (A) Following DSBs, biophysical changes occur in chromatin and chromatin contacts with PML NBs are compromised, resulting in microbody formation *via* a fission mechanism from preexisting NBs. Secondly, DNA-repair processes regulate PML NB number as loss of ATM, ATR, Chk2 or NBS1 inhibits the increase in PML NB number following DNA damage. (B) PML phosphorylation. Kinases involved in DNA-repair pathways such as Chk2 and ATR can directly phosphorylate PML. ATM mediates PML phosphorylation through Chk2, but it is unknown whether ATM can directly phosphorylate PML. Phosphorylation of PML may disrupt PML NBs, but PML NBs may also be disrupted by further changes in chromatin mediated by ATM through phosphorylation of Kap1. Adapted from Dellaire *et al.* (2006) [119].


A
Inhibition of
ATM, ATR,
Chk2 or NBS1
Biophysical
Response
DNA Repair
dependent
DNA
DSB
Chromatin contacts
Changes in chromatin structure


B
Changes in
chromatin
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NBS1
Kap1
Chk2
ATR

CHAPTER 2: MATERIALS AND METHODS

2.1 REAGENTS

All reagents were from Sigma-Aldrich unless stated otherwise.

2.2 CELL LINES AND DRUG TREATMENTS

Cell lines used in this study are as follows: Phoenix helper-free retrovirus producer cell line (gift from C. McCormick, Dalhousie University, Halifax, Nova Scotia), normal human fibroblasts (NHF) (GM05757; Coriell Cell Repository) and osteosarcoma (U2OS) cells expressing short hairpin RNA (shRNA) against GFP or Kap1 (gift from Y. Shiloh, Tel Aviv University, Tel Aviv, Israel). Phoenix and U2OS cells were grown in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution. U2OS cells expressing GFP or Kap1 shRNA were grown in complete medium supplemented with 1 μg/mL puromycin. NHF and subsequent cell lines derived from them were grown in alpha-minimum essential medium eagle (α-MEME) supplemented with 15% FBS and 1% penicillin-streptomycin-glutamine solution. All cell lines were cultured at 37°C in a 5% CO_2 atmosphere.

To induce DSBs, cells were treated with 20 μM etoposide (VP16) for 30 minutes, washed in phosphate-buffered saline (PBS) (WISENT Inc.), and left to recover for the indicated periods of time.

2.3 CELL LINES CREATED BY RETROVIRAL TRANSDUCTION

All retroviral vectors were transfected into Phoenix cells using the Calcium Phosphate ProFection® Mammalian Transfection System (Promega), as described by the manufacturer. The following cell lines were transduced with the resulting retrovirus

produced from the packaging cell line. The scheme of retroviral transductions is described in Figure 15.

An immortalized normal human fibroblast cell line (Tert-NHF) was generated by retroviral transduction of an early passage of NHF using a pBabe-Hygro-hTert vector [331] encoding the catalytic subunit of telomerase, followed by selection in 75 ug/mL hygromycin (BioShop). Cell lines stably expressing GFP shRNA or Kap1 shRNA were created through retroviral transduction of Tert-NHF cells using pRetrosuper-Puro vectors encoding shRNA against GFP or Kap1 (gift from Y. Shiloh, Tel Aviv University, Tel Aviv, Israel), respectively, followed by selection in 1 ug/mL puromycin. Tert-NHF Kap1 shRNA (Tert-NHF ΔKAP1) cell lines stably expressing wild-type or mutant Kap1 were created by retroviral transduction with the vector pCLXSN expressing HA-tagged wild- type Kap1, mutant Kap1 S824A or Kap1 S824D (gift from Y. Shiloh, Tel Aviv University, Tel Aviv, Israel), respectively, and underwent selection with 1 ug/mL puromycin and 200 ug/mL neomycin.

2.4 IMMUNOFLUORESCENCE

Cells grown on coverslips were treated with VP16 to induced DSBs. Cells were fixed in 2% paraformaldehyde (PFA; Electron Microscopy Sciences) (EMS) in PBS at room temperature (RT) for 10 minutes (min), followed by three 5 min washes in PBS. Cells were quenched in 25 mM ammonium chloride for 5 min to reduce autofluorescence, permeabilized in PBS/0.5% Triton-X100 for 5 min, followed by three 5 min washes in PBS.

Cells were blocked in 5% normal donkey serum in PBS. Cells were then immunolabeled with polyclonal rabbit anti-PML (1:300) (Santa Cruz Biotech; SC-5621) or mouse anti-KRIP1 for Kap1 (1:500) (BD Biosciences; 610680) for 1 h at RT, followed by three 5 min washes in PBS. Cells were further labeled with secondary antibodies conjugated to Alexa Fluor 488 (1:200) (A21206) or Alexa Fluor 568 (1:200) (A10037) (Molecular Probes, Invitrogen) for 30 min at RT, followed by three 5 min washes in PBS. Vectashield mounting medium (Vector Laboratories, Inc.) containing 4,6'diamidino-2-phenylindole (DAPI) or 1 μg/mL DAPI in PBS with mounting medium containing 90% glycerol (Bioshop) and 2% propyl gallate in PBS was used to stain DNA and mount coverslips on slides.

Fluorescent images were taken using a 40X 1.4 NA oil-immersion objective lens on a Zeiss Axioplan 2 motorized upright fluorescence microscope fitted with a Zeiss Axiocam HRc digital camera. Images were acquired using AxioVision 4.6 (Zeiss). Further image processing and analysis was done using ImageJ v.1.42 (National Institutes for Health) (NIH) and Photoshop 8.0 (Adobe).

2.5 STATISTICAL ANALYSIS

To determine the mean PML NB number, immunofluorescence images of PML from multiple focal planes were used to generate an average-intensity Z-projection in Image J v1.42 (NIH). For each experiment, PML NBs were counted in 30 cells for each time point and nucleus size was used to normalize NB number. The ratio of the area of each nucleus divided by the mean area of the nucleus in a dataset was used to normalize NB number between experiments. Normalization of NB number was needed as PML NB

variation during the cell cycle and with ploidy reduces variability between datasets [119]. Experiments were repeated as indicated. Error analysis was expressed as standard error for single experiments (SE) (n = 30) or standard error of the mean (SEM) for repeated experiments (3 x 30 cells, n = 90). Statistical significance between datasets was generated using a pairwise *t* test using Excel software (Microsoft).

2.6 WESTERN BLOT ANALYSIS OF WHOLE CELL LYSATES

Whole cell lysates were prepared after treatment in two ways. To assess the integrity of DNA-damage signaling pathways in the Tert-NHF cell line, whole cell lysates were prepared in 2X SDS loading buffer (20% glycerol, 125 mM Tris-HCl pH 6.8, 4% sodium dodecyl sulfate [SDS], 0.2% bromophenol blue, 10% β-mercaptoethanol) supplemented with the phosphatase inhibitors NaF (10 mM) and β-glycerolphosphate (40 mM). These lysates were boiled for 5 min followed by sonication at a low output for 10-15 seconds to shear the genomic DNA (Misonix Sonicator 3000).

All other whole cell lysates were prepared using a denaturing lysis buffer (10% glycerol, 62.5 mM Tris-HCl pH 6.8, 2% SDS) supplemented with the phosphatase inhibitors NaF (1mM) and Na_3VO_4 (1mM). Hot lysis buffer was added to the cells, immediately followed by boiling for 10 min and sonication as described above. When the lysates were ready to be used, bromophenol blue and β-mercaptoethanol were added to the lysate to a final concentration of 0.05% and 5%, respectively.

Proteins in whole cell lysates were resolved by SDS-polyacrylamide gel electrophoresis and blotted to polyvinylidene difluoride (PVDF) (BioRad or iBlot; Invitrogen) or nitrocellulose (iBlot; Invitrogen). To confirm intact DNA-damage

signaling pathways and levels of Kap1, proteins were visualized using primary antibodies specific to Kap1 (BD Biosciences; 610680), phosphorylated Ser 824 on Kap1 (Bethyl Laboratories, Inc; A300-767A), ATM (GeneTex; GTX70107), phosphorylated Ser 1981 on ATM (Cell Signaling Technology; 4526), Chk2 (Cell Signaling Technology; 2662), phosphorylated Thr 68 on Chk2 (Cell Signaling Technology; 2661), phophorylated Ser 139 on γ-H2A.X (Upstate Cell Signaling Solutions; 05-636) and β-actin (Sigma-Aldrich; A2228). Secondary antibodies used were against mouse (A6782) or rabbit (A6154) from Sigma Aldrich. An enhanced chemiluminescent kit (Thermo Scientific; 34080) followed by exposure to X-ray film (Kodak) was used to detect the antigen-antibody complexes. Densitometry was performed using Image J v.1.42 software (NIH) to determine the expression levels of certain proteins and normalize these levels to the loading control β-actin, to obtain relative protein levels.

2.7 FLOW CYTOMETRY FOR CELL-CYCLE ANALYSIS

Cells were harvested and pelleted by centrifugation at 4000 rpm for 5 min. The cells were washed in cold PBS and pelleted again as described above. Cells were fixed in 70% ethanol on ice at -20°C overnight or longer. On the day of analysis, cells were pelleted, washed in PBS containing 0.2 mg/mL RNase, and pelleted again. Cells were resuspended in PBS-RNase containing 50 μg/mL propidium iodide to a final cell concentration of $3x10^6$ cells/mL and kept on ice for at least 30 min until analysis. Cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences), and peak areas were calculated using Modfit LT 3.0 software (Verity).

2.8 MICCROCOCCAL NUCLEASE DIGESTION

Micrococcal nuclease (MNase) digestions were carried out as described by Ziv *et al.* (2006) [307] with minor modification. Briefly, $1x10^7$ cells were harvested, pelleted and washed twice in PBS. Nuclei were isolated in a buffered solution (340 mM sucrose, 15 mM Tris-HCl pH 7.5, 15 mM NaCl, 60 mM KCl, 10 mM DTT, 0.15 mM spermine, 0.5 mM spermidine, Sigma protease-inhibitor cocktail, 0.25% TritonX-100) at 4°C for 10 min with periodic vortexing, followed by three washes in the buffered solution. Nuclei were digested at 25°C with MNase (Roche Applied Science) at a concentration of 7 U per 250 µL of MNase digestion buffer (60 mM KCl, 15 mM NaCl, 15 mM Tris-HCl pH 7.5, 0.25 M sucrose, 1 mM $CaCl_2$). Aliquots (50 µL) were taken at 5, 10, 15, 20 and 30 min and added directly to 0.5 M EDTA pH 8 to terminate the reaction. A SDS-PrK solution (5% SDS, 1mg/mL Proteinase K) was added to digest away the protein. The resulting solution was diluted in MNase buffer (5 mM Tris-HCl pH 7.5, 25 mM $CaCl_2$) and genomic DNA was purified by phenol-chloroform extraction, followed by ethanol precipitation. The resulting DNA fragments and 1 kB ladder (Fisher Scientific) were resolved on a 1.5% agarose gel containing ethidium bromide (EtBr).

MNase profiles were obtained from UV-fluorescence images of EtBr-stained agarose gels (Licor), followed by analysis of the images using the profiler plugin from Image J v1.42 (NIH). The resulting peaks in these profiles indicate the band fluorescence intensity of each oligonucleosome (N1-N4).

2.9 ELECTRON SPECTROSCOPIC IMAGING

Cells were processed and imaged as described by Dellaire *et al.* (2004) [120]. Briefly, cells on fully confluent coverslips were fixed, quenched and permeabilized in the same manner as regular immunofluorescence. Cells were further fixed in 1% gluteraldehyde (EMS) for 5 min, followed by three 5 min washes in PBS at RTs. Cells were then washed 5X 10 min in distilled water (GIBCO, Invitrogen) to remove excess PBS and prevent salt crystallization in subsequent steps. Cells were then dehydrated in a series of graded ethanol washes (30%, 50%, 70%, 90%, 100%), each for 30 min.

Following dehydration, cells were embedded in an epoxy resin, Quetol 651 (EMS). Cells were first infiltrated with the Quetol 651 resin alone for approximately two hours, followed by 2X 2 h incubations in a Quetol 651 mix (35% v/v Quetol 651, 54% v/v NMA, 11% v/v NSA, 2% v/v DMP-30 hardener; EMS). Coverslips were placed in permanox dishes (Nunc) with Quetol 651 mix and placed in a 65°C incubator for 48 h in order for the resin to harden.

The embedded cells were sent to the lab of Dr. David Bazett-Jones (The Hospital for Sick Children, Toronto, Ontario), where the following steps were performed. The blocks of resin containing the cells of interest were sectioned to 70 nm using a Leica ultramicrotome, from which the resulting sections were colleted on a mesh copper grid (EMS). The sections were imaged using a Tecnai 20 transmission electron microscope (FEI) equipped with an energy-filtering spectrometer (Gatan). Energy-filtered electron micrographs of nitrogen and phosphorus were collected, false-colour labeled (nitrogen – green, phosphorus – red) and combined in Photoshop 8.0 (Adobe) to highlight

chromatin. More detailed protocols on electron spectroscopic imaging can be seen in Bazett-Jones and Hendzel (1999) [332] and Dellaire *et al.* (2004) [120].

The composite elemental maps for nitrogen and phosphorus were then analyzed for thickness of nuclear-lamina-associated chromatin using Image J v1.42 software (NIH). Pixel measurements were converted into microns (μm) and then averaged (n = 10). Statistical significance between cell lines was generated using a pairwise *t* test in Excel (Microsoft).

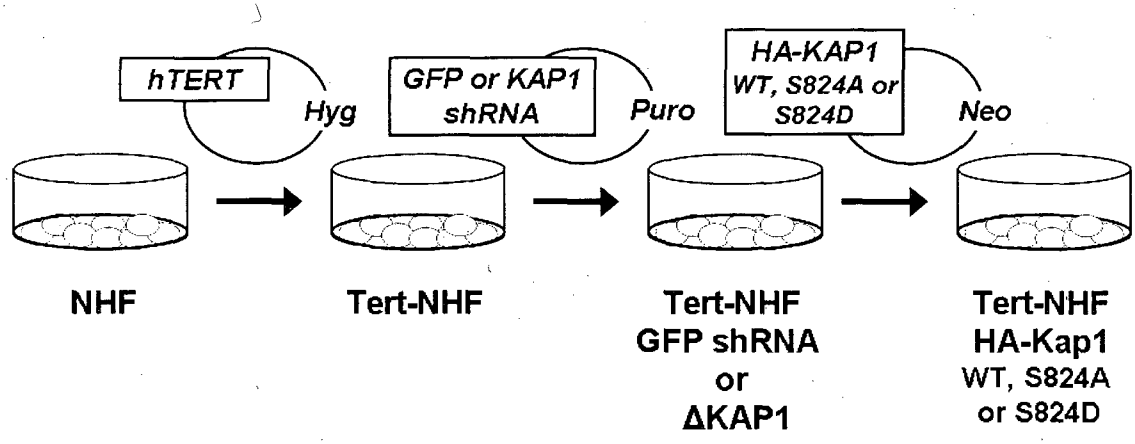


Figure 15. Retroviral transduction of cell lines used in this study. NHF cells were transduced with a retrovirus expressing the catalytic subunit of human telomerase (hTERT). Immortalized Tert-NHF cells were then sequentially transduced with retroviruses encoding a shRNA directed against GFP or Kap1, followed by re-introduction of HA-tagged wild-type Kap1 or mutant Kap1 (S824A or S824D). Antibiotic selection at each stage of transduction is indicated.

CHAPTER 3: RESULTS

3.1 GENERATING A TERT-IMMORTALIZED NHF CELL LINE

This study heavily relied on the use of a well-characterized cell line to achieve stable knockdown of Kap1 through RNAi followed by re-introduction of wild-type or mutant Kap1 to allow the study of the effects of Kap1 on PML NB number before and after DSBs. Previous studies looking at the response of PML NB to DSB had used primary human fibroblasts, WI38 [321], GM05757 [119]. I chose the GM05757 NHF cell line, which has been used in numerous studies involving DNA-damaging agents [333-336]. Untransformed cells exhibit cellular senescence after several rounds of replication, which is triggered by the shortening of telomeres [337-339]. Ectopic expression of the catalytic subunit of telomerase (TERT) can extend the replicative life span of cells by aiding in the maintenance of telomeres [331, 340, 341]. Therefore, I immortalized the NHF cell line by retroviral transduction of human TERT using low passage cells.

Prior to creating other stable cell lines through RNAi or ectopic gene expression, I sought to characterize the DNA-repair pathways and PML NB behaviour of the Tert-NHF cells (Figure 16). The integrity of DNA-damage signaling pathways was assessed using etoposide (VP16) in a single pulsed dose (20 μM, 30 min) to create ~75-100 DSBs, equivalent to ~2G of IR (Figure 16A) [119]. VP16 inhibits topoisomerase II (TopoII), an enzyme that passes through a DNA double helix causing a transient DSB to control DNA topology [342]. When TopoII is inhibited by VP16, normally transient DSBs created by the enzyme persist as lesions that are then converted to DSBs [342]. ATM, Kap1 and Chk2 were present at all time points, indicating that the genes for these proteins were not affected by TERT integration into the genome (Figure 16A). Activation of ATM was seen after 30 min VP16 treatment, as evidence by phosphorylation of ATM on serine

residue 1981 (Figure 16A). Downstream targets of ATM were also activated following VP16 treatment. H2A.X, Chk2 and Kap1 were phosphorylated on serine residue 139 (Figure 16A), threonine residue 68 (Figure 16A) and serine residue serine 824, respectively (Figure 16A), with peak phosphorylation occurring at 30 min post-VP16 treatment.

PML NB behaviour after VP16 treatment was also assessed. Following 3 h recovery from VP16 treatment, PML NB number was significantly higher compared to the no DNA damage control ($p<0.05$) (Figure 16B). These results were similar to the response of non-immortalized NHFs to VP16 treatment [119]. Therefore, TERT immortalization did not affect either the DNA-damage signaling or PML NB behaviour of this cell line, making the Tert-NHF cell line suitable to use for the creation of other cell lines.

3.2 LOSS OF KAP1 CAUSES AN INCREASE IN PML NB NUMBER IN A CELL-TYPE INDEPENDENT MANNER AND DOES NOT RELATE TO CHANGES IN CELL CYCLE DISTRIBUTION

ATM and ATR are implicated in the regulation of PML NB number [119]. It is possible that ATM and/or ATR cause changes in chromatin structure affecting PML NB integrity. Indeed, PML NB number is affected by changes in chromatin [102, 114]. Furthermore, loss of Kap1, a protein regulated by ATM, has been shown to cause global decondensation of chromatin [307]. Therefore, to identify the chromatin-dependent control of PML NB number, I wanted to determine if PML NB number would be affected by the loss of Kap1.

A stable Kap1-knockdown cell line was achieved by transducing retrovirus encoding shRNA against Kap1 into Tert-NHF cells to knock down Kap1 by RNAi. There was an 80% knockdown of Kap1 protein in the Tert-NHF ΔKAP1 cells compared to Tert-NHF cells alone or with a control GFP shRNA (Figure 17). Immunofluorescence analysis was used to further confirm Kap1 knockdown (Figure 18B) compared to the GFP control (Figure 18A) and to assess PML NB number (Figure 18). Without DNA damage, PML NB number was significantly higher in ΔKAP1 cells (~20 ± 0.5 NBs) compared the GFP control (~12 ± 0.5 NBs) ($p<0.05$) (Figure 19; control).

Kap1 was re-introduced into a Tert-NHF ΔKAP1 cell line by transducing these cells with retrovirus encoding HA-tagged wild-type Kap1 that is not affected by Kap1 shRNA. Kap1 protein levels increased back to 70% of control levels (Figure 17). Without DNA damage, PML NB number in these cells was ~14NBs (± 0.3 NBs), a similar level as seen in the GFP control (~12 ± 0.5 NBs), indicating that the increase in PML NBs seen in ΔKAP1 cells was specific to the depletion of Kap1 (Figure 19; control).

The same phenomenon was also observed in U2OS cells, where the depletion of Kap1 by 91% using RNAi led to a significant increase in PML NB number (~13 ± 0.8 NBs) compared to U2OS cells expressing the GFP shRNA control (8 ± 1 NB) ($p<0.05$) (Figure 20). Thus, depletion of Kap1 by RNAi leads to a cell-type independent increase in PML NB number.

PML NB number can vary during the cell cycle; in particular, PML NBs increase in number in S-phase [114]. Therefore, I used propidium iodide and flow cytometry to see if the loss of Kap1 affects the fraction of cells in S-phase, which could contribute to an increase in PML NB number. The cell-cycle profiles looked similar between Tert-NHF

and Tert-NHF ΔKAP1 cells (Figure 21A). Of more importance, the percentage of cells in S-phase was similar for Tert-NHF cells (13 ± 2 %) and ΔKAP1 (17 ± 2 %) (p = 0.28) (Figure 21B). Therefore, the increase in PML NB number due to loss of Kap1 does not depend on cell type or does not rise from changes in cell cycle distribution.

3.3 LOSS OF KAP1 ALTERS CHROMATIN STRUCTURE

Kap1 is involved in chromatin organization and loss of Kap1 leads to increased nuclease sensitivity of chromatin in U2OS cells, which is interpreted as relaxation of chromatin [307]. I wanted to observe if this is the case in our Tert-NHF ΔKAP1 cell line. Basal chromatin condensation was evaluated by micrococcal nuclease (MNase) digestion, which cleaves the linker DNA between nucleosomes resulting in chromatin fragments of multiples of ~146 bp [3] corresponding to fragments with different numbers of nucleosomes. The mono-, di-, and tri-nucleosome DNA fragments that are resolved by agarose gel electrophoresis reflect chromatin accessibility to MNase, a surrogate measure of chromatin compaction. MNase accessibility was greater in the Tert-NHF ΔKAP1 cells, as smaller DNA fragments were produced earlier in the MNase digestion (Figure 22A). Profiles from the gels illustrate more clearly that in Tert-NHF ΔKAP1 cells, smaller oligonucleosomes were generated from the 10 and 30 minutes of MNase digestion in figure 8A compared to those from Tert-NHF cells alone (Figure 22B). This finding suggests that loss of Kap1 mediates an enhanced sensitivity of chromatin to MNase.

Although chromatin relaxation associated with loss of Kap1 can be shown on a molecular level by an increased sensitivity to MNase (Figure 22A) [307], changes in chromatin ultrastructure as a result of the loss of Kap1 have never been demonstrated.

Therefore, I employed electron microscopy to visualize chromatin on an ultrastructural level in Tert-NHF ΔKAP1 cells. Electron spectroscopic imaging (ESI) has the ability to distinguish between protein and DNA-based structures such as chromatin [120, 332]. Merged nitrogen (green) and phosphorus (red) elemental maps allow the identification of chromatin (yellow) (Figure 23A). Condensed chromatin can be seen most easily at the periphery of human nuclei adjacent to the nuclear lamina. Large regions of dense chromatin are seen in Tert-NHF cells alone, whereas Tert-NHF ΔKAP1 cells show more decondensed chromatin at the nuclear periphery (white arrows, Figure 23A). As a consequence, chromatin at the nuclear lamina was significantly thinner in Tert-NHF ΔKAP1 cells (0.20 ± 0.03 μm) compared to Tert-NHF cells (0.35 ± 0.05 μm) ($p<0.05$) (Figure 23B). Therefore, loss of Kap1 appears to cause decondensation of chromatin at the ultrastructural level.

3.4 EVIDENCE FOR A KAP1 PHOSPHORYLATION-DEPENDENT MECHANISM REGULATING CHANGES IN PML NB NUMBER

Loss of Kap1 causes chromatin relaxation [307] and regulates PML NB number as demonstrated above. Chromatin relaxation also occurs when Kap1 is phosphorylated by ATM on serine residue 824 following DNA damage, which most likely alters the association of Kap1 with chromatin [307]. If Kap1 itself regulates PML NB number, perhaps phosphorylation of Kap1 also regulates PML NB number. Therefore, to test this hypothesis, I determined PML NB number following DNA damage in ΔKAP1 cells complemented with Kap1 phospho-mutants.

First, Kap1 phospho S824A or S824D mutants were re-introduced into Tert-NHF ΔKAP1 cells by retroviral transduction using KAP1 cDNAs resistant to KAP1 RNAi [307].

The Kap1 S824A mutant cannot be phosphorylated, as the serine residue at position 824 is substituted with an alanine, which lacks a hydroxyl group. The Kap1 S824D mutant is a phospho-mimetic mutant, where the substitution of a serine with an aspartic acid mimics the charge after phosphorylation. The expression of these mutants was 80% of the levels of wild-type Kap1 prior to knockdown (Figure 17).

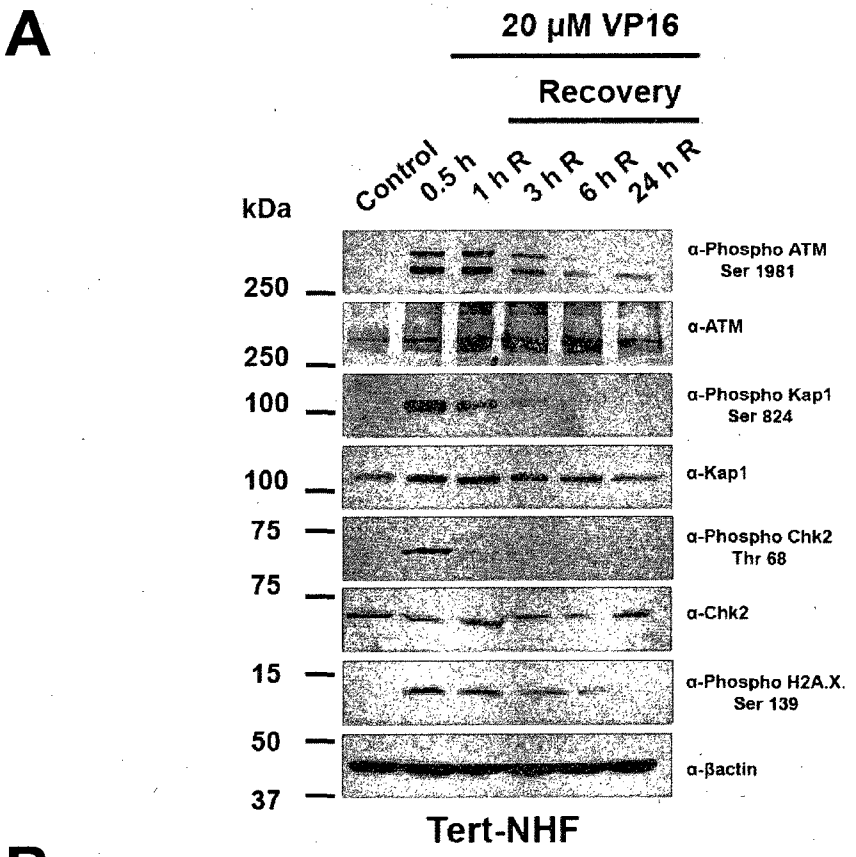
I next examined these cell lines for PML NB number by immunofluorescence analysis (Figure 24). If Kap1 phosphorylation is important for regulating PML NB number, I would expect that, prior to DNA damage, the Kap1 S824D mutant would have higher PML NB number compared to the GFP shRNA cells and the Kap1 S824A mutant, as the S824D mutant mimics phosphorylation of Kap1. Indeeed, PML NB number was significantly higher in Kap1 S824D mutant cells (~17 ± 0.5 NBs) compared to the GFP shRNA control (~12 ± 0.5 NBs) and Kap1 S824A mutant cell (~14 ± 0.5 NBs) prior to DNA damage ($p<0.05$) (Figure 25; control). This finding suggests that phosphorylation of Kap1 is important in regulating PML NB.

3.5 FOLLOWING DNA DAMAGE, PML NB NUMBER FURTHER INCREASES WITH LOSS OF KAP1 OR MUTANT KAP1

In a NHF cell line, PML NB number increases in response to DSBs and, specifically following VP16 treatment, PML NB number peaks at 3 h recovery [119]. This trend was confirmed following immunofluorescence analysis of PML NB number in GFP shRNA cells and HA-tagged wild-type Kap1 cells, where there is an increase from ~12 ± 0.5 and 14 ± 0.5 NBs to ~22 ± 0.5 and 24 ± 0.5 NBs, 3 h post VP16 treatment respectively ($p<0.05$) (Figure 19; control & 3 h R). If Kap1 were the only factor regulating PML NB number prior to and following VP16 treatment, PML NB number in ΔKAP1 cells would

remain at a constant level following DNA damage. However, PML NB number in ΔKAP1 cells further increased from 20 ± 0.5 NBs to 24 ± 0.5 NBs ($p<0.05$) (Figure 25; control & 3 h R). PML NB number also further increased in both of the Kap1 phospho-mutants, with body number in Kap1 S824A and S824D increasing from ~14 ± 0.5 and 17 ± 0.3 NBs to ~19 ± 0.5 NBs 3 h post-VP16 treatment ($p<0.05$) (Figure 25; control & 3 h R). Both Kap1 phospho-mutants had approximately the same number of PML NBs (~19 ± 0.5 NBs) 3 h post-VP16 treatment (Figure 25; control & 3 h R). Together these data indicate that some other factors or mechanisms could be controlling PML NB number following DNA damage, in addition to Kap1.

Figure 16. ATM DNA-damage signaling pathways and PML NB behaviour following etoposide (VP16) treatment remain unperturbed. (A) Assessment of ATM, Kap1, Chk2 and H2A.X phosphorylation and protein levels. Cellular extracts were blotted with the indicated antibodies at various recovery times following 30 min of VP16 treatment to induce DSBs. (B) PML NB number increases after DNA damage. Mean PML NB number is compared over time after VP16 treatment and indicated periods of recovery. Error bars represent standard error (n = 30) (*$p<0.05$ between control and 3 h recovery) (R = recovery).


A
20 μM VP16
Recovery
Control
0.5 h
1 h R
3 h R
6 h R
24 h R
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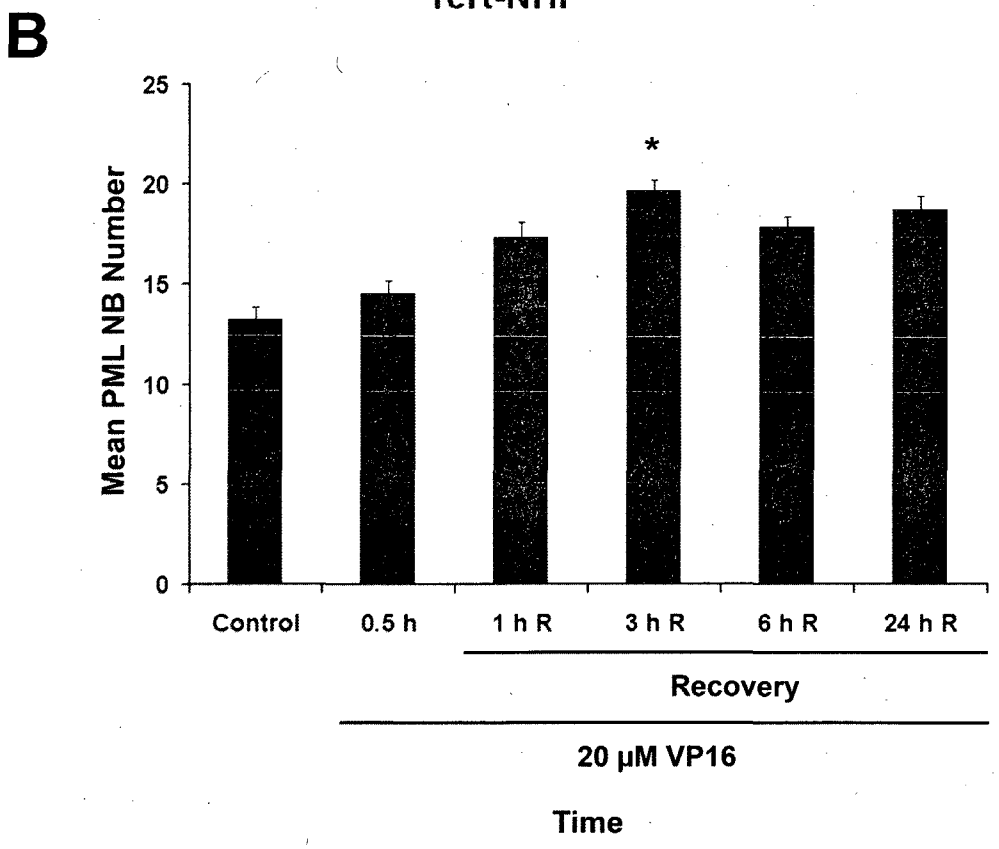
250
250
100
100
75
75
15
50
37
α-Phospho ATM Ser 1981
α-ATM
α-Phospho Kap1 Ser 824
α-Kap1
α-Phospho Chk2 Thr 68
α-Chk2
α-Phospho H2A.X. Ser 139
α-βactin
Tert-NHF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Mean PML NB Number
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15
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0.5 h
1 h R
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Recovery
20 μM VP16
Time

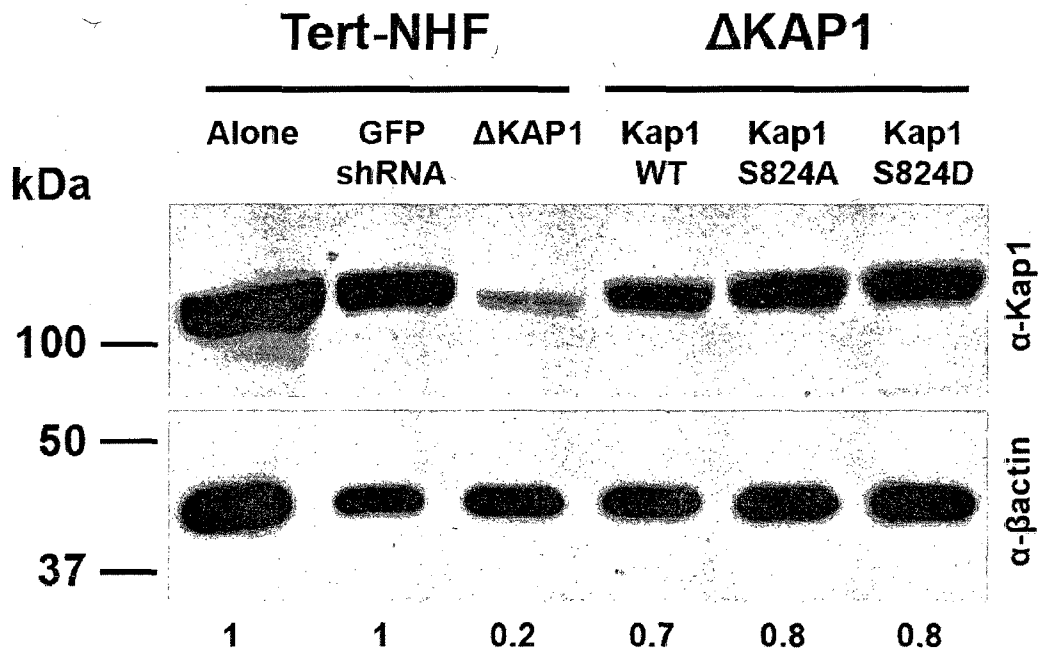


Figure 17. Relative Kap1 protein levels in cells lines with different constitutions of Kap1. Cellular extracts were obtained from Tert-NHF GFP shRNA or ΔKAP1 cells followed by ectopic expression of wild-type or mutant Kap1 in a ΔKAP1 cell line. Confirmation of Kap1 protein levels was done by western blot analysis using an antibody against Kap1, with β-actin as a loading control. Relative protein levels are indicated.

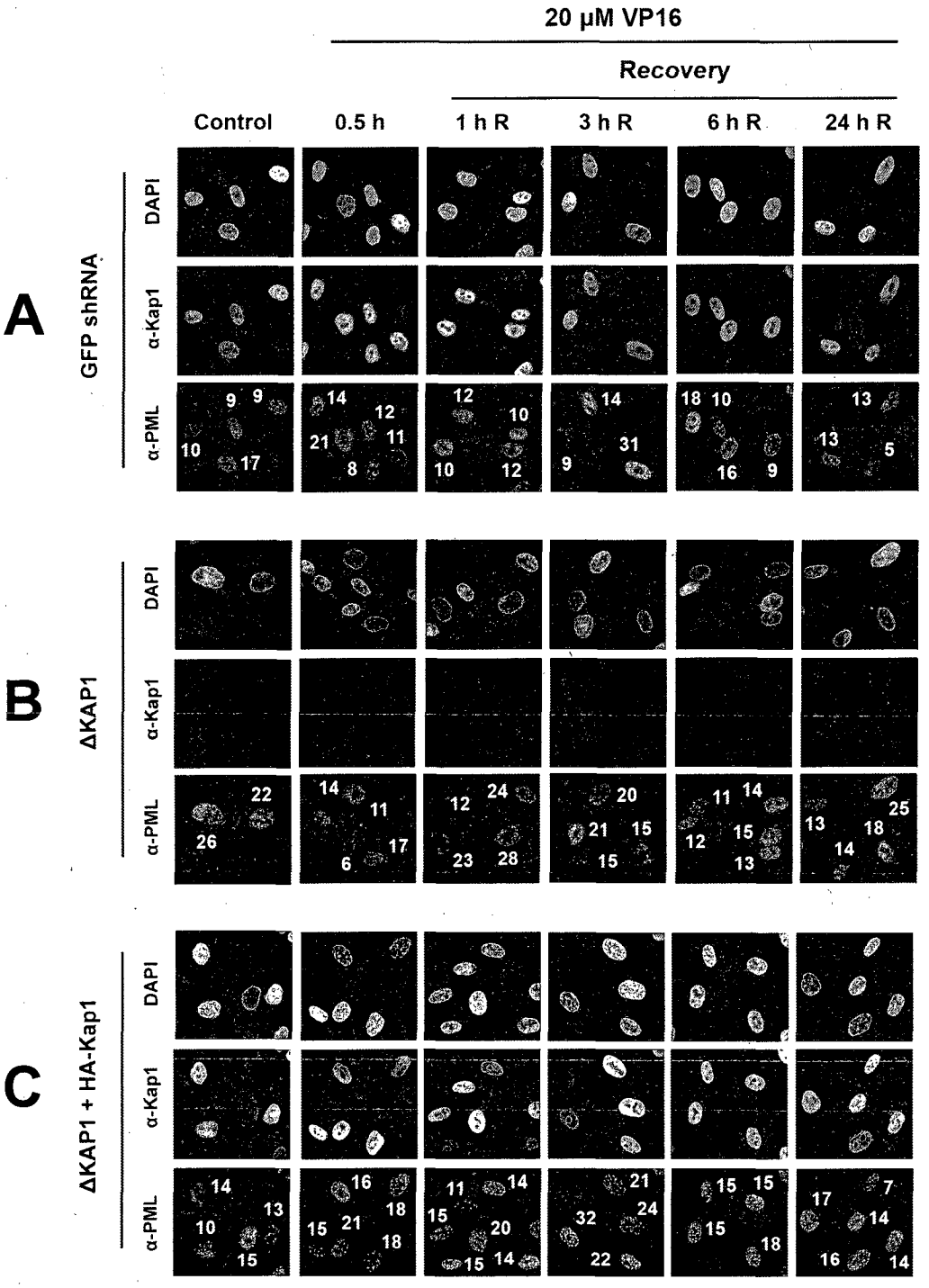


Figure 18. Representative immunofluorescence images of Tert-NHF cell lines with wild-type Kap1 or loss of Kap1. Normal levels of Kap1 can be seen in (A) GFP shRNA cells and (C) ΔKAP1 + HA-Kap1 cells. Loss of Kap1 is observed in (B) ΔKAP1 cells. Images were analyzed for levels of Kap1 and PML NB number in average-intensity Z-projections after VP16 treatment and indicated periods of recovery. DNA was stained with DAPI to view the nucleus (R = recovery).

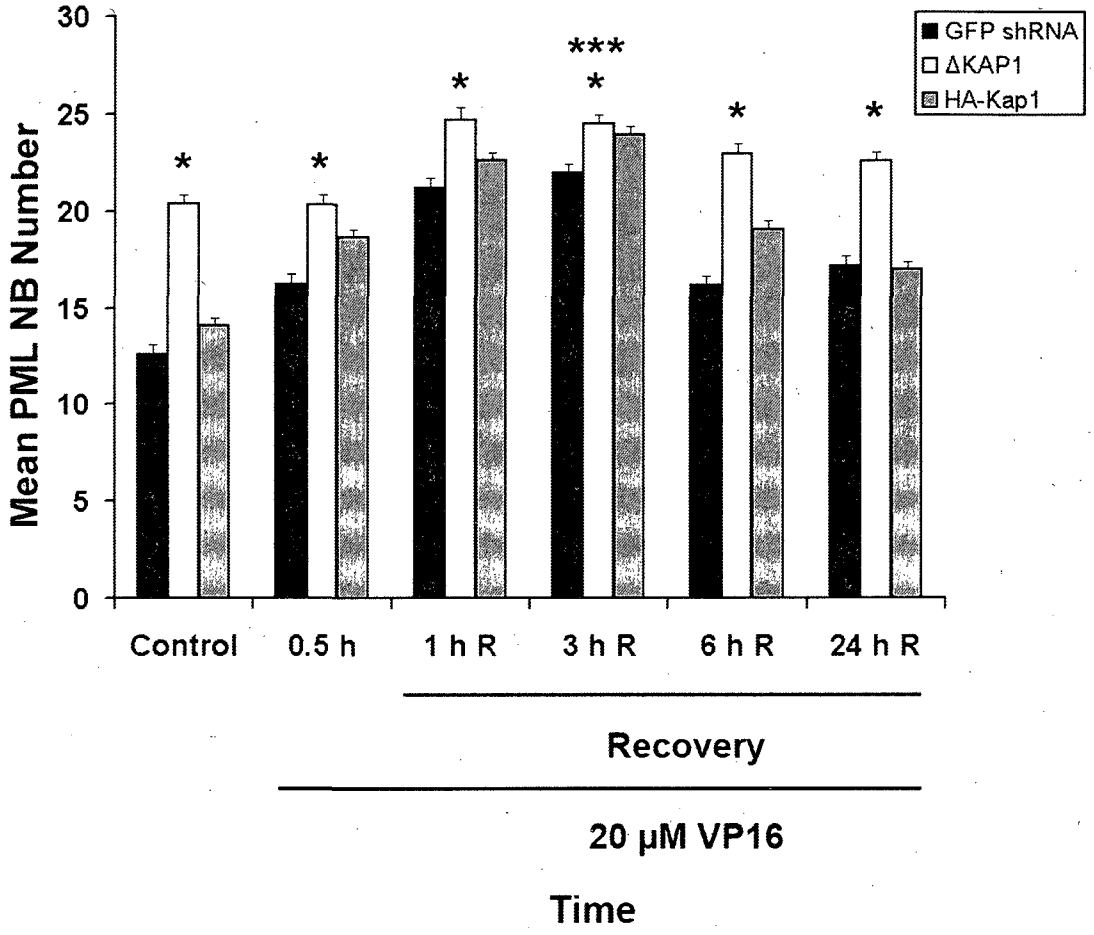


Figure 19. PML NB number increases with loss of Kap1 before and after DNA damage. (A) Comparison of mean PML NB number over time after VP16 treatment and recovery for the indicated periods of time in Tert-NHF treated with GFP shRNA, ΔKAP1 cells and in a ΔKAP1 cell line following the re-introduction of HA-tagged wild-type Kap1. Error bars represent standard error of the mean (n = 90) (*$p<0.05$ between cell lines, ***$p<0.05$ between control and 3 hr recovery within the cell line) (R = recovery).

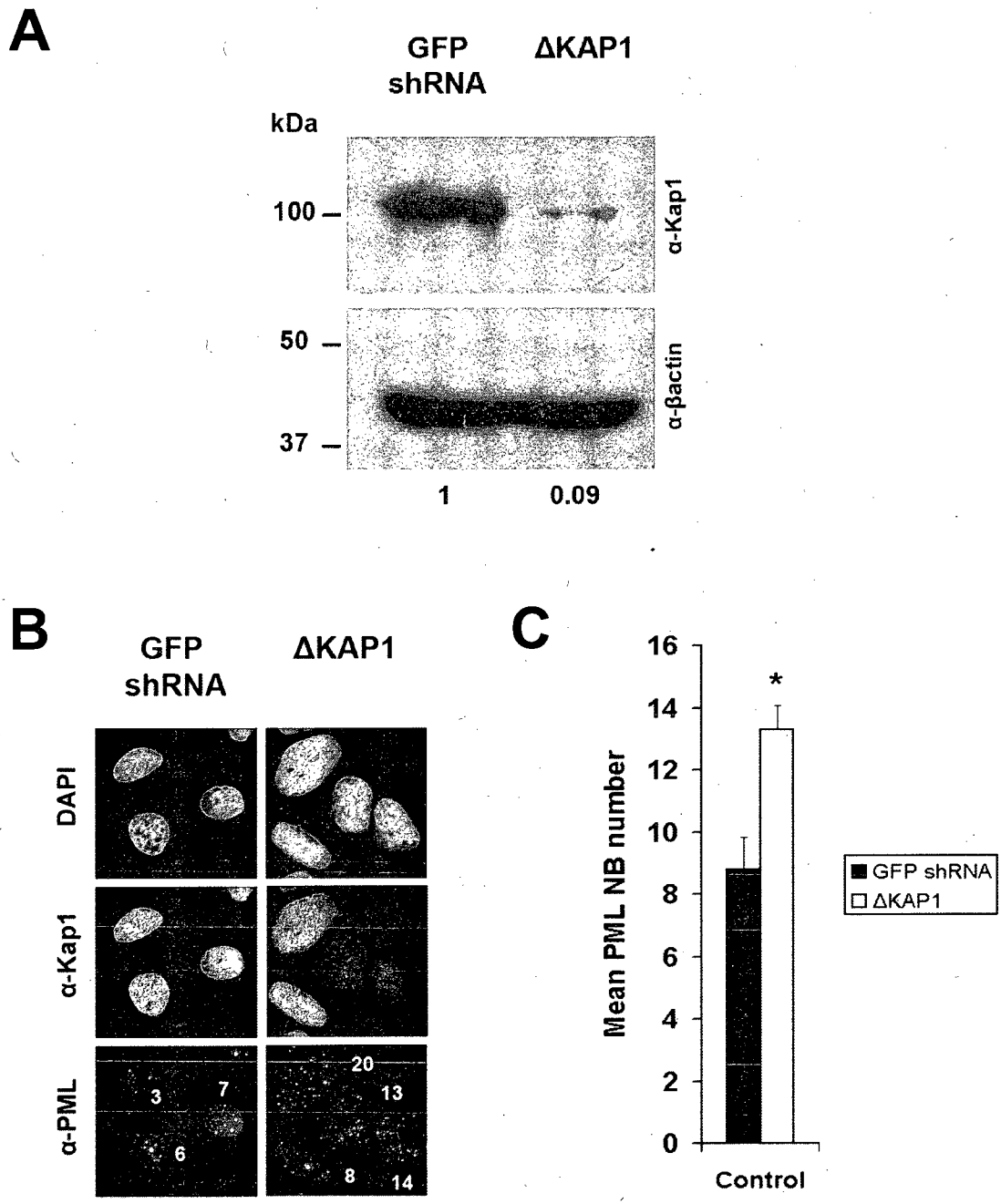


Figure 20. Characterization of a U2OS cell line for Kap1 protein and PML NB number with normal levels of Kap1 and upon loss of Kap1. (A) Western blot analysis for Kap1 expression in U2OS cell lines with GFP shRNA or ΔKAP1. Relative protein levels are indicated. (B) Immunofluorescence analysis of Kap1 and PML NB number in average-intensity Z projections of U2OS cell lines with GFP shRNA or ΔKAP1. (C) Comparison of mean PML NB number without DNA damage. Error bars represent standard error (n = 30) (*$p < 0.05$).

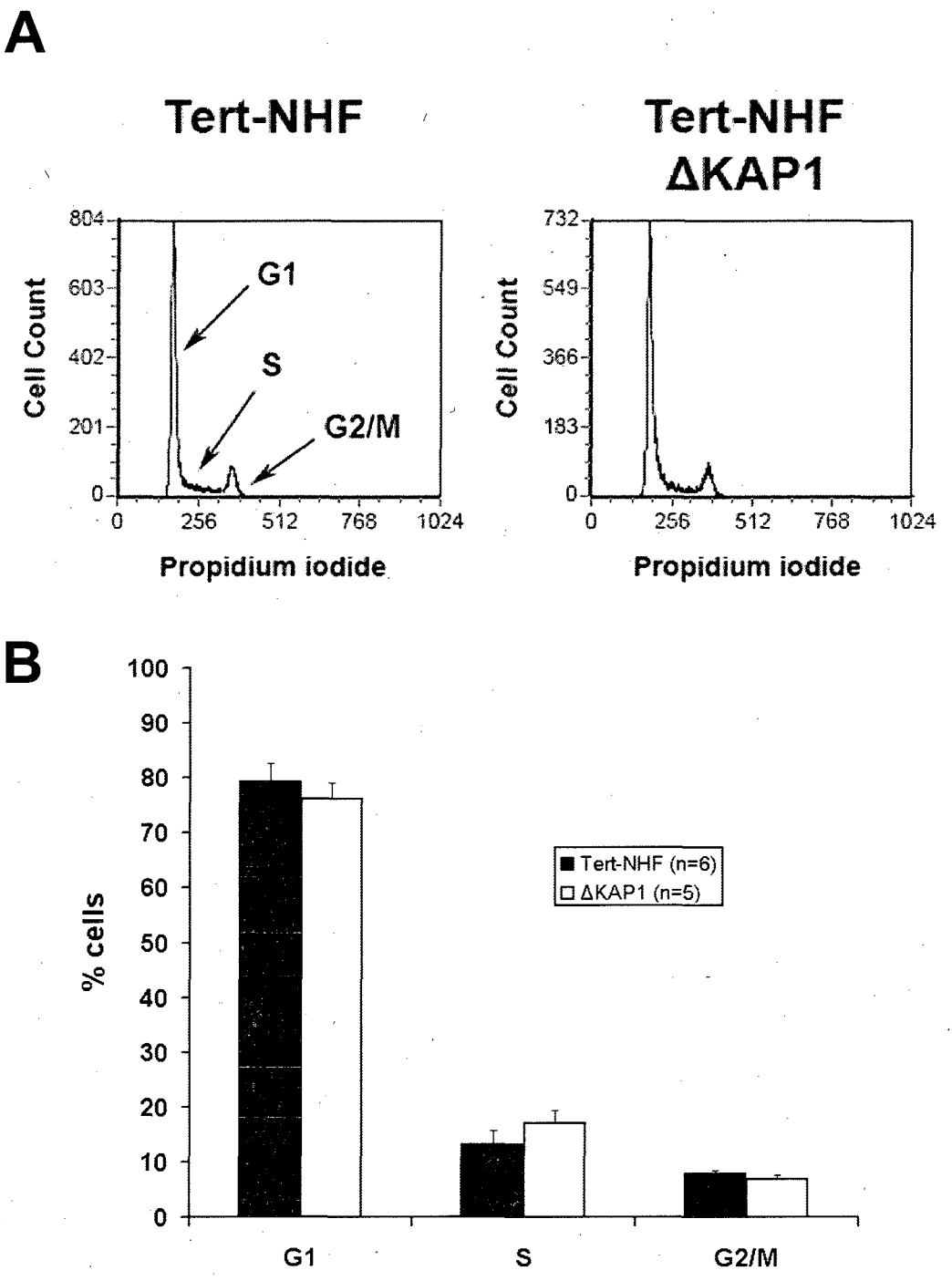
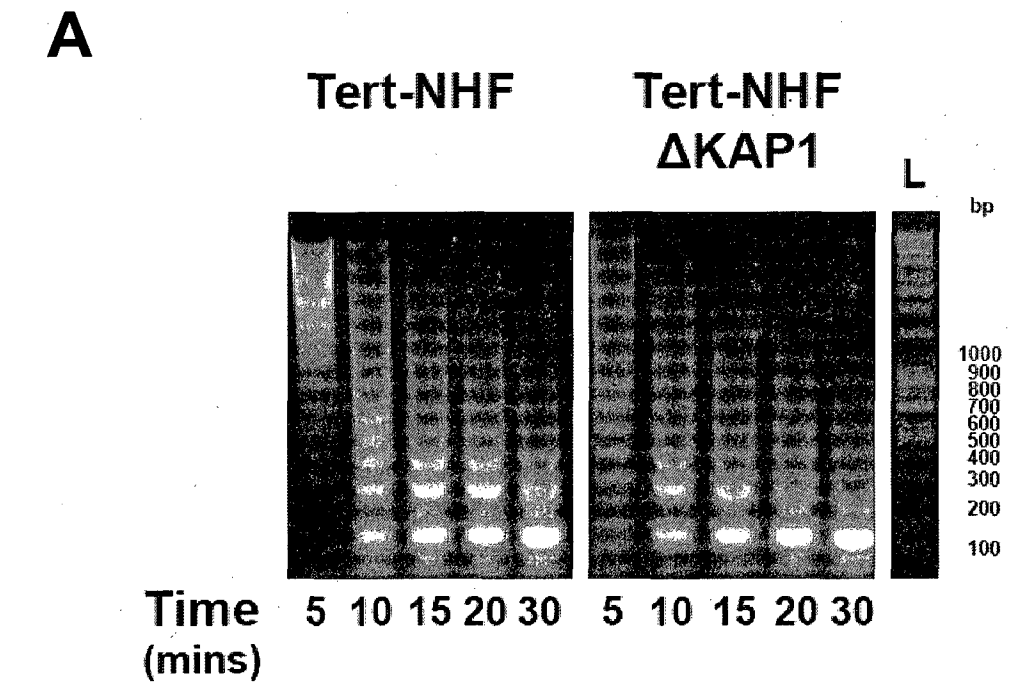


Figure 21. Loss of Kap1 does not activate an S-phase cell-cycle checkpoint. (A) Flow cytometry profiles of Tert-NHF and Tert-NHF ΔKAP1 cells with no DNA damage. Propidium iodide (PI) represents DNA content (x axis), whereas the number of cells exhibiting a specific fluorescence of PI represents cell count (y axis). (B) Expression of the data shown in (A) as percentage of cells in each phase of the cell cycle (G1, S, G2/M). The area of under each peak in the flow cytometry profile was used to determine the percentage of cells in each phase of the cell cycle. (p=0.28 S-phase).



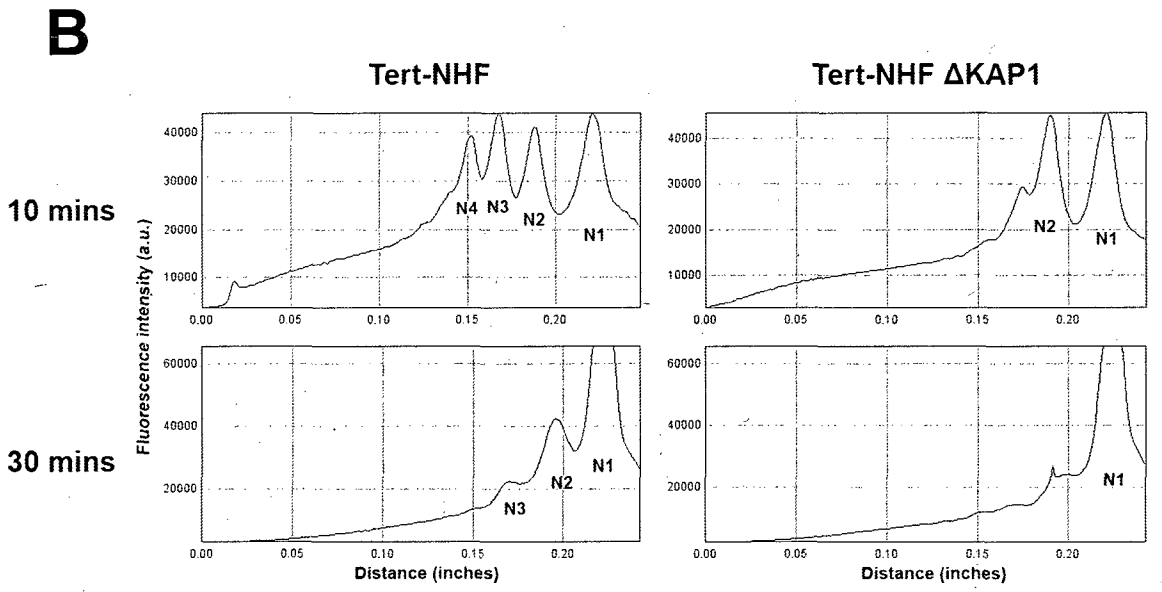


Figure 22. Loss of Kap1 induces chromatin decondensation. (A) Chromatin accessibility was assessed by MNase digestion in Tert-NHF or Tert-NHF ΔKAP1. The chromatin from untreated cells was subjected to MNase digestion for the indicated periods of time and the resulting DNA fragments and DNA ladder (L) in basepairs (bp) were resolved on a 1.5% agarose gel. (B) Profiles of the indicated digestion time points in the cell lines indicated in (A). Fluorescence intensity is expressed in arbitrary units (a.u.). N1-N4 denotes the number of nucleosomes in each oligonucleosome.

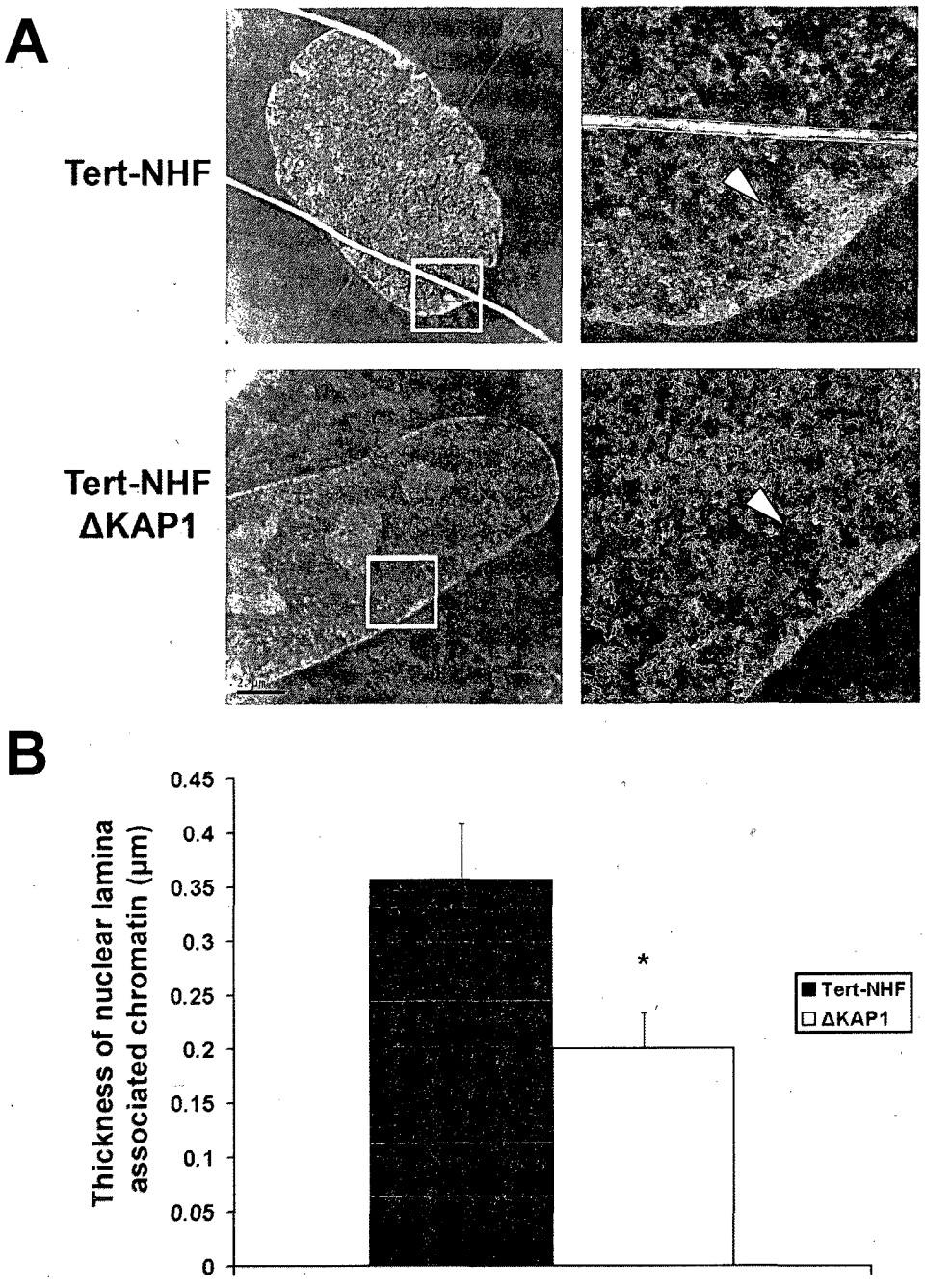


Figure 23. Change in nuclear-lamina-associated chromatin with loss of Kap1. (A) ESI micrograph of a single Tert-NHF cell alone or with ΔKAP1. Cells were embedded and sectioned to 70 nm. Elemental maps were generated using ESI. Phosphorus (red) and nitrogen (green) were merged, resulting in the image seen where chromatin is indicated in yellow due to its high phosphorus and nitrogen content. The outlined area was magnified in the coloured panel. Nuclear-lamina-associated chromatin is indicated by the white arrow heads. (B) Thickness of chromatin at the nuclear lamina in Tert-NHF and Tert-NHF ΔKAP1. Thickness of the chromatin expressed in microns (μm) was obtained from numerous measurements (n=10) on the image in (A) for each cell line (*$p < 0.05$).

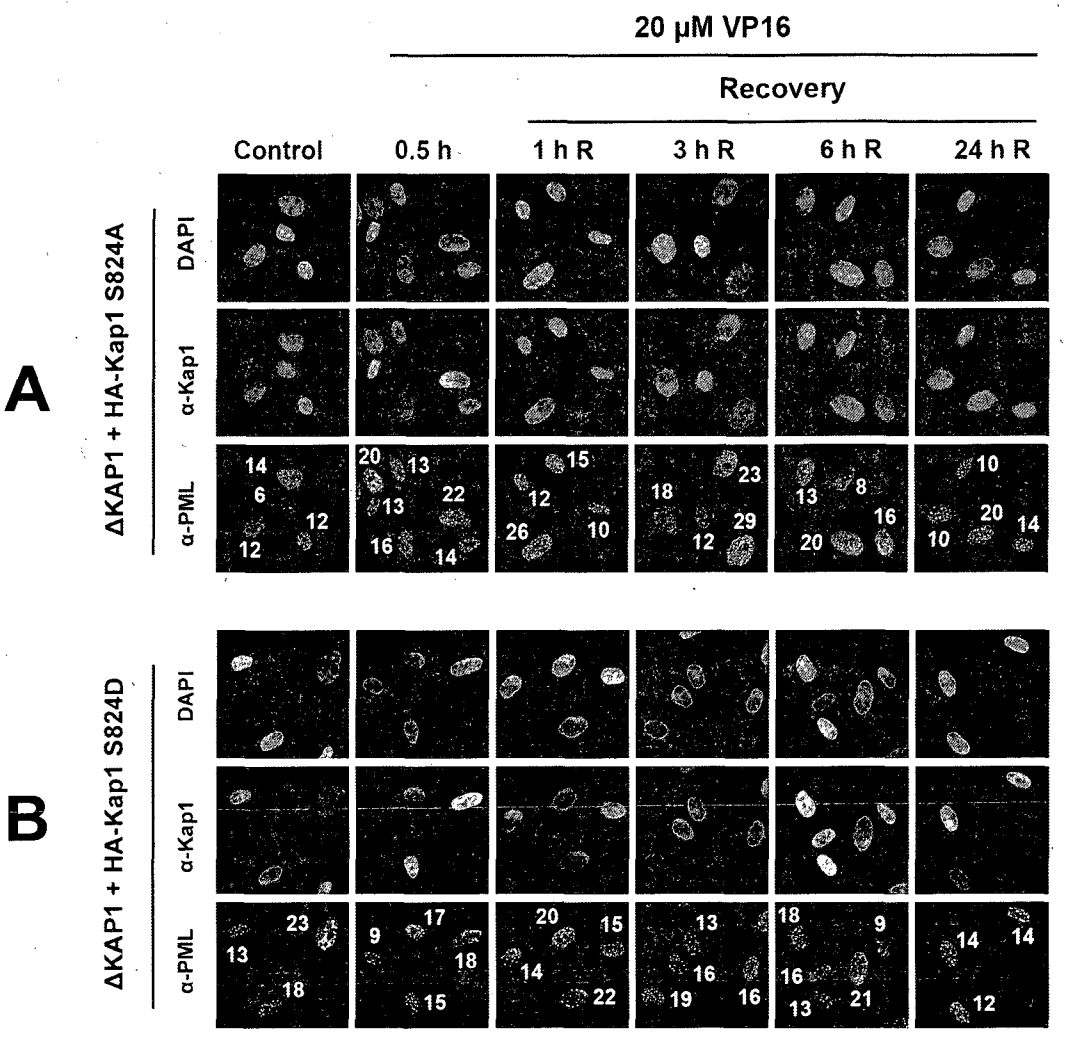


Figure 24. Representative immunofluorescence images of Tert-NHF cell lines with mutant Kap1. Mutant Kap1 is expressed in the (A) ΔKAP1 + HA-Kap1 S824A or (B) S824D cells. Images were analyzed for levels of Kap1 and PML NB number in average-intensity Z-projections after VP16 treatment and indicated periods of recovery. DNA was stained with DAPI to view the nucleus (R = recovery).

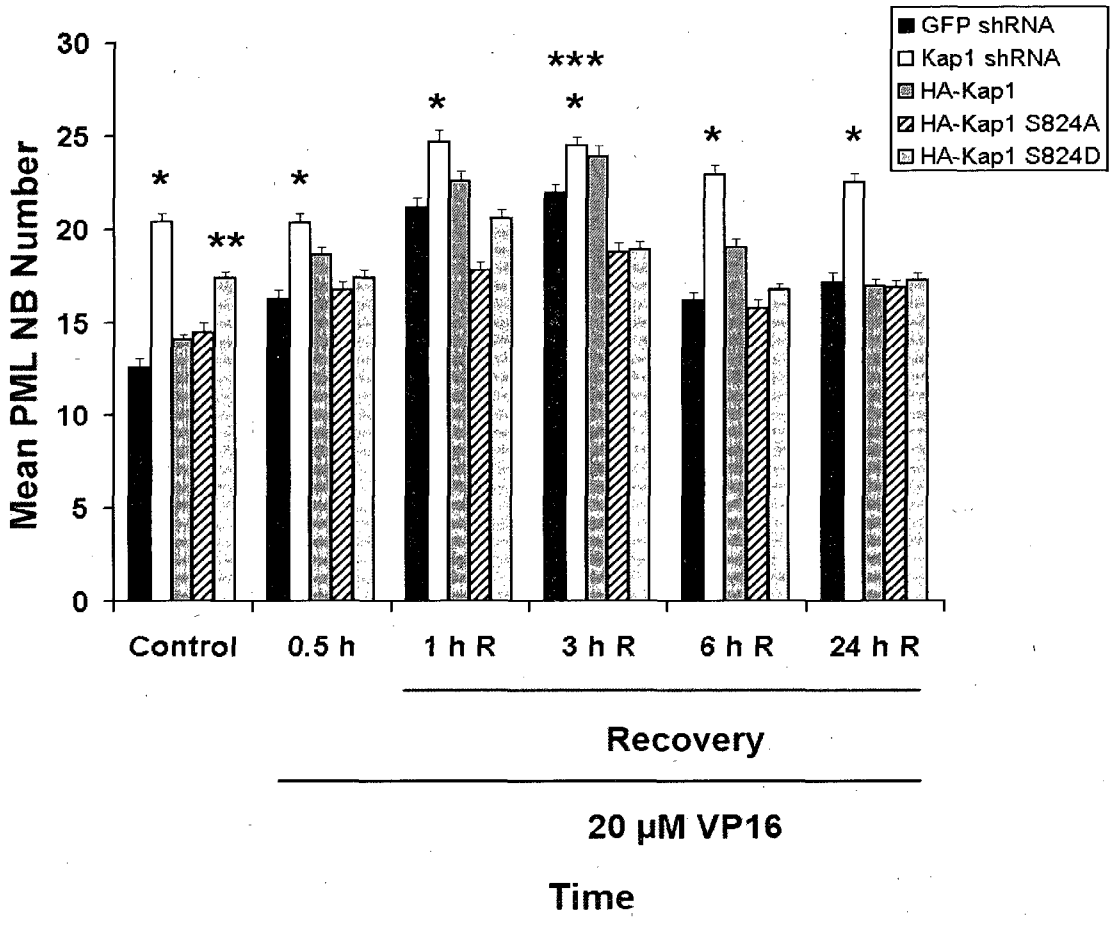


Figure 25. Expression of mutant Kap1 indicates that PML NB number is regulated by phosphorylation of Kap1. (A) Comparison of mean PML NB number over time after VP16 treatment and recovery for the indicated periods of time in Tert-NHF cells with GFP shRNA or ΔKAP1, following re-introduction of HA-tagged mutant S824A or S824D Kap1 in ΔKAP1 cells. Error bars represent standard error of the mean (n = 90) (*$p<0.05$ between cell lines, **$p<0.05$ between GFP shRNA/HA-Kap1/Kap1 S824A and Kap1 S824D, ***$p<0.05$ between control and 3 hr recovery within the cell line) (R = recovery).

CHAPTER 4: DISCUSSION

4.1 SUMMARY

PML NBs mediate signals in response to DNA damage to facilitate DNA repair, cell- cycle control and apoptosis [149]. Furthermore, PML NBs act as DNA damage sensors by increasing in number following DNA damage [119, 120]. This phenomenon is regulated by ATM and ATR kinases [119]. How ATM and ATR regulate the increase in PML NBs following DNA damage may be through changes in chromatin structure, due to the intimate relationship between chromatin and PML NBs [102, 119]. Kap1 is an ATM/ATR substrate that facilitates chromatin condensation, and can contribute to the decondensation of chromatin when Kap1 is either phosphorylated by ATM on serine residue 824 or depleted [37, 307]. I have found a novel pathway involving Kap1 in the regulation of PML NB number in part through Kap1-mediated changes in chromatin structure (Figure 26).

4.2 A KAP1-DEPENDENT MECHANISM REGULATING PML NB NUMBER

Kap1 is a protein that normally regulates chromatin structure along with other proteins such as HP1 and KRAB-ZFP to facilitate the formation of heterochromatin and gene silencing [37]. Loss of Kap1 or phosphorylation of Kap1 by ATM on serine residue 824 mediates global decondensation of chromatin [307], although Kap1 can be phosphorylated by ATR as well [310]. PML NBs respond to changes in chromatin structure by increasing in number [102, 119]. As ATM and ATR regulate PML NB number following DNA damage [119], it is possible that ATM and/or ATR mediate PML NB number *via* Kap1.

To my surprise, I found that Kap1 was directly implicated in the control of PML NB number. I observed an increase in PML NB number in the absence of DNA damage

in Tert-NHF depleted of Kap1 by RNAi (Tert-NHF ΔKAP1) (Figure 18, 19). This effect is not dependent on cell type, as an increase in PML NB number prior to DNA damage was also seen in the U2OS cell line after ΔKAP1 (Figure 20). Furthermore, this effect on PML NB number is specific to Kap1, as re-introduction into Tert-NHF ΔKAP1 cells of an HA-tagged wild-type Kap1, resistant to Kap1 shRNA reduced PML NB number to a level similar to that seen in the GFP shRNA control cells (Figure 18, 19). This is the first study linking Kap1 to the control of PML NB number (Figure 26).

4.2.1 HOW DOES KAP1 CONTROL PML NB NUMBER?

There are several of possibilities as to how Kap1 controls PML NB number. One possibility is that loss of Kap1 activates intra-S checkpoints in the cell cycle, resulting in an increase in PML NBs. PML NBs have been shown to increase two-fold during S-phase [114]. Therefore, the increase in PML NB number in cells lacking Kap1 might be due to an increase of cells in S-phase. However, there were similar numbers of cells in S-phase in Tert-NHF and Tert-NHF ΔKAP1 cells (Figure 21). A similar observation was made by Ziv and colleagues (2006) [307] as cells lacking Kap1 did not affect activation of cell-cycle checkpoints, even upon DNA damage, compared to cells with control GFP shRNA [307]. However, these published data are expressed as G1/G2 ratio, and the percentage of cells in S-phase is lost. It is necessary to confirm this lack of cell-cycle effect in ΔKAP1 cells by specifically looking at cells in S-phase. Nonetheless, a significant increase of cells in S-phase would be required to alter PML NB number, which was not observed.

A more likely possibility as to how PML NB number is regulated by Kap1 is through chromatin structure. PML NBs make extensive contacts with chromatin [102] and

increase in number in response to changes in chromatin topology, such as the PML NB response during S phase [114]. During S-phase, topological changes occur in the replicating chromatin, where chromatin under topological stress retracts from the PML NB pulling it apart [114]. In my study, decondensation of chromatin with loss of Kap1 was demonstrated by an increased accessibility of chromatin to nuclease digestion (Figure 22) [307] and by electron spectroscopic imaging (Figure 23). In cells lacking Kap1, chromatin surrounding the NB decondenses and could lead to PML NBs being pulled apart, increasing body number through a mechanism similar to the PML NB increase during S-phase [114].

The increase in PML NB number that occurs during early S-phase suggests that PML NBs are primarily associated with euchromatin rather than heterochromatin [114], which replicates later in S-phase [114]. Therefore, an increase in euchromatin domains would allow for an increase in PML NB number [114]. Kap1 maintains heterochromatin structure by acting as an intermediary factor that facilitates condensation of chromatin and gene silencing through interactions with other heterochromatin proteins such as HP1, SETDB1, Mi-2α in the NuRD HDAC and KRAB-ZFP [37, 48, 49]. Loss of Kap1 and decondensation of chromatin may increase the number of euchromatin domains able to associate with PML NBs, which might lead to an increase PML NB number. Therefore, PML NBs may make contacts with heterochromatin *via* Kap1.

A number of studies have looked at the association of PML NBs and heterochromatin. Many of these studies have linked PML NBs with HP1, a binding partner of Kap1 [162, 163, 343], thus implicating PML NBs in heterochromatin remodeling. PML NB associated protein Daxx interacts with alpha thalassemia/mental retardation syndrome X-linked (ATRX), a SWI/SNF chromatin remodeler, which interacts with HP1

to facilitate remodeling and/or condensation of chromatin specifically in G2 [116, 163]. HP1 is also required for ALT-associated PML NBs [343] and has also been shown to interact with Sp100, another PML NB associated protein [162]. Studies have also shown that PML NBs associate with heterochromatin at nucleoli [147] and with heterochromatin foci linked to cellular senescence [193]. As for a direct association of PML NBs with Kap1, novel Kap1-containing foci have been observed adjacent to PML NBs [344, 345]. These Kap1-containing foci colocalize with a number of Kap1 interacting proteins, such as KRAB-ZFP and HP1α/β, which are important in maintaining heterochromatin structure [37, 344]. Why Kap1 foci are juxtaposed with PML NBs remains unclear, but this association suggests that PML NBs may play a role in Kap1-mediated repression, perhaps *via* sumoylation of Kap1 [344]. The links between PML NBs, Kap1 and other heterochromatin factors such as HP1 suggest that PML NBs may play a role in the formation of heterochromatin. Loss of Kap1 causes decondensation of chromatin as Kap1 protein interactions are compromised, resulting in an increase in PML NBs, perhaps by a fission mechanism. If this PML NB behaviour is truly a heterochromatin effect, loss of other heterochromatin proteins such as HP1, HDACs, HMETs or KRAB-ZFPs may result in similar increases in PML NB number.

Furthermore, this phenomenon may be specific to nuclear-lamina-associated chromatin. In cells lacking Kap1, chromatin at the nuclear lamina was thinner compared to that in normal Tert-NHF cells (Figure 23). At the nuclear periphery, chromatin is compact and silenced, and HP1 has been associated with nuclear lamina proteins [346], which could indirectly implicate Kap1 in the compaction of chromatin at the nuclear periphery.

4.3 PML NB NUMBER IS CONTROLLED BY PHOSPHORYLATION OF KAP1

It is evident that Kap1 directly plays a role in regulating PML NB number. However, Kap1 can undergo post-translational modifications following DNA damage. Following DNA damage, ATM phosphorylates Kap1 on serine residue 824, resulting in global decondensation of chromatin [307]. Cells lines expressing phospho-inhibitory (S824A) or phospho-mimetic (S824D) Kap1 mutants in place of normal Kap1 were used to assess whether phosphorylation of Kap1 could regulate PML NB number. Phosphorylation of Kap1 is important in regulating PML NB number as, prior to DNA damage, PML NB number was higher in Kap1 S824D mutant cells compared to GFP shRNA control and Kap1 S824A mutant cells (Figures 24, 25). Similar to the loss of Kap1, the phospho-mimetic Kap1 mutant also causes decondensation of chromatin [307]. Therefore, phosphorylation of Kap1 may also contribute to the increase in PML NB number as chromatin decondenses, allowing PML NBs to pull apart (Figure 26).

Following DNA damage, both S824A and S824D Kap1 mutants had similar levels of PML NBs, except at 1 h post DNA damage where PML NB peaks for the Kap1 S824D mutant. However, PML NB number normally peaks at 3 h post DNA damage [119] (Figure 25). Phosphorylation of Kap1 by ATM occurs within 1 h of DNA damage [307]. Therefore, phosphorylation of Kap1 and decondensation of chromatin may be early events that contribute to the increase in PML NB number following DNA damage.

Phosphorylation of Kap1 by ATM is involved not only with decondensation of chromatin but also with gene expression and DNA repair. Kap1 phosphorylation by ATM promotes the expression of genes controlling the cell cycle (p21, Gadd45α) and apoptosis (Bax, Bak, Noxa) in response to DNA damage, and expression of these genes is

controlled by a sumoylation/phosphorylation switch on Kap1 [308]. As Kap1 foci and PML NBs are in close proximity and PML NBs contain lots of SUMO-1, PML NB could play a role in this sumolylation/phosphorylation switch or help transduce the signals for cell- cycle arrest or apoptosis. Additionally, phosphorylated Kap1 colocalizes with several proteins at DNA-damage foci, such as 53BP1, γ-H2A.X, BRCA1 and TopBP1 [310]. These proteins are localized at PML NBs at later time points following DNA damage [101, 119]. Although the majority of repair has occurred by this time, PML NBs may allow association of phosphorylated Kap1 and other proteins to transduce signals for cell-cycle arrest or apoptosis and/or mediate post-translational modifications of DNA-repair factors or even Kap1 itself. Perhaps PML NBs mediate Kap1 phosphorylation, dephosphorylation or sumoylation.

As an added twist, it has been suggested that Kap1 plays a role in the repair of damage that occurs in heterochromatin [246]. DSBs that occur within heterochromatin require ATM signaling for their repair, as loss of Kap1 and other heterochromatin proteins such as HP1 or HDACs alleviates the requirement for ATM in DSB repair [246]. This finding suggests that ATM signaling perturbs heterochromatin *via* Kap1 allowing better access to lesions for efficient DNA repair [246]. As PML NBs associate with heterochromatin and Kap1 as discussed above, perhaps PML NBs take part in the repair of DSBs in heterochromatin.

4.4 EVIDENCE OF A KAP1-INDEPENDENT MECHANISM REGULATING PML NB NUMBER

Other factors may be involved in the increase in PML NBs post DNA damage. Following DNA damage, PML NB number further increased in cells with ΔKAP1 alone or with Kap1 mutants (Figure 25). This increase suggests that another mechanism or pathway, other than phosphorylation of Kap1, contributes to the increase in PML NBs following DNA damage (Figure 26). These other factors may be further changes in chromatin [303], phosphorylation of PML by Chk2, ATR or ATM (Figure 14) [198], or post-translational modification of other PML NB associated proteins. Further changes in chromatin structure may be due to the DNA damage itself [303] or mobilization of other heterochromatin proteins including HP1β [311]. PML and other PML NB components are modified following DNA damage, which may contribute to the increase in PML NB number by disrupting protein interactions at the PML NB. Other PML NB components that may be modified by ATM/ATR are cell-cycle related proteins or DNA-repair proteins including BRCA1, NBS1, H2A.X, or even ATM/ATR mediator proteins 53BP1 or TopBP1 [220, 255]. Therefore, following DNA damage, phosphorylation of Kap1 and other factors may play a role in regulating PML NB number.

4.5 FUTURE DIRECTIONS

This study implicates a Kap1-dependent mechanism in the regulation of PML NB number through changes in chromatin structure (Figure 26). Following DNA damage, Kap1 phosphorylation and other ATM/ATR mediators or effectors may play a role in the increase in PML NB number (Figure 26). Although Kap1 is directly implicated in the

control of PML NB number, the exact mechanism remains unclear. Many questions arise from this study.

4.5.1 DOES ΔKAP1 AFFECT S-PHASE?

Although there was no increase of S-phase cells to contribute to the increase in PML NB number with loss of Kap1, it is necessary to confirm this observation using a different technique in addition to the typical cell-cycle analysis. To obtain a typical cell-cycle profile, propidium iodide is used, which intercalates with DNA [347]. The stage of the cell cycle is interpreted from the DNA content of the cell as it correlates with the fluorescence of propidium iodide. A specific way to assess S-phase of the cell cycle is to use bromodeoxyuridine (BrdU) incorporation. BrdU is a synthetic nucleoside that is analogous to thymidine. BrdU incorporates into replicating cells in S phase and can be detected using an antibody against BrdU with a fluorescent tag [348]. By using BrdU, we can detect cells in early, mid and late S-phase and thus this technique may be able to detect any S-phase delay in ΔKAP1 cells.

4.5.2 WHAT IS THE SPATIAL ORGANIZATION OF KAP1 IN NUCLEI AND HOW DOES LOSS OF KAP1 AFFECT CHROMATIN MOBILITY?

Kap1 seems to act through changes in chromatin structure to regulate PML NB number. Micrococcal nuclease (MNase) digestion is a molecular technique to assess chromatin structure [307], which showed that loss of Kap1 causes decondensation of chromatin by increasing accessibility to MNase (Figure 22) [307]. Electron spectroscopic imaging (ESI), a microscopic technique that allows visualization of ultrastructural changes in chromatin [120], demonstrated that the nuclear-lamina-associated chromatin was thinner in ΔKAP1 cells than in control cells (Figure 23), suggesting decondensation of heterochromatin at the nuclear envelope. Chromatin at the nuclear envelope and nuclear

lamina is largely condensed and transcriptionally silent [346, 349]. It would be interesting to see specifically where Kap1 is located in chromatin and its dispersal, using gold immuno-detection of Kap1 and phospho-mimetic Kap1 by ESI. Live-cell microscopy could also be used to observe changes in chromatin mobility in cells with and without ΔKAP1, as loss of physical constraints on chromatin such as nuclear compartments increase chromatin mobility [350].

There are many studies suggesting that the Kap1 effect on PML NB number may be specific to heterochromatin, as Kap1 is important in maintaining heterochromatin structure [37]. Furthermore, PML NBs have been associated with heterochromatin structure [116, 162, 163, 343]. Does loss of Kap1 specifically affect heterochromatin, or is there a global effect on chromatin structure? Southern blot analysis can be done on the MNase digestions from ΔKAP1 cells. Probes specific for euchromatin (Alu repeats) [13], heterochromatin (LINE1 repeats) [13], telomeres (telomeric repeats) [351] and pericentromeric chromatin (α-satellites) [352] can be used to delineate which type of chromatin Kap1 may be affecting.

4.5.3 DOES LOSS OF KAP1 AFFECT GENOMIC STABILITY AND/OR DNA REPAIR?

Although Kap1 affects chromatin structure, how does Kap1 cause an increase in PML NB number? Does Kap1 have a role in maintaining genome stability? One way to assess this is by looking at sister-chromatid exchange (SCE). Does loss of Kap1 increase SCE as loss of PML increases SCE? SCE can be assessed by BrdU incorporation [353]. For examples, to measure SCE, cells can be treated with BrdU for 24 h followed by growth in the absence of BrdU for 24 h and then collect cells in mitosis, cytospin and look for exchange of the BrdU labeled chromatid with the unlabeled chromatid. Another

component of genome stability is translocation frequency. Does loss of Kap1 cause a higher propensity for translocations? This can be assessed by spectral karyotyping, which simultaneously visualizes all pairs of chromosomes in different colours using fluorescently-labeled probes specific for each chromosome [354].

Kap1 has also been implicated in DSB repair associated with heterochromatin [246]. If Kap1 aids in the repair of DSBs in heterochromatin, are PML NBs involved? This can be assessed through immunofluorescence analysis looking for colocalization of Kap1, PML and phosphorylated H2A.X, which occurs at the site of the DSBs. Lastly, is the increase in PML NB number specific to heterochromatin? Do loss of other heterochromatin proteins such as HP1 or HDACS increase PML NB number? This can be done by RNAi to knockdown levels of these heterochromatin proteins followed by immunofluorescence anlysis for PML NB number.

4.6 CONCLUSIONS

Changes in chromatin structure induced by loss or phosphorylation of Kap1 plays a role in regulating PML NB number, but a number of details wait to be elucidated. For example, there is also a Kap1-independent mechanism that leads to a further increase in PML NB after DNA damage. The components of the Kap1-independent mechanism after DNA damage could involve other aspects of DNA-repair signaling and/or chromatin remodeling as discussed above. Although partially elucidated in this study, the mechanisms behind the increase in PML NB number following DNA damage have proved to be complex. The further study of the control of PML NB number will aid in the use of PML NBs as a biomarker for the DNA-damage response in cancer cells.

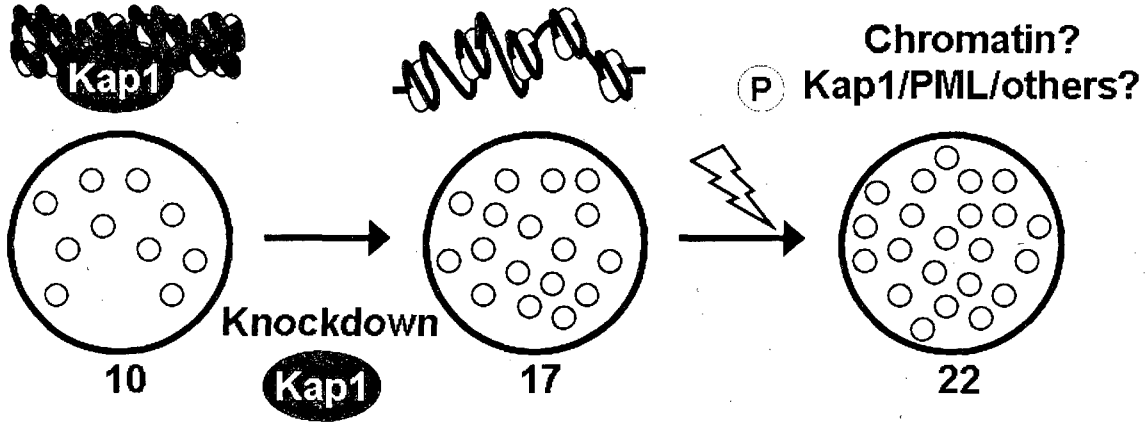


Figure 26. Model for the regulation of PML NB number by Kap1. In an undamaged cell, there is a basal number of PML NBs, as Kap1 is unperturbed. Chromatin is condensed as Kap1 protein-protein interactions remain intact. Kap1 is directly implicated in PML NB control. When Kap1 is lost, PML NB number increases as chromatin becomes decondensed due to perturbations in Kap1 protein-protein interactions. PML NB number further increases following DNA damage in cells lacking Kap1. Phosphorylation of Kap1 does play a role in PML NB number control after DNA damage. However, there is a Kap1-independent mechanism controlling PML NB number post-DNA damage. This mechanism may involve further changes in chromatin structure, PML phosphorylation or post-translational modification of other PML NB components.

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