DISSERTATION

TANKYRASE 1 INFLUENCES TELOMERE RECOMBINATION, STABILITY OF THE NHEJ PROTEIN DNA-PKCS AND GENOMIC INTEGRITY

Submitted by

Ryan Christopher Dregalla

Graduate Degree Program in Cell & Molecular Biology

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 2011

Doctoral Committee:

Advisor: Susan M. Bailey

Howard L. Liber
Michael M. Weil
Jennifer K. Nyborg
Paul J. Laybourn

ABSTRACT

TANKYRASE 1 INFLUENCES TELOMERE RECOMBINATION, STABILITY OF THE NHEJ PROTEIN DNA-PKCS AND GENOMIC INTEGRITY

The Poly(ADP-ribosyl)ating Polymerase (PARP) family of enzymes has gained considerable attention recently due to the success of inhibiting their activities in breast cancers with BRCA 1/2 deficient backgrounds. PARPs serve as key regulators of protein recruitment, stability and activity in specific intracellular pathways including DNA-repair, telomere stability, transcription factor regulation and mitotic integrity. The PARP family member, PARP-5a, otherwise known as tankyrase 1 is unique in that it lacks a DNA-binding domain and interacts with proteins specifically. First found to regulate telomere length by promoting access to telomerase, tankyrase 1 has since become associated with a multitude of critical cellular processes.

In our studies investigating the role of DNA-dependent Protein Kinase catalytic subunit (DNA-PKcs) and tankyrase 1 at telomeres, we find that tankyrase 1 is required for the suppression of sister chromatid recombination events at the telomere and that the leucine zipper domain of DNA-PKcs is necessary for accurate end-capping function. Interestingly, during our investigation we also identified a link between the stability of the DNA-PKcs protein and tankyrase 1.

We find that under conditions in which tankyrase 1 is depleted or catalytically inhibited, DNA-PKcs becomes a substrate for proteasome mediated degradation. The depletion of tankyrase 1 by siRNA-mediated knockdown or PARP inhibition resulted in the failure of DNA-PKcs function in both telomere end-capping and the DNA damage response following exposure to ionizing radiation; i.e., increased sensitivity to ionizing radiation-induced cell killing, mutagenesis, chromosome aberrations and telomere fusions. Further, we find that the loss of DNA-PKcs is not coupled with depletion of Ku70, Ku80 or the PI3-kinase ATM, illustrating that tankyrase 1 acts to regulate DNA-PKcs specifically. Taken together, we identify important and novel roles of tankyrase 1 with implications not only for DNA repair and telomere biology, but also for cancer and aging.

ACKNOWLEDGEMENTS

For the past three and a half years, I have learned a tremendous amount from my advisor, committee members, lab mates, friends and family. The support system created by these people and the associated educational experiences has helped me become the scientist, educator and family member I am today.

First, I would like to acknowledge Dr. Susan Bailey in her accomplishments as a mentor, educator and scientist. It has been a tremendous privilege having you as my mentor and work in an environment where I was provided with the freedom to conduct scientific experiments with novelty; allowing me to grow as an individual and continuously evolve my scientific perspective. This experience has facilitated my passion for science and for that, I am eternally thankful.

To all committee members, Drs. Howard Liber, Michael Weil, Jennifer Nyborg and Paul Laybourn, I wish to express my gratitude for your time and efforts on my behalf. Each of you has dedicated significant periods of time consulting with me regarding experimental design and data interpretation. In addition, several of you have been instructors for the classes I took throughout my graduate career. Each of you have all played an essential role in my development as a scientist and have contributed to my talents as an educator.

To all fellow lab members (encompassing most of the MRB 4th floor, past and present), thank you for your continuous support and input. At one point or another, I have consulted each of you in regards to experimental design, assay execution, trouble-shooting, data interpretation and general criticisms. Thank you for your time and honesty.

A special thanks to my friends and family, who have had a tremendous impact on my life-perspective, attitude and core values. I sincerely appreciate the contributions of my mother and siblings, who have always been supportive of my endeavors, providing assistance and advice along the way. A whole-hearted 'thank-you' to my soon-to-be wife, Jillian, for stepping into my life, encouraging my dreams and believing in everything that I do. Forever & ever. Hayden, you have been my motivation throughout the past 7 years of life. I don't know where I would be without you, thank you.

For the sake of honesty, I would like to thank Steve Fowler, the owner and operator of the 'Maya Cove' for providing hope; you have shown me that keeping true to yourself is the only way to achieve your dreams. Furthermore, your establishment and the 'associated crew' are of the finest quality, creating an atmosphere where you always feel 'at home' but are still required to find your way home shortly after midnight, strong work.

TABLE OF CONTENTS

CHAPTER 1

Introduction

1.1.0 Telomeres guard chromosomal stability

Telomeres are hexameric-repeat sequences that exist at the terminal ends of eukaryotic chromosomes. In vertebrates, leading-strand telomeric DNA is composed of a 'TTAGGG' hexameric-repeat sequence ranging in size from approximately 2 - 15 kilobases (kb) in length and is universally accepted as a 'non-coding', untranslated region of linear chromosomes [1]. Telomeres serve two critical functions at the terminal ends of the chromosome. First, telomeres act as a buffer region safeguarding coding DNA and thereby solving the 'end replication problem'; the chronic chromosomal shortening per replication phase of the cell cycle as a consequence of the inability of DNA polymerases to synthesize nucleotide polymers in the 3' to 5' direction. Second, telomeres solve the end-protection problem by assuming a specific conformation that serves to preserve the integrity of linear chromosome ends and prevent them from being recognized as a double strand break (DSB) by DNA-repair machinery, protecting against chromosome fusions and genomic instability [2-4].

1.1.1 Telomeres solve the end-replication problem

Each round of cell division in organisms containing linear chromosomal DNA is coupled with the 'end replication problem' [5, 6]. Following each DNA replication phase of the cell cycle, the telomere is shortened due to the inability of DNA polymerases to replace the excised RNA primer with 3' - 5' DNA synthesis. In addition, nuclease

activities further recesses 5' terminal ends to generate a 3' single-stranded overhang. Generation of the 3' single-stranded overhang, which ranges from 50 – 500 bases in length and is crucial for effective telomere-end capping [7-9], but does pose a pitfall through the shortening of the chromosome [5]. However, telomeres serve as a 'buffer region' between essential coding DNA and the chronic chromosomal shortening per round of DNA replication, coding regions of the chromosomes DNA are not initially impacted.

Telomere length maintenance/telomerase

Cells that contain a ribonucleoprotein termed telomerase posses the ability to lengthen the 3' single-stranded overhang de novo during each replication cycle, allowing for stable telomere length over multiple divisions. Thus, cells that express the appropriate components of the telomerase nucleoprotein are able to divide without limitation [10, 11]. Telomerase positive cells express detectable levels of the protein component, a telomere-specific reverse transcriptase known as (hTERT) [12]. Using a telomere RNA component (hTERC) integrated within TERT, telomerase is able to extend the 3' single stranded overhang on each end of the chromosome [12].

Most somatic cells do not possess telomerase [13, 14] or a method for alternative lengthening of the telomere (ALT [15]). The exceptions are germ line and adult stem cells which contain active telomerase [16-18]. However, the enzymatic activity detected within these telomerase positive cell-types is not robust enough to protect against gradual erosion of the telomeres over a life-time [12]. Thus, there is a finite number of divisions a cell can undergo before replication is no longer possible without invading coding DNA

[19, 20]. This limit is reached sooner in somatic, telomerase and ALT negative cells compared to germ line and adult stem cells.

Consequences of critically short/dysfunctional telomeres

The limited number of divisions that can be achieved by a cell lacking telomerase activity is termed the 'Hayflick Limit' that once reached, requires the cell to initiate a stable, non-dividing state termed senescence [19]. In cells lacking a method for telomere elongation, the Hayflick limit serves as an effective method to suppress the passage of mutations accumulated within a single cell to progeny daughter cells in subsequent divisions and increased carcinogenic potential. The inactivation of key tumor suppressor genes which are tasked with the maintaining appropriate cell proliferation can result in failed senesce. As a consequence, the cell enters 'crisis', where critically eroded telomeres result in unstable chromosome ends that react with adjacent chromosome ends forming rearrangements (dicentrics and translocations). Cells that are to survive 'crisis' are required to engineer a method of telomere elongation by either, telomerase activation or ALT; posing a risk for carcinogenic development as most tumor cells have become immortalized, with an infinite capacity to divide [21-25].

1.1.2 Telomeres solve the end-protection problem

Linear chromosomes require an end-capping mechanism to avoid activation of the DNA-damage response (DDR) (reviewed in [26]). Generally, the DDR works in conjunction with cell cycle checkpoints to ensure DNA-damage induced over the course of the cell cycle is accurately repaired and thus does not persist into replication and/or mitotic division. DNA damage persisting throughout the cell cycle can result in increased mutation and genomic instability [27].

Shelterin prevents DDR

The results of studies have demonstrated that the accurate formation of telomeres in a capped conformation is dependent on telomeric DNA in complex with a series of double and single-stranded DNA-binding protein complexes to avoid recognition by the DDR. Protein complexes that bind telomeric DNA also interact with one another to form the telomere-protein core unit of 'shelterin'. Effective telomere end-capping is achieved by the combined efforts of shelterin components, six proteins that maintain the integrity of telomeric DNA in a 'capped' conformation [28]. The core of the shelterin complex is composed of two dimerized telomere duplex-binding proteins; the Telomere Repeat binding Factors 1 (TRF1) and TRF2 in homodimer conformations. In addition to being the primary DNA-binding proteins of double-stranded telomeric DNA, the TRF1 and TRF2 homodimers are necessary for the recruitment of the remaining four shelterin components: TRF1 & TRF2 Interacting Nuclear Protein 2 (TIN2), Repressor/Activator Protein 1 (Rap1 (human ortholog)), Tripeptidyl-peptidase 1 (TPP1) and the single-stranded 3' overhang binding protein POT1 [29]. In association with these recruited proteins, the homodimers TRF1 and TRF2 are referred to as the TRF1 & TRF2 complexes. In complex, TRF1 and TRF2 are designated the generic roles of telomere length control and telomere maintenance, respectively (Figure 1B & 1C [30]) [31-34].

Shelterin shields the terminal chromosome ends from recognition by DDR-associated protein machinery (reviewed in [35]). The TRF1 & TRF2 complexes are essential for the integrity of the double-stranded 'T-loop' component of the 'capped' telomere. Following a homologous recombination-like event, the single-stranded overhang invades the telomere duplex and binds to the complementary C-rich telomere

strand. The resulting 'capped' telomere is composed of a telomere-loop (T-loop) bound by TRF1 & TRF2 and a triple stranded region at the site of the recombination event where a small stretch of single-stranded DNA is displaced, forming the displacement loop (D-loop) bound by the single-stranded-binding protein Protection Of Telomeres 1 (POT1) (Figure 1A [30]). The telomere-end cap comprised of the T- and D-loops protects the telomere from processing by DDR machinery [36]. Under conditions in which shelterin proteins are rendered dysfunctional, the telomere can become 'uncapped', recognized as a DSB by DDR machinery and processed as such, resulting in cytogenetically visible telomere-based fusion phenotypes (reviewed in [26]). Telomere uncapping is associated with the misregulation of one or more shelterin protein components. Telomere-based fusions can occur between different chromosomes and/or adjacent sister chromatids as a consequence of failed end-capping and thus, contribute to genomic instability [37-39].

Other telomere associated proteins that interact with shelterin and play a regulatory role and/or aid in telomere stability include: DNA-repair/signaling proteins ERCC1, Apollo, Ku70, Ku80/86, DNA-PKcs, Mre11, RAD51, the 9-1-1 complex, PARP1 and PARP2; replication associated helicases RecQ & WRN, chromatin modifier HP1 and regulatory proteins tankyrase 1 and tankyrase 2 [40-50]. By various methods, each of these 'telomere-associated' proteins aid in sustaining the appropriate function of POT1 and the TRF1 & TRF2 complexes in their telomeric duties (Figure 1B & 1C [30]).

1.1.3 Tankyrase 1 functions as an accessory component of 'Shelterin'

Tankyrase 1 is a ubiquitously expressed member of the poly(ADP-ribosyl)ating polymerase (PARP) family [51], first characterized as an accessory shelterin component

where is serves to remove TRF1 from the telomere, providing access to telomerase [52]. Upon TRF1 poly(ADP-ribosyl)ation (pADPr/PARsylation) via tankyrase 1, TRF1 is released from the telomere, rapidly ubiquinated and subsequently degraded [52-54]. Hence, tankyrase 1 is thought to be a negative regulator of TRF1 and therefore, a positive regulator of telomere length. Studies have demonstrated the over expression of tankyrase 1 results in telomere elongation in telomerase positive cells where no effect on telomere length was observed in telomerase negative backgrounds. These findings illustrate the role of tankyrase 1 at the telomere is to catalytically modify TRF1 via PARsylation, thereby allowing access to the telomere by telomerase and elongation of the telomere [55].

Misregulation of the TRF1 telomere dissociation dynamics by tankyrase 1 has detrimental consequences. Tankyrase 1 knockdown has been found to result in extensive sister chromatid fusions [39] and in some cell lines, mitotic arrest [39, 56, 57]. Sister chromatid fusions result from persistent association of cohesion complexes between sister chromatids, keeping the telomeric ends in close proximity throughout DNA replication and G2 phase of the cell cycle. Depletion of tankyrase 1 results in failure of TRF1 dissociation from the telomere, thus it is thought that the chromatid ends undergo recombination events, which ultimately result in covalent fusions between sister chromatids [39]. It has been shown the cell death phenotype associated with tankyrase 1 knockdown is associated with anaphase bridge formation in HeLa cells and subsequent mitotic arrest [57].

1.1.4 DNA-PKcs is required for telomere end-capping in mammalian cells

Recognized as the catalytically active kinase subunit of the DNA-dependent Protein Kinase holoenzyme (DNA-PK), DNA-PKcs is essential for appropriate mammalian telomere capping, at the leading strand in particular [37]. Functionally null, truncated forms of the DNA-PKcs protein in the SCID mouse were found to contribute to telomere uncapping and abundant telomere-telomere and telomere-DSB fusion phenotypes [37, 38]. Though the exact biochemical role of DNA-PKcs at the telomere is not currently understood, it has been shown that polymorphic variants of the protein also results in mass telomere uncapping ([58, 59] , Chapter 2).

BALB/c and SCID mice contain single-nucleotide polymorphisms (SNPs) in the protein kinase, DNA-activated, catalytic polypeptide (*Prkdc)* allele, resulting in variant forms of the translated DNA-dependent protein kinase catalytic subunit (DNA-PKcs) protein. The SCID cell line contains a nonsense mutation (Y4045X) [60] that results in a truncated form of the protein in addition to the two SNPs identified within the BALB/c mouse: one within the leucine zipper domain (R2140C) and the other within the phosphoinositol kinase 3-related kinase (PIKK) domain (M3844V) [61-63] (Figure 2, adapted from [64]). In each case, the fibroblasts from SCID and BALB/c mice show significantly elevated frequencies of telomere-based fusions compared to fibroblasts from the C57BL/6 mouse containing the 'common' *Prkdc* allele. Although the SCID mouse cell line provided the initial evidence for the dependence of accurate mammalian telomere capping on the DNA-PKcs protein [38], investigation of the BALB/c cell line suggests a full length, polymorphic variant of DNA-PKcs is insufficient for accurate telomere capping. This is speculated to be the consequence of deficient function of the

domains possessing the SNPs, reducing the functional capacity of the DNA-PKcs protein. Taken together, it has been established that DNA-PKcs is required for telomere capping. Further, specific domains of DNA-PKcs are essential for DNA-PKcs-mediated telomere capping.

1.1.5 Consequences of uncapped/dysfunctional telomeres

Deficiency in core shelterin components results in telomere dysfunction, chromosomal aberrations and genomic instability (reviewed in [35]). Exposed chromosome ends as a consequence of telomere uncapping activates DDR machinery and the telomere is processed as a DSB. Thus, high frequencies of telomere-DSB and telomere-telomere fusions are recognized as a consequence of telomere uncapping [26]. Of the Shelterin components, deficiency of TRF2 results in the processing of the telomere by classical non-homologous end-joining (C-NHEJ), resulting in increased frequencies of telomere-based fusions [34, 65, 66]. Therefore, TRF2 is thought to protect the telomere from recognition as a DSB via NHEJ machinery; when lost, end-joining repair is prominent and reflected in the telomere-based fusions [31, 34]. Additionally, TRF2 is thought to play a role in T-loop formation [67]. Failure to accurately reform the capped-end following replication activates the DDR and NHEJ mediated repair results in telomere-based chromosome-chromosome fusions [34, 65, 66]

The consequences of TRF2 inhibition or expression of a dominant negative form have been shown to result in multiple different phenotypes including apoptosis and cellular senescence. In certain cell types, activation of DDR via ATM in response to exposed telomere ends activates the p53 mediated apoptotic pathway [31, 65, 68]. While in other cell types including human fibroblasts, TRF2 dysfunction results in cell

morphological characteristics that resemble shortened telomere induced cellular senescence [66]. Cumulatively, these studies demonstrate telomere integrity and protection from end fusions is largely dependent on TRF2 function at the telomere and its ability to block ATM-mediated DDR activation.

In the case of TRF1 deficiency, the effects investigated thus far have been partial to the telomere length regulation and telomerase activity. Recent evidence has demonstrated cell cycle arrest following the conditional deletion of TRF1 in mouse embryonic fibroblasts [69]. This study shows TRF1 deficiency activates the DDR in an ATM/ATR-dependent fashion, resulting in multitelomeric signals (duplicated telomeres) and telomere fusion phenotypes, particularly between sister chromatids. In support of other studies that suggest telomeres are fragile sites for DNA-replication that lead to incomplete replication, breaks and gaps [70], these findings suggest the loss of TRF1 leads to an increase of stalled replication forks, ATR association and DDR initiation. Interestingly, the induction of chromatid fusion phenotypes at the telomere as a consequence of TRF1 deletion mirrors the effect of tankyrase 1 knockdown, where persistant cohesion association of sister chromtids leads to telomere fusions between chromatids [39]. Thus, it is suspected the appropriate regulation of TRF1 with the telomere is critical in the downstream regulation of cohesion association with sister chromatids at the telomere.

Deficiencies in the POT1/Pot1 protein has been shown to result in multiple telomere instability phenotypes. In the mice, Pot1a and Pot1b are each expressed and play redundant roles; interestingly only Pot1a knockout results in embryonic lethality whereas Pot1b knockouts were viable. Pot1a knockdown in leads to substantial increases

in telomeric recombination events between sister chromatids known as telomere-sister chromatid exchanges (T-SCEs), implying the 3' telomeric-overhangs act as a potential substrate for homologous recombination in the absence of this single-stranded binding protein [71, 72]. Interestingly, depletion of POT1 can also lead to telomere-based fusions, but to a *far less* extent than those observed in TRF2 depletion [72]. The phenotype more frequently associated with POT1 depletion was the accumulation of telomere dysfunction-induced foci (TIFs) at the telomere during G1 of the cell cycle [73]. TIFs are comprised of colocalized p53-binding protein 1 (BP531), the double strand-break foci marker γ-H2AX and TRF1 at the telomere. In this study, TIFs were shown to be stably carried throughout the cell cycle for multiple divisions without becoming substrates for end-joining repair machinery and telomere fusion phenotypes [73]. Further, expression of POT1 defective in it's ability to bind DNA has been shown to elongate telomeres in telomerase positive backgrounds suggesting a role in telomere length regulation along with TRF1 [74, 75].

It is known that NHEJ proteins are required for appropriate telomere end-capping [38] and may be recruited to the telomere by POT1 [38, 73]. In the event of POT1 depletion, NHEJ components are not recruited to the exposed DNA-ends and thus, fail to perform their function, which in turn results in persistent TIF formation [73]. POT1 may recruit NHEJ proteins and protect the 3' overhang, facilitating the regression of the 5' end to generate an adequate 3' end for accurate D-loop formation. Loss of the POT1 protein results in the association of ATR with the exposed end and initiation of the DNA-damage response [2]. On the other hand, TRF2 appears to serve a NHEJ repressing function by inhibiting ATM-dependent DDR initiation. Therefore, when TRF2 is lost,

recruited NHEJ machinery performs its 'full' function, resulting in the telomere fusions observed under TRF2 knockdown conditions; when coupled with POT1 knockdown and the ATM and ATR pathways initiate the DDR concurrently resulting in elevated telomere-based fusions [73].

Cumulatively, the resulting defects produced by deficiencies and/or misregulated Shelterin components will result in telomere reactivity and chromosomal instability by various fusion and recombination events. Chromosome- and chromatid-type fusions create unstable anaphase bridges that are forcefully resolved via random breakage between the two fused chromosomes, resulting in the asymmetrical distribution of genetic material between daughter cells. The recipient daughter cells no longer contain stable genomes and consequently become prone to carcinogeneic development. Further, fusions occurring in the religation events following these anaphase bridge breaks are capable of forming small deletions and translocation of genetic material across chromosomes. Consistent with classical tumorigenic models, deletions of tumor suppressor genes serve as a means for inactivation of the tumor suppressor gene function. Translocation events between chromosomes are also known to result in a wide array of fusion proteins, potentially activating proto-oncogenes (reviewed in [76]).

1.2.0 Non-homologous end-joining (NHEJ) in genomic stability

Seemingly contradictory, accurate end-capping of mammalian telomeres relies on the NHEJ protein DNA-PKcs, providing a role for DNA-PKcs in maintaining genomic stability beyond end-joining [37, 38]. While the role of DNA-PKcs in NHEJ has been well characterized; functioning to maintain genomic stability throughout the cell cycle as the catalytically active kinase component of DNA-PK in classical non-homologous end-

joining (C-NHEJ) DSB-repair [77], the exact role of DNA-PKcs at the telomere is not understood.

In the C-NHEJ pathway, DNA-PKcs is recruited to the Ku70/Ku80 heterodimeric complex, localized on each side of the DSB, where it phosphorlyates serine/theonine residues in a regulatory and auto-regulatory fashion. Once DNA-PKcs is complexed with the Ku70/Ku80 heterodimer (Ku), the heterodimer translocates along the DNA-strand approximately one helical turn, allowing for the formation of the functional DNA-PK holoenzyme [78]. Formation of identical DNA-PK complexes directly opposed to one-another, acts to 'synapse' the two ends of the DSB in close proximity, allowing for repair of the DSB following the completion of DSB end-processing (Reviewed in [79, 80].

The DNA-PK holoenzyme recruits multiple accessory proteins, to do the work of end-processing and religation of the DSB ends. Currently known DNA-PK accessory proteins include: XRCC4, Ligase IV, DNA-Polymerase μ & λ, a poly-nucleotide kinase (PNK), Artemis (nuclease function) and XLF/Cernunnos (reviewed in [79]). The mechanism of recruitment for each protein is not well understood but each contributes to DNA-PK/NHEJ function. In the case of Artemis, it is speculated the DNA-PKcs:Artemis complex forms an active exo/endonuclease, capable of processing the complex DNA ends in preparation for religation [81, 82]. Further processing of the damaged ends are formed via DNA-polymerase, PNK and XLF/Cernunnos before the DNA-PK enzyme becomes phosphorylated via DNA-PKcs kinase activity. Though the 'initiating event' is currently unknown, it is recognized that Ku70/80 phosphorylation and DNA-PKcs autophosporylation events are essential for appropriate DNA-PK regulation and dissociation from the DSB, allowing for end-joining to occur. In this final, critical step,

DNA-PK-dependent NHEJ requires the activity of XRCC4/LigaseIV once the Ku and DNA-PKcs components dissociate. Hence, C-NHEJ is referred to as a DNA-PK/LigaseIV-dependent end-joining method of repair. Barring extensive and complicated damage, C-NHEJ is an efficient, rapid method of DSB repair that operates throughout the cell cycle. However, C-NHEJ is coupled with deletion events per end-joining event, which can contribute to increased mutagenesis and genomic instability (reviewed in [79]).

An alternative, DNA-PK/LigaseIV-independent method of DSB end-joining is known to exist in cells incapable of performing C-NHEJ [83]. Generally, this 'back-up' pathway operates when functional forms of Ku70/80, Ligase IV or DNA-PKcs are absent. This alternative non-homologous end-joining (A-NHEJ) process is poorly characterized but is known to be PARP-1-dependent and function independently of DNA-PK and Ligase IV [84-87]. Recent evidence has illuminated PARP-1 and, potentially Ligase III as the primary, necessary players in the A-NHEJ pathway [83]. Interestingly, PARP-1, Ligase III, XRCC1 and DNA-Polymerase β are known to be components involved in base excision repair and single-strand break repair. Although capable of end-joining, A-NHEJ is slower in the end-joining process compared to C-NHEJ. Additionally, it is more prone to end-joining error (mis-joining) and either fails to achieve end-joining resulting in terminal deletions or results in chromosomal translocations/rearranges [88-90].

1.3.0 PARPs in genomic stability

PARPs are specific to higher eukaryotes, a family of Poly(ADP-ribosyl)ating Polymerases (PARP) that use NAD^+ as a substrate to modify 'self' and recipient proteins

posttranslationally by the addition of several hundred ADP-ribose groups (pADPr) in a highly branched fashion. Each pADPr-monomer has a net 'negative-two' charge and thus, is capable of inducing major conformational changes in recipient proteins, in many cases altering protein function [91, 92]. Further, the heavy negative charge imparted by this modification drives non-covalent, electrostatic protein-protein interactions and in some cases, electrostatic repulsion. The ability of pADPr to facilitate of both attraction and repulsion requires close regulation to ensure the necessary and appropriate functions are achieved as a result of protein pADPr modification [93]. In the event that hypo-PARsylation, or even complete dePARsylation of a modified protein is required to perform a specific function, the PARP counter enzyme, poly(ADP-ribos)yl glycohyrolase (PARG) activity is fundamental (reviewed in [94]). *In vitro* studies have suggested PARG activity is rapid enough to dePARsylate all pADPr-modified proteins intracellularly within a 1-2 minute time frame [95]. It is important to recognize the dynamic nature of PARP-based protein modification and the effects of PARsylation in regulation of the recipient protein.

The 'original' poly(ADP-ribosyl)ating polymerase (PARP), termed PARP-1, was identified as a nuclear DNA-binding protein with the capacity to recognize the chemical structure of nicks in single-stranded DNA (single-stranded breaks (SSB)) and modify histone structure around the damage site [96]. The innate catalytic function of all PARP family members is to modify substrate proteins posttranslationally, including itself, by the addition of several hundred poly(ADP-ribose) groups (pADPr). However, additional domains possessed by PARPs are highly variable from one PARP to another, each with unique binding domain enabling interaction with a wide range of substrates [93].

Of the PARP family members, only a select few are capable of DNA-interaction (Figure 3 A [93]). Others possess domains that function specifically in facilitating the PARPs interaction with particular target proteins for pADPr modification, thereby achieving some regulation of the pADPr-recipient function. The result of the heterogeneity observed in the function domains across the PARP family is an indicator of the wide array of roles attributed to PARP activity. Included in this unique family of pADPr posttranslational modifiers are PARP-1, PARP-2, PARP-3, vault (VPARP) and the tankyrases family including PARP-5a and PARP-5b [97].

PARP-1 is a known chromatin modifier and plays a crucial role in DNA-repair. PARP-1 recognizes and binds to SSBs through its' DNA-binding domain (DBD) and subsequent pADPr modification of histones H1-H2B relaxes the nucleosome to allow PARP-1 access to the damaged DNA. Once stably interacting with the damage site, PARP-1 becomes hypo-PARsylated by the combination of auto-modification and poly(ADP-ribosyl) glycohydrolayse (PARG) activity ([97, 98]). The hypo-autoPARsylated state of PARP-1 at the SSB acts to recruit additional DNA-repair proteins necessary for SSB repair. Amongst the first to be recruited to hypo-PARsylated PARP-1 is the XRCC1/LigaseIII complex [97], followed by DNA-polymerase β (Pol β) . It is has been reported that failure of PARP-1 to dissociate from the DNA-damage site in a timely manner blocks the association of Pol β with the break [98, 99]. It appears PARP-1 dissociation from the DNA is dependent on the activation of PARP-1 auto-PARsylation. Stimulated by DNA-binding, PARP-1 auto-modification results in a heavy negative charge imparted by each ADP-monomer [93, 97]. The modification drives an electrostatic repulsion between PARP-1 and the DNA-strand, allowing for access by Pol

β. This event in its entirety is descriptive of the PARP-1 dependent process of base-excision repair (BER) and potentially nucleotide excision repair (NER). Furthermore, PARP-1 plays an essential role in mitotic integrity and DSB-repair via the A-NHEJ pathway as mentioned previously [93, 97].

PARP-2 is the only other family member that contains a DNA-binding domain (DBD), however its' role *in vivo* is currently poorly characterized. The model of PARP-2 function is one of playing a 'back-up' or 'accessory' role to PARP-1. In the event of PARP-1 depletion, residual PARP activity at sites of DNA-damage is due to PARP-2 activity [100]. Further, double knockout of PARP-1/PARP-2 results in the intensification of PARP-1 depletion phenotypes. Nonetheless, PARP-2 appears to play a role in DNA-repair, both with and without PARP-1 activity.

PARP-3 is unique in that it is recognized as a cytoplasmically active PARP. PARP-3 is thought to be a component of the centrosome [97] and appears to function, at least in part, with PARP-1. Until very recently, the exact role of PARP-3 in any cellular process was entirely uncharacterized [93, 97]. A recent study demonstrated PARP-3 automodification accelerates classical non-homologous end-joining DNA-repair via the recruitment of the pADPr binding protein, aprataxin and poly-nucleotide kinase-like factor (APLF) [101]. It is assumed that PARP-3 automodification (in a hypoPARsylated state) recruits APLF to the double-strand break. This is suspected to be downstream of DNA-PK holoenzyme dissociation from the DSB and leads to rapid resolution of the lesion as APLF forms a complex with XRCC4 and Ligase IV, retaining the XRCC4/Ligase IV complex bilaterally at the DSB [101]. Thus, XRCC4-dependent alignment of the processed ends and Ligase IV resolution of the lesion is promoted by

APLF localization to the DSB via PARP-3 autoPARsylation activity [101]. This particular activity of a PARP family member is not novel in nature, as PARP-1 similarily uses automodification for protein recruitment in SSB-repair and potentially in the alternative NHEJ pathway. However, it is the first characterized role of PARP-3 intracellularly and provides additional evidence for the involvement of PARPs as 'accessory proteins' in DNA-repair pathways.

The vault particle-interacting PARP, termed PARP-4/VPARP is the largest of member of the PARP family. The specific role of the ribonucleoprotein vault particles in cellular processes is not currently understood and thus, the function of PARP-4 is unknown. Vault particles are known to interact with the major vault protein (MVP), telomerase-associated protein (TEP) and untranslated vault mRNA (VRNA). The role of PARP-4 in association with vault complexes may be in the subcellular localization of the ribonucleoprotein in complex with either MVP, TEP and VRNA [93, 97].

Tankyrases are the most distinguishable members of the PARP family, sharing no homology with other PARP family members, with the exception of their catalytic PARP domain. These unique PARP family enzymes are further categorized into a subset of proteins: PARP-5a and PARP-5b, also termed tankyrase 1 and tankyrase 2 respectively. The difference between the two proteins is marginal; the N-terminus of tankyrase 1 contains a Histidine-Proline-Serine repeat that is lacking entirely in tankyrase 2 [93, 97] (Figure 3 B [104]). Aside from this difference, tankyrases share more than 85% amino acid homology, possessing equivalent functional domains [93]. Tankyrases lack DBDs but contains twenty-four ankyrin-like repeat domains (ANK) that are critical for interaction with substrate proteins. Further, tankyrases are the only PARPs that posses a

sterile alpha motif domain (SAM), used for tankyrase-tankyrase multimerization (oligomerization) [102, 103] (Figure 3 [93, 104]). It is due to these unique domains within the PARP-5 group of PARPs that tankyrases are recognized primarily as having a regulatory role via pADPr modification of specific receptor proteins.

Of the two tankyrases, tankyrase 1 is the best characterized. Initially identified as a nuclear, shelterin-associated protein, tankyrase 1 was first found to regulate TRF1 via PARsylation; inducing a change in the TFR1 homodimer affinity for telomeric DNA and facilitating dissociation presumably by electrostatic repulsion. Once dissociated from the telomere, PARsylated TRF1 is subject to E3 ligase ubiquitination and subsequent degradation if not de-PARsylated via PARG. Tankyrase 1-dependent dissociation of TRF1 from the telomere has two functional implications. First, TRF1 must be removed from the telomere for the progression of the replication fork during DNA replication. Second, cells that contain active telomerase require access to the telomere to perform elongation of the telomere's single-stranded 3' overhang. TRF1 release initiates t-loop destabilization and telomerase access/association with the telomere [33, 51].

Tankyrase 1 has now been found to play a functional role in a multitude of cell processes, other than at the telomere [104]. Tankyrase-dependent PARsylation of pADPr-acceptor proteins is required for accurate cellular function throughout the cell cycle. Tankyase-dependent pADPr-modification of substrate proteins has been shown to play a role in protein stabilization, scaffolding and activation. To briefly illustrate examples of these characteristics, tankyrase 1 pADPr modification is required for accurate spindle pole formation by stabilization and scaffolding of the nuclear mitotic apparatus (NuMA) protein during mitosis [56, 105, 106]; whereas tankyrase 1 pADPr

modification is essential in the activation of the transcription factor β –catenin [107]. Interestingly, NuMA is dependent on pADPr modification as a method for appropriate protein network organization and noncovalent scaffolding with the immediately adjacent NuMA and tankyrase proteins [104, 105]. Tankyrase 1 is responsible for the PARsylation of the β-catenin sequestering protein axin, resulting in subsequent ubiquitination and degradation of the axin-dependent sequestering complex [107]. Hence, tankyrase 1 plays an indirect regulatory role in the level of intracellular β-catenin by negatively regulating the corresponding sequestering complex [107].

In addition to regulating intracellular proteins, tankyrase 1 plays an auto-regulatory role via PARsylation. Once the pADPr modification of an acceptor protein is initiated, tankyrases multimerize by interacting with the SAM domain of adjacent tankyrases to accelerate processivity of the growing pADPr chain. To dissociate from the tankyrase oligimer, individual tankyrases auto-PARsylate, inducing a conformational change that disrupts the tankyrase-tankyrase SAM interaction. If not de-PARsylated by the activity of PARG and APD-ribose lyase (removes the final ADP monomer), pADPr modified tankyrases dissociate and become a vulnerable substrate for E3 ligase-mediated ubiquitination and subsequent proteasome-mediated degradation [108]. Hence, tankyrase stability is regulated via auto-PARsyaltion.

1.4.0 An unanticipated relationship: PARPs and DNA-PKcs

Prior sections have provided evidence for roles of PARPs and DNA-PKcs at both DNA-damage sites and in telomere stability. Thus, there are likely instances where both a PARP and DNA-PKcs co-localize to specific sites. One of these sites is the telomere. In mammalian cells, DNA-PKcs is required to accurately cap the telomere [38].

Likewise, tankyrase 1 is required for the release of TRF1 from the telomere during replication. The role of both tankyrase 1 and DNA-PKcs are essential to maintain appropriate length and function of telomeres [38, 51]. Beyond the telomere, DNA-PKcs plays a role in DNA-repair, as do a variety of PARP family members.

In DNA-repair, PARP-1 and PARP-2 are speculated to have redundant roles in BER, possibly NER, as well as alternative NHEJ pathways. Similarly, DNA-PKcs localizes to DNA-DSBs following the initial recruitment of the Ku 70/80 heterodimer, forming the active DNA-PK holoenzyme. It is controversial as to whether or not PARP-1 competes with the Ku heterodimer for double-strand break ends, or if they co-localize at sites to achieve a common goal. A pivotal IP study suggested that PARP-1 and DNA-PKcs have a brief period of interaction intracelluarlly [109].

1.4.1 Evidence for DNA-PKcs dependence on PARP

Following the identification of PARP-1 in complex with DNA-PKcs, further *in vitro* analysis revealed a potential functional attribute to this interaction. *In vitro* combination of DNA-PKcs with PARP-1 showed a shift in DNA-PKcs molecular weight only when incubated with NAD^+, the substrate for PARP catalytic activity. Further, incubation with PARP-1 and NAD^+ resulted in the up-regulation of DNA-PKcs kinase activity on itself and on a variety of substrates *in vitro* [109]. Interestingly, these findings strongly suggest that pADPr modification has a positive impact on the catalytic activity of DNA-PKcs. This implication has not been verified by any other study to date. In fact, the question has only become more convoluted as there is no evidence supporting the involvement of PARP-1 in DNA-PKcs function. Further, PARP-1 and DNA-PKcs each act in what are believed to be independent, non-related end-joining processes.

Although a specific *in vivo* protein-protein interaction between PARP-1 and DNA-PKcs has not been revealed, the initial findings are relevant in broadening the scope of pAPDr influence and are suggestive of the mechanism by which DNA-PKcs protein is regulated. Importantly, identification of DNA-PKcs pADPr modification *in vitro* demonstrated DNA-PKcs is capable of becoming PARsylated [109]. In addition, immunoprecipitation of pADPr residues (in whole cell lysate) identified DNA-PKcs as a PARsylated member of the proteaome [110], supporting the findings of Ruscetti and collegues [109]. Given the high rate of intracellular PARG activity, PARsylated proteins are short lived. Literature has shown that PARsylated proteins are closely regulated, as the modification generally plays a functional role [92, 93, 97, 104]. Interestingly, DNA-PKcs has been found to contain pADPr interacting motifs (non-covalent interactions with pADPr residues on modified proteins) in addition to covalent pADPr modification, allowing for non-covalent protein-protein interactions [111]; suggestive of a method for PARP-mediated protein-protein scaffolding, mirroring the mechanism for complex formation observed in the NuMA model [56, 105, 106].

Here, we investigate the impact of single-nucleotide polymorphisms in the the leucine zipper domain and phophatidylinositol-3-kinase like-kinase (PIKK) domain of the murine *Prkdc* allele to determine their influence in DNA-PKcs end-capping capabilities. Furthermore, we find that tankyrasse 1 is responsible for contributing to genome stability, playing a novel, though indirect role in NHEJ DNA-repair. Surprisingly, we find genomic instability phenotypes emerge as a consequence of tankyrase 1 depletion or catalytic inhibition that reflect hallmarks of DNA-PKcs deficiencies at the telomere and in DNA-repair. We are the first to report that DNA-PKcs

is regulated by the catalytic PARP activity of tankyrase 1. The studies presented here investigate the mechanisms by which DNA-PKcs becomes PARsylated, and begin to uncover the functional role of this modification.

1.5.0 Figures

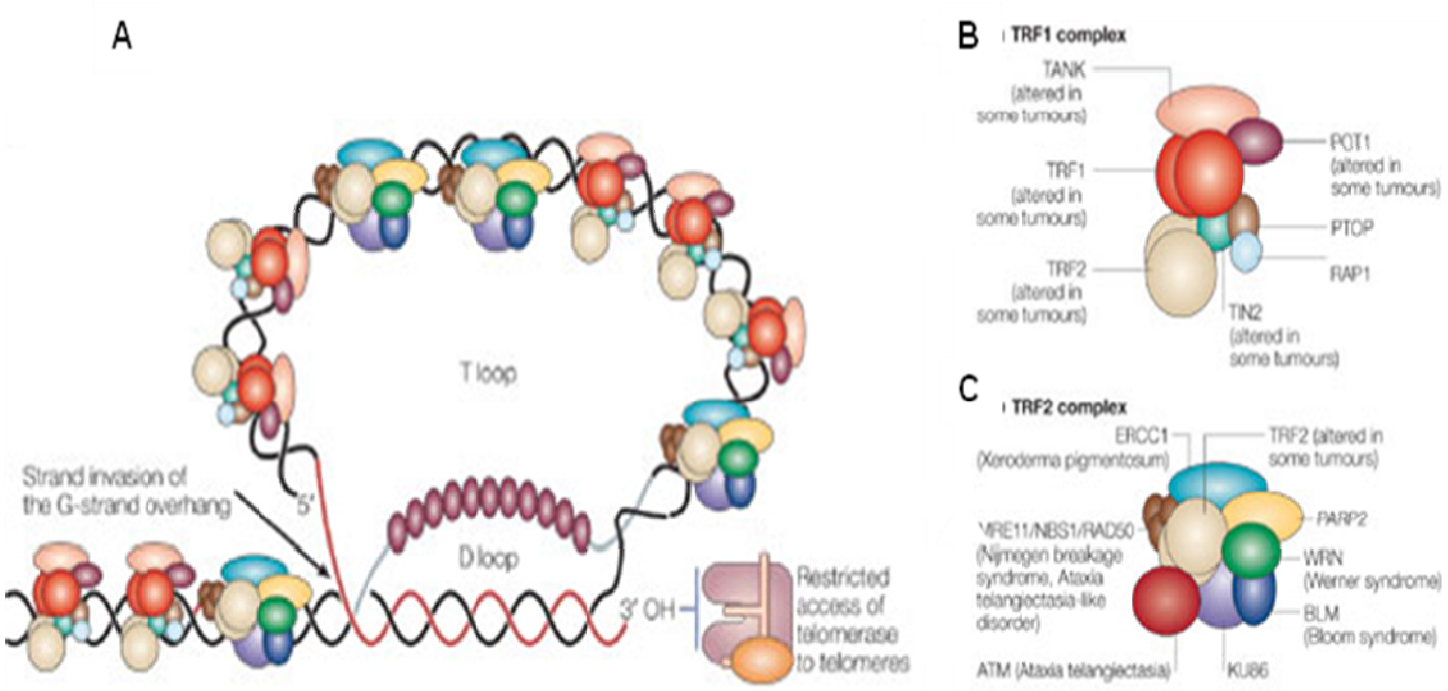


Figure 1. Telomere structure and associated protein components of Shelterin. **(A)** Depiction of telomeric DNA and shelterin protein components in the formation of the T-loop and D-loop in appropriate telomere capping. **(B)** Components of the TRF1 complex **C.** Components of the TRF2 complex [30].

Blasco, 2005

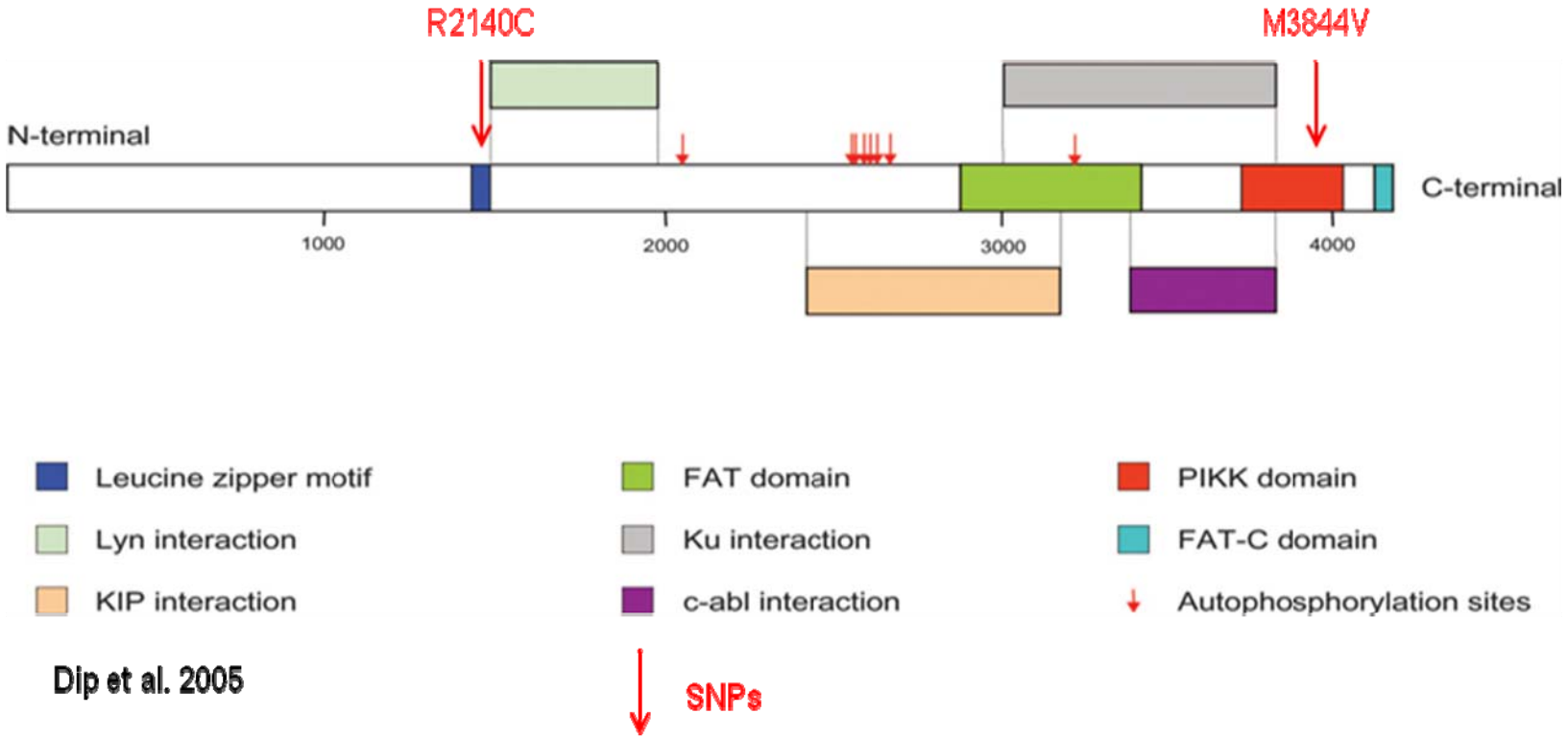


Figure 2. Domains of the DNA-PKcs protein. Single-nucleotide polymorphisms are illustrated in their approximate positions within the relative domains. Image is adapted from Dip et al., 2005 [64]

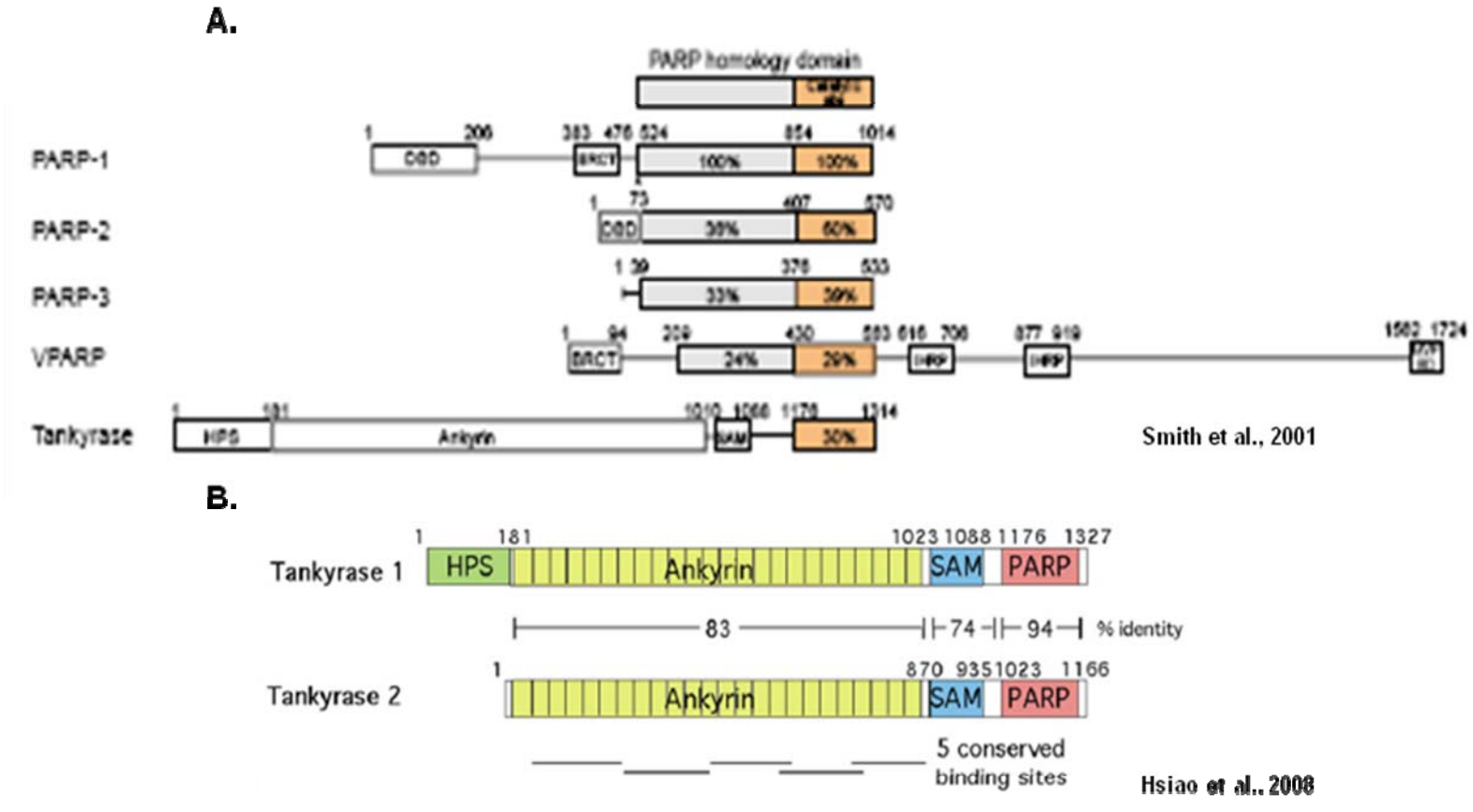


Figure 3. PARP family members and functional domains. (A) Tankyrases lack DNA-binding and BRCT domains that are common in DNA-repair proteins and possessed by PARP-1 and PARP-2. Tankyrases are unique to the PARP family in that they contain ankyrin repeat domains and a sterile alpha motif. [93]. **(B)** The 'HPS' repeat at the N-terminus is the only component that separates tankyrase 1 from tankyrase 2 [104].

REFERENCES

1.6.0 References

1. de Lange T, Shiue L, Myers RM, Cox DR, Naylor SL, Killery AM, Varmus HE: **Structure and variability of human chromosome ends**. *Mol Cell Biol* 1990, **10**(2):518-527.
2. de Lange T: **How telomeres solve the end-protection problem**. *Science* 2009, **326**(5955):948-952.
3. Meyne J, Ratliff RL, Moyzis RK: **Conservation of the human telomere sequence (TTAGGG)n among vertebrates**. *Proc Natl Acad Sci U S A* 1989, **86**(18):7049-7053.
4. Moyzis RK, Buckingham JM, Cram LS, Dani M, Deaven LL, Jones MD, Meyne J, Ratliff RL, Wu JR: **A highly conserved repetitive DNA sequence, (TTAGGG)n, present at the telomeres of human chromosomes**. *Proc Natl Acad Sci U S A* 1988, **85**(18):6622-6626.
5. Chakhparonian M, Wellinger RJ: **Telomere maintenance and DNA replication: how closely are these two connected?** *Trends Genet* 2003, **19**(8):439-446.
6. Lingner J, Cech TR: **Telomerase and chromosome end maintenance**. *Curr Opin Genet Dev* 1998, **8**(2):226-232.
7. Huffman KE, Levene SD, Tesmer VM, Shay JW, Wright WE: **Telomere shortening is proportional to the size of the G-rich telomeric 3'-overhang**. *J Biol Chem* 2000, **275**(26):19719-19722.
8. Makarov VL, Hirose Y, Langmore JP: **Long G tails at both ends of human chromosomes suggest a C strand degradation mechanism for telomere shortening**. *Cell* 1997, **88**(5):657-666.
9. Wellinger RJ, Sen D: **The DNA structures at the ends of eukaryotic chromosomes**. *Eur J Cancer* 1997, **33**(5):735-749.
10. Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu CP, Morin GB, Harley CB, Shay JW, Lichtsteiner S, Wright WE: **Extension of life-span by introduction of telomerase into normal human cells**. *Science* 1998, **279**(5349):349-352.
11. Jiang XR, Jimenez G, Chang E, Frolkis M, Kusler B, Sage M, Beeche M, Bodnar AG, Wahl GM, Tlsty TD *et al*: **Telomerase expression in human somatic cells does not induce changes associated with a transformed phenotype**. *Nat Genet* 1999, **21**(1):111-114.
12. Collins K, Mitchell JR: **Telomerase in the human organism**. *Oncogene* 2002, **21**(4):564-579.
13. Greider CW, Blackburn EH: **Identification of a specific telomere terminal transferase activity in Tetrahymena extracts**. *Cell* 1985, **43**(2 Pt 1):405-413.
14. Greider CW, Blackburn EH: **The telomere terminal transferase of Tetrahymena is a ribonucleoprotein enzyme with two kinds of primer specificity**. *Cell* 1987, **51**(6):887-898.

15. Bryan TM, Englezou A, Dalla-Pozza L, Dunham MA, Reddel RR: **Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines**. *Nat Med* 1997, **3**(11):1271-1274.
16. Flores I, Benetti R, Blasco MA: **Telomerase regulation and stem cell behaviour**. *Curr Opin Cell Biol* 2006, **18**(3):254-260.
17. Harrington L: **Does the reservoir for self-renewal stem from the ends?** *Oncogene* 2004, **23**(43):7283-7289.
18. Lansdorp PM: **Role of telomerase in hematopoietic stem cells**. *Ann N Y Acad Sci* 2005, **1044**:220-227.
19. Harley CB, Futcher AB, Greider CW: **Telomeres shorten during ageing of human fibroblasts**. *Nature* 1990, **345**(6274):458-460.
20. Hayflick L, Moorhead PS: **The serial cultivation of human diploid cell strains**. *Exp Cell Res* 1961, **25**:585-621.
21. Shay JW, Bacchetti S: **A survey of telomerase activity in human cancer**. *Eur J Cancer* 1997, **33**(5):787-791.
22. Bryan TM, Englezou A, Gupta J, Bacchetti S, Reddel RR: **Telomere elongation in immortal human cells without detectable telomerase activity**. *Embo Journal* 1995, **14**(17):4240-4248.
23. Murnane JP, Sabatier L, Marder BA, Morgan WF: **Telomere dynamics in an immortal human cell line**. *Embo Journal* 1994, **13**(20):4953-4962.
24. Neumann AA, Reddel RR: **Telomere maintenance and cancer -- look, no telomerase**. *Nat Rev Cancer* 2002, **2**(11):879-884.
25. Chan SW, Blackburn EH: **New ways not to make ends meet: telomerase, DNA damage proteins and heterochromatin**. *Oncogene* 2002, **21**(4):553-563.
26. Chan SS, Chang S: **Defending the end zone: studying the players involved in protecting chromosome ends**. *FEBS Lett* 2010, **584**(17):3773-3778.
27. McClintock B: **The Stability of Broken Ends of Chromosomes in Zea Mays**. *Genetics* 1941, **26**(2):234-282.
28. de Lange T: **Shelterin: the protein complex that shapes and safeguards human telomeres**. *Genes Dev* 2005, **19**(18):2100-2110.
29. Chen Y, Yang Y, van Overbeek M, Donigian JR, Baciu P, de Lange T, Lei M: **A shared docking motif in TRF1 and TRF2 used for differential recruitment of telomeric proteins**. *Science* 2008, **319**(5866):1092-1096.
30. Blasco MA: **Telomeres and human disease: ageing, cancer and beyond**. *Nat Rev Genet* 2005, **6**(8):611-622.
31. Karlseder J, Broccoli D, Dai Y, Hardy S, de Lange T: **p53- and ATM-dependent apoptosis induced by telomeres lacking TRF2**. *Science* 1999, **283**(5406):1321-1325.
32. Smogorzewska A, van Steensel B, Bianchi A, Oelmann S, Schaefer MR, Schnapp G, de Lange T: **Control of human telomere length by TRF1 and TRF2**. *Mol Cell Biol* 2000, **20**(5):1659-1668.
33. van Steensel B, de Lange T: **Control of telomere length by the human telomeric protein TRF1**. *Nature* 1997, **385**(6618):740-743.
34. van Steensel B, Smogorzewska A, de Lange T: **TRF2 protects human telomeres from end-to-end fusions**. *Cell* 1998, **92**(3):401-413.

35. Palm W, de Lange T: **How shelterin protects mammalian telomeres**. *Annu Rev Genet* 2008, **42**:301-334.
36. Wang RC, Smogorzewska A, de Lange T: **Homologous recombination generates T-loop-sized deletions at human telomeres**. *Cell* 2004, **119**(3):355-368.
37. Bailey SM, Cornforth MN, Kurimasa A, Chen DJ, Goodwin EH: **Strand-specific postreplicative processing of mammalian telomeres**. *Science* 2001, **293**(5539):2462-2465.
38. Bailey SM, Meyne J, Chen DJ, Kurimasa A, Li GC, Lehnert BE, Goodwin EH: **DNA double-strand break repair proteins are required to cap the ends of mammalian chromosomes**. *Proc Natl Acad Sci U S A* 1999, **96**(26):14899-14904.
39. Hsiao SJ, Smith S: **Sister telomeres rendered dysfunctional by persistent cohesion are fused by NHEJ**. *J Cell Biol* 2009, **184**(4):515-526.
40. Deng Z, Dheekollu J, Broccoli D, Dutta A, Lieberman PM: **The origin recognition complex localizes to telomere repeats and prevents telomere-circle formation**. *Curr Biol* 2007, **17**(22):1989-1995.
41. Francia S, Weiss RS, Hande MP, Freire R, d'Adda di Fagagna F: **Telomere and telomerase modulation by the mammalian Rad9/Rad1/Hus1 DNA-damage-checkpoint complex**. *Curr Biol* 2006, **16**(15):1551-1558.
42. Garcia-Cao M, O'Sullivan R, Peters AH, Jenuwein T, Blasco MA: **Epigenetic regulation of telomere length in mammalian cells by the Suv39h1 and Suv39h2 histone methyltransferases**. *Nat Genet* 2004, **36**(1):94-99.
43. Hsu HL, Gilley D, Blackburn EH, Chen DJ: **Ku is associated with the telomere in mammals**. *Proc Natl Acad Sci U S A* 1999, **96**(22):12454-12458.
44. Hsu HL, Gilley D, Galande SA, Hande MP, Allen B, Kim SH, Li GC, Campisi J, Kohwi-Shigematsu T, Chen DJ: **Ku acts in a unique way at the mammalian telomere to prevent end joining**. *Genes Dev* 2000, **14**(22):2807-2812.
45. Lenain C, Bauwens S, Amiard S, Brunori M, Giraud-Panis MJ, Gilson E: **The Apollo 5' exonuclease functions together with TRF2 to protect telomeres from DNA repair**. *Curr Biol* 2006, **16**(13):1303-1310.
46. Opresko PL, von Kobbe C, Laine JP, Harrigan J, Hickson ID, Bohr VA: **Telomere-binding protein TRF2 binds to and stimulates the Werner and Bloom syndrome helicases**. *J Biol Chem* 2002, **277**(43):41110-41119.
47. Tarsounas M, Munoz P, Claas A, Smiraldo PG, Pittman DL, Blasco MA, West SC: **Telomere maintenance requires the RAD51D recombination/repair protein**. *Cell* 2004, **117**(3):337-347.
48. van Overbeek M, de Lange T: **Apollo, an Artemis-related nuclease, interacts with TRF2 and protects human telomeres in S phase**. *Curr Biol* 2006, **16**(13):1295-1302.
49. Zhu XD, Kuster B, Mann M, Petrini JH, de Lange T: **Cell-cycle-regulated association of RAD50/MRE11/NBS1 with TRF2 and human telomeres**. *Nat Genet* 2000, **25**(3):347-352.
50. Zhu XD, Niedernhofer L, Kuster B, Mann M, Hoeijmakers JH, de Lange T: **ERCC1/XPF removes the 3' overhang from uncapped telomeres and**

represses formation of telomeric DNA-containing double minute chromosomes. *Mol Cell* 2003, **12**(6):1489-1498.
51. Smith S, Giriat I, Schmitt A, de Lange T: **Tankyrase, a poly(ADP-ribose) polymerase at human telomeres**. *Science* 1998, **282**(5393):1484-1487.
52. Seimiya H, Muramatsu Y, Smith S, Tsuruo T: **Functional subdomain in the ankyrin domain of tankyrase 1 required for poly(ADP-ribosyl)ation of TRF1 and telomere elongation**. *Mol Cell Biol* 2004, **24**(5):1944-1955.
53. Seimiya H, Smith S: **The telomeric poly(ADP-ribose) polymerase, tankyrase 1, contains multiple binding sites for telomeric repeat binding factor 1 (TRF1) and a novel acceptor, 182-kDa tankyrase-binding protein (TAB182)**. *J Biol Chem* 2002, **277**(16):14116-14126.
54. Chang W, Dynek JN, Smith S: **TRF1 is degraded by ubiquitin-mediated proteolysis after release from telomeres**. *Genes Dev* 2003, **17**(11):1328-1333.
55. Cook BD, Dynek JN, Chang W, Shostak G, Smith S: **Role for the related poly(ADP-Ribose) polymerases tankyrase 1 and 2 at human telomeres**. *Mol Cell Biol* 2002, **22**(1):332-342.
56. Chang P, Coughlin M, Mitchison TJ: **Tankyrase-1 polymerization of poly(ADP-ribose) is required for spindle structure and function**. *Nat Cell Biol* 2005, **7**(11):1133-1139.
57. Dynek JN, Smith S: **Resolution of sister telomere association is required for progression through mitosis**. *Science* 2004, **304**(5667):97-100.
58. Williams ES, Klingler R, Ponnaiya B, Hardt T, Schrock E, Lees-Miller SP, Meek K, Ullrich RL, Bailey SM: **Telomere dysfunction and DNA-PKcs deficiency: characterization and consequence**. *Cancer Res* 2009, **69**(5):2100-2107.
59. Fabre KM, Ramaiah L, Dregalla RC, Desaintes C, Weil MM, Bailey SM, Ullrich RL: **Murine Prkdc Polymorphisms Impact DNA-PKcs Function**. *Radiat Res* 2011.
60. Araki R, Fujimori A, Hamatani K, Mita K, Saito T, Mori M, Fukumura R, Morimyo M, Muto M, Itoh M *et al*: **Nonsense mutation at Tyr-4046 in the DNA-dependent protein kinase catalytic subunit of severe combined immune deficiency mice**. *Proc Natl Acad Sci U S A* 1997, **94**(6):2438-2443.
61. Okayasu R, Suetomi K, Yu Y, Silver A, Bedford JS, Cox R, Ullrich RL: **A deficiency in DNA repair and DNA-PKcs expression in the radiosensitive BALB/c mouse**. *Cancer Res* 2000, **60**(16):4342-4345.
62. Ullrich RL, Bowles ND, Satterfield LC, Davis CM: **Strain-dependent susceptibility to radiation-induced mammary cancer is a result of differences in epithelial cell sensitivity to transformation**. *Radiat Res* 1996, **146**(3):353-355.
63. Yu Y, Okayasu R, Weil MM, Silver A, McCarthy M, Zabriskie R, Long S, Cox R, Ullrich RL: **Elevated breast cancer risk in irradiated BALB/c mice associates with unique functional polymorphism of the Prkdc (DNA-dependent protein kinase catalytic subunit) gene**. *Cancer Res* 2001, **61**(5):1820-1824.
64. Dip R, Naegeli H: **More than just strand breaks: the recognition of structural DNA discontinuities by DNA-dependent protein kinase catalytic subunit**. *FASEB J* 2005, **19**(7):704-715.

65. de Lange T: **Protection of mammalian telomeres**. *Oncogene* 2002, **21**(4):532-540.
66. Smogorzewska A, de Lange T: **Different telomere damage signaling pathways in human and mouse cells**. *Embo Journal* 2002, **21**(16):4338-4348.
67. Stansel RM, de Lange T, Griffith JD: **T-loop assembly in vitro involves binding of TRF2 near the 3' telomeric overhang**. *Embo Journal* 2001, **20**(19):5532-5540.
68. Celli GB, de Lange T: **DNA processing is not required for ATM-mediated telomere damage response after TRF2 deletion**. *Nat Cell Biol* 2005, **7**(7):712-718.
69. Martinez P, Thanasoula M, Munoz P, Liao C, Tejera A, McNees C, Flores JM, Fernandez-Capetillo O, Tarsounas M, Blasco MA: **Increased telomere fragility and fusions resulting from TRF1 deficiency lead to degenerative pathologies and increased cancer in mice**. *Genes Dev* 2009, **23**(17):2060-2075.
70. Sfeir A, Kosiyatrakul ST, Hockemeyer D, MacRae SL, Karlseder J, Schildkraut CL, de Lange T: **Mammalian telomeres resemble fragile sites and require TRF1 for efficient replication**. *Cell* 2009, **138**(1):90-103.
71. Baumann P: **Are mouse telomeres going to pot?** *Cell* 2006, **126**(1):33-36.
72. Hockemeyer D, Daniels JP, Takai H, de Lange T: **Recent expansion of the telomeric complex in rodents: Two distinct POT1 proteins protect mouse telomeres**. *Cell* 2006, **126**(1):63-77.
73. Hockemeyer D, Sfeir AJ, Shay JW, Wright WE, de Lange T: **POT1 protects telomeres from a transient DNA damage response and determines how human chromosomes end**. *Embo Journal* 2005, **24**(14):2667-2678.
74. Bunch JT, Bae NS, Leonardi J, Baumann P: **Distinct requirements for Pot1 in limiting telomere length and maintaining chromosome stability**. *Mol Cell Biol* 2005, **25**(13):5567-5578.
75. Loayza D, De Lange T: **POT1 as a terminal transducer of TRF1 telomere length control**. *Nature* 2003, **423**(6943):1013-1018.
76. Quintas-Cardama A, Cortes J: **Molecular biology of bcr-abl1-positive chronic myeloid leukemia**. *Blood* 2009, **113**(8):1619-1630.
77. Kurimasa A, Kumano S, Boubnov NV, Story MD, Tung CS, Peterson SR, Chen DJ: **Requirement for the kinase activity of human DNA-dependent protein kinase catalytic subunit in DNA strand break rejoining**. *Mol Cell Biol* 1999, **19**(5):3877-3884.
78. Yoo S, Dynan WS: **Geometry of a complex formed by double strand break repair proteins at a single DNA end: recruitment of DNA-PKcs induces inward translocation of Ku protein**. *Nucleic Acids Research* 1999, **27**(24):4679-4686.
79. Dobbs TA, Tainer JA, Lees-Miller SP: **A structural model for regulation of NHEJ by DNA-PKcs autophosphorylation**. *DNA Repair (Amst)* 2010, **9**(12):1307-1314.
80. Lempiainen H, Halazonetis TD: **Emerging common themes in regulation of PIKKs and PI3Ks**. *Embo Journal* 2009, **28**(20):3067-3073.
81. Britton S, Frit P, Biard D, Salles B, Calsou P: **ARTEMIS nuclease facilitates apoptotic chromatin cleavage**. *Cancer Res* 2009, **69**(20):8120-8126.

82. Yannone SM, Khan IS, Zhou RZ, Zhou T, Valerie K, Povirk LF: **Coordinate 5' and 3' endonucleolytic trimming of terminally blocked blunt DNA double-strand break ends by Artemis nuclease and DNA-dependent protein kinase**. *Nucleic Acids Research* 2008, **36**(10):3354-3365.
83. Mansour WY, Rhein T, Dahm-Daphi J: **The alternative end-joining pathway for repair of DNA double-strand breaks requires PARP1 but is not dependent upon microhomologies**. *Nucleic Acids Research* 2010, **38**(18):6065-6077.
84. Guirouilh-Barbat J, Huck S, Bertrand P, Pirzio L, Desmaze C, Sabatier L, Lopez BS: **Impact of the KU80 pathway on NHEJ-induced genome rearrangements in mammalian cells**. *Mol Cell* 2004, **14**(5):611-623.
85. Guirouilh-Barbat J, Rass E, Plo I, Bertrand P, Lopez BS: **Defects in XRCC4 and KU80 differentially affect the joining of distal nonhomologous ends**. *Proc Natl Acad Sci U S A* 2007, **104**(52):20902-20907.
86. Ma JL, Kim EM, Haber JE, Lee SE: **Yeast Mre11 and Rad1 proteins define a Ku-independent mechanism to repair double-strand breaks lacking overlapping end sequences**. *Mol Cell Biol* 2003, **23**(23):8820-8828.
87. Nevaldine B, Longo JA, Hahn PJ: **The scid defect results in much slower repair of DNA double-strand breaks but not high levels of residual breaks**. *Radiat Res* 1997, **147**(5):535-540.
88. Simsek D, Jasin M: **Alternative end-joining is suppressed by the canonical NHEJ component Xrcc4-ligase IV during chromosomal translocation formation**. *Nat Struct Mol Biol* 2010, **17**(4):410-416.
89. Yan CT, Boboila C, Souza EK, Franco S, Hickernell TR, Murphy M, Gumaste S, Geyer M, Zarrin AA, Manis JP *et al*: **IgH class switching and translocations use a robust non-classical end-joining pathway**. *Nature* 2007, **449**(7161):478-482.
90. Zhu C, Mills KD, Ferguson DO, Lee C, Manis J, Fleming J, Gao Y, Morton CC, Alt FW: **Unrepaired DNA breaks in p53-deficient cells lead to oncogenic gene amplification subsequent to translocations**. *Cell* 2002, **109**(7):811-821.
91. Althaus FR, Richter C: **ADP-ribosylation of proteins. Enzymology and biological significance**. *Mol Biol Biochem Biophys* 1987, **37**:1-237.
92. Ueda K, Hayaishi O: **ADP-ribosylation**. *Annu Rev Biochem* 1985, **54**:73-100.
93. Smith S: **The world according to PARP**. *Trends Biochem Sci* 2001, **26**(3):174-179.
94. Okita N, Ashizawa D, Ohta R, Abe H, Tanuma S: **Discovery of novel poly(ADP-ribose) glycohydrolase inhibitors by a quantitative assay system using dot-blot with anti-poly(ADP-ribose)**. *Biochem Biophys Res Commun* 2010, **392**(4):485-489.
95. Juarez-Salinas H, Sims JL, Jacobson MK: **Poly(ADP-ribose) levels in carcinogen-treated cells**. *Nature* 1979, **282**(5740):740-741.
96. Bouchard VJ, Rouleau M, Poirier GG: **PARP-1, a determinant of cell survival in response to DNA damage**. *Exp Hematol* 2003, **31**(6):446-454.
97. Ame JC, Spenlehauer C, de Murcia G: **The PARP superfamily**. *Bioessays* 2004, **26**(8):882-893.

98. Lindahl T, Satoh MS, Poirier GG, Klungland A: **Post-translational modification of poly(ADP-ribose) polymerase induced by DNA strand breaks**. *Trends Biochem Sci* 1995, **20**(10):405-411.
99. Sukhanova MV, Khodyreva SN, Lebedeva NA, Prasad R, Wilson SH, Lavrik OI: **Human base excision repair enzymes apurinic/apyrimidinic endonuclease1 (APE1), DNA polymerase beta and poly(ADP-ribose) polymerase 1: interplay between strand-displacement DNA synthesis and proofreading exonuclease activity**. *Nucleic Acids Research* 2005, **33**(4):1222-1229.
100. Ame JC, Rolli V, Schreiber V, Niedergang C, Apiou F, Decker P, Muller S, Hoger T, Menissier-de Murcia J, de Murcia G: **PARP-2, A novel mammalian DNA damage-dependent poly(ADP-ribose) polymerase**. *J Biol Chem* 1999, **274**(25):17860-17868.
101. Rulten SL, Fisher AE, Robert I, Zuma MC, Rouleau M, Ju L, Poirier G, Reina-San-Martin B, Caldecott KW: **PARP-3 and APLF function together to accelerate nonhomologous end-joining**. *Mol Cell* 2011, **41**(1):33-45.
102. De Rycker M, Price CM: **Tankyrase polymerization is controlled by its sterile alpha motif and poly(ADP-ribose) polymerase domains**. *Mol Cell Biol* 2004, **24**(22):9802-9812.
103. De Rycker M, Venkatesan RN, Wei C, Price CM: **Vertebrate tankyrase domain structure and sterile alpha motif (SAM)-mediated multimerization**. *Biochem J* 2003, **372**(Pt 1):87-96.
104. Hsiao SJ, Smith S: **Tankyrase function at telomeres, spindle poles, and beyond**. *Biochimie* 2008, **90**(1):83-92.
105. Chang P, Coughlin M, Mitchison TJ: **Interaction between Poly(ADP-ribose) and NuMA Contributes to Mitotic Spindle Pole Assembly**. *Mol Biol Cell* 2009, **20**(21):4575-4585.
106. Sbodio JI, Chi NW: **Identification of a tankyrase-binding motif shared by IRAP, TAB182, and human TRF1 but not mouse TRF1. NuMA contains this RXXPDG motif and is a novel tankyrase partner**. *J Biol Chem* 2002, **277**(35):31887-31892.
107. Huang SM, Mishina YM, Liu S, Cheung A, Stegmeier F, Michaud GA, Charlat O, Wiellette E, Zhang Y, Wiessner S *et al*: **Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling**. *Nature* 2009, **461**(7264):614-620.
108. Yeh TY, Meyer TN, Schwesinger C, Tsun ZY, Lee RM, Chi NW: **Tankyrase recruitment to the lateral membrane in polarized epithelial cells: regulation by cell-cell contact and protein poly(ADP-ribosyl)ation**. *Biochem J* 2006, **399**(3):415-425.
109. Ruscetti T, Lehnert BE, Halbrook J, Le Trong H, Hoekstra MF, Chen DJ, Peterson SR: **Stimulation of the DNA-dependent protein kinase by poly(ADP-ribose) polymerase**. *Journal of Biological Chemistry* 1998, **273**(23):14461-14467.
110. Gagne JP, Isabelle M, Lo KS, Bourassa S, Hendzel MJ, Dawson VL, Dawson TM, Poirier GG: **Proteome-wide identification of poly(ADP-ribose) binding proteins and poly(ADP-ribose)-associated protein complexes**. *Nucleic Acids Research* 2008, **36**(22):6959-6976.

111. Pleschke JM, Kleczkowska HE, Strohm M, Althaus FR: **Poly(ADP-ribose) binds to specific domains in DNA damage checkpoint proteins**. *J Biol Chem* 2000, **275**(52):40974-40980.

CHAPTER 2

The role of DNA-PKcs in telomeric end-capping is dependent on the leucine zipper domain

The following was published in:
Fabre KM, Ramaiah L, Dregalla RC, Desaintes C, Weil MM, Bailey SM, Ullrich RL: **Murine Prkdc Polymorphisms Impact DNA-PKcs Function**. *Radiat Res* 2011.
All figures in the following chapter are original productions of the dissertation author.

2.1.0 Introduction & Background

Genomic stability is largely dependent on timely and accurate repair of DNA damage. In mammalian cells, an important contribution to both classical non-homologous end-joining (C-NHEJ) and telomere function is the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) activity. Although the general role of DNA-PKcs is well appreciated, the function and contribution of specific domains within the large (470 kDa protein) are not well characterized. Deficiency of DNA-PKcs associated with specific polymorphic forms of the *Prkdc* allele, such as the functionally null form expressed in the severe combined inmmunodeficient mouse (SCID), have been shown to impact telomere end-capping function as well as NHEJ-mediated DNA-repair following exposure to ionizing radiation (IR) [1-4]. Cytogenetic studies have revealed hallmarks of DNA-PKcs protein deficiency, resulting in failed telomere end-capping and manifesting as telomere-telomere and telomere-DSB fusion events [2, 5]. Further, studies investigating radiation sensitivity and carcinogenic potential in mouse models possessing single-nucleotide polymorphisms (SNPs) in the *Prkdc* allele, such as BALB/c find them

to be more prone to lung and mammary adenocaricomas following low dose irradiation ($\leq$ 0.5 Gy) with either low-linear energy transfer (LET) γ-rays or high LET neutrons [6].

Chromosome aberrations & rearrangements can be detected with state-of-the-art cytogenetic techniques like Fluorescent *in situ* Hybridization (FISH) and Chromosome Orientation Fluorescent *in situ* Hybridization (CO-FISH) [7-9], which are particularly useful in IR studies for characterizing chromosomal instability phenotypes in the form of chromosome aberrations (gaps, breaks, and fusions), indicating genomic instability within the cell population (excludes microsatellite instability). FISH is a technique that is useful in labeling specific chromosomes and chromosome sequences through probe hybridization to particular genes, allowing for detection of chromosomal rearrangements/translocation events. Further, FISH can be used to cytogenetically identify centromeres (CM-FISH) [10, 11] as well as telomere sequences at the terminal ends of the chromosome [12]. However, this particular cytogenetic technique is restricted by its ability to distinguish between chromatid-specific sequences. Modification of the FISH technique, where newly synthesized daughter strand incorporated bromo-deoxy-uridine (BrdU) in each round of the cell cycle provided a mechanism for strand-specific degradation, producing single-stranded chromatids that could be labeled based on the orientation of their sequence [9]. This modified version of FISH is sensitive to the detection of inversions [8, 13] and can distinguish between telomeres synthesized during either leading or lagging strand synthesis [1]. Using this technique, it is possible to identify telomere based fusions and differentiate between telomere-double-strand break fusions and telomere-telomere fusions, at end-points that were formerly impossible using traditional FISH [14].

Here, we investigate the roles played by specific SNPs within the *Prkdc* allele and appropriate DNA-PKcs function in conserving genomic stability. The impact of variant forms of the *Prkdc* allele on DNA-PKcs protein function and therefore, chromosome stability, telomere-uncapping and NHEJ dysfunction was evaluated in a variety of mouse cell lines possessing different *Prkdc* allele variants.

The C57BL/6 murine cell line was used as a positive control for the 'wild-type' *Prkdc* allele and genetic background. The murine severe combined immunnodeficient (SCID) cell line served as a negative control for DNA-PKcs function (null). The murine SCID cell line is homozygous for a functionally null form of the DNA-PKcs protein resulting from a T-to-A transversion mutation, creating an tyrosine to an ochre stop codon (UAA) conversion in the C-terminus (Y4045X) and the loss of the final 88 amino acids [15-17]. Although the SCID *Prkdc* allele is stably expressed and the essential phosphatidylinositol 3-related-kinase (PIKK) motifs are conserved, the translated protein is less abundant within SCID cells compared to the common C57BL/6 mouse [17]. This indicates the reduction in the level of DNA-PKcs protein in SCID cells is the result of a posttranscriptional or posttranslational defect. The truncated form of DNA-PKcs is paired with reduced kinase activity, though it is not known whether it is a consequence of lowered protein levels or loss of kinase function. As a consequence, the truncated DNA-PKcs variant in SCID cells is defective in NHEJ function and telomere end-capping, resulting in the significant increase of cytogenetically visible telomere-telomere and telomere-DSB fusions [2, 14].

The BALB/c cell line contains two single-nucleotide polymorphisms (SNPs) within the *Prkdc* allele; one in the phosphatidylinositol 3' kinase-related kinase domain

and the other in the protein-interacting leucine zipper domain [18-20]. The A-to-G transition creates a SNP within the *Prkdc* kinase domain in exon 81, codon 3844 results in an amino acid conversion from methionine to valine (M3844V), potentially impacting the kinase activity of the DNA-PKcs protein [20]. The leucine zipper domain possesses a SNP resulting from a C-to-T base transition in exon 48, codon 2140, resulting in an arginine to cysteine amino acid conversion (R2140C). The leucine zipper domain influences protein interactions with DNA and protein substrates [21]. In regards to the BALB/c murine strain, it has been proposed the IR sensitivity and susceptibility to carcinogenesis may be connected to the SNP in the leucine zipper (R2140C) of the *Prkdc* allele; impacting its ability to interact with specific proteins, DNA and potentially accurate tertiary structure [20]. Cumulatively, the SNPs within the BALB/c cell line *Prkdc* gene results in the significant elevation in chromosome aberrations as the result of telomere and DSB-based fusions in response to low-doses of low LET γ-ray irradiation [14, 18-20, 22]. As in SCID cells, the expression level of the *Prkdc* allele in BALB/c cells is normal but levels of the DNA-PKcs protein is reduced along with the corresponding kinase function [20].

In an effort to determine if the IR-induced instability phenotypes observed in the BALB/c mouse result from the SNPs in the *Prkdc* domains or more from the BALB/c background itself, we generated two congenic mouse strains containing the different *Prkdc* alleles on either a C57BL/6 (wild-type) or BALB/c background. In the case of the *C.B6-Prkdc*^{B6} (C.B6) congenic mouse, the wild-type *Prkdc* allele (C57BL/6) control was crossed onto a BALB/c background [3]. Conversely, the *B6.C-Prkdc*^{BALB} (B6.C) mouse contains the BALB/c *Prkdc* allele crossed onto the C57BL/6 background [3]. We also

utilized cells derived from the inbred LEWES/EiJ (LEWES) mouse strain, containing only the BALB/c SNP within the *Prkdc* alleles PIKK domain (M3844V) [23]. DNA-repair capabilities and telomeric end-capping function in the various mouse cell lines possessing variant forms of the *Prkdc allele* were determined by CO-FISH analysis to identify the impact of the various SNPs and their corresponding domain(s) (Figure 1). We provide additional evidence that supports SNPs in specific Prkdc domains have consequences in telomere capping efficiency, opposed to the genetic background on which it exists. Further, we provide the first evidence that suggests an essential role for the *Prkdc* leucine zipper domain in accurate DNA-PKcs-mediated end-capping.

2.2.0 Results

2.2.1 BALB/c and SCID mouse cell lines display major chromosome instability phenotypes.

C57BL/6 mice possess the common *Prkdc* allele. SCID mice contain a truncated, functionally 'null' form of DNA-PKcs (significantly lower protein levels and activity [18]), whereas BALB/c express a variant form of DNA-PKcs protein owing to *Prkdc* SNPs within the leucine zipper domain (R2140C) and the PIKK domain (M3844V). CO-FISH analysis of BALB/c and SCID cell lines following 0 and 1 Gy γ-ray irradiation showed a significant increase in telomere uncapping phenotypes (telomere-DSB fusions) compared to the C57BL/6 wild-type control ($p < 0.05$) (Figure 2). These results are consistent with previous reports regarding telomere end-capping failure and DSB-repair via NHEJ in both cell lines as a result of the variant *Prkdc* allele present in each cell type [6, 15-17, 19, 20].

2.2.2 Role of BALB/c *Prkdc* SNPs in dealing with DNA ends

Congenic Mouse Strains and Telomere Integrity

Following 1 Gy γ-ray irradiation and CO-FISH analysis, our control C57BL/6 cell line responded with statistically insignificant increases in DSB-telomere fusions over the unirradiated population of C57BL/6 cells ($p > 0.05$). The implication of the control study is that the common *Prkdc* allele on the C57BL/6 background is sufficient for maintaining genomic stability by NHEJ function and telomere end-capping. The congenic C.B6 cell line contains the common *Prkdc* allele (as in C57BL/6), on a BALB/c background. Under non-irradiated conditions, C.B6 telomere-based fusions resemble the C57BL/6 background. Following 1 Gy γ-ray irradiation, the frequency of telomere-DSB fusions did not significantly increase over the non-irradiated C.B6 population, resembling the frequency observed in C57BL/6 1 Gy irradiated cells ($p > 0.05$). Based on these observations, we conclude that the C.B6 cell line responds to radiation-induced DSBs in similar fashion as the C57BL/6 cell line, *suggesting* it is the *Prkdc* allele, not the mouse background that influences the cells ability to maintain functional, capped telomeres and accurately repair DSBs.

CO-FISH analysis of telomere stability and function in the B6.C congenic cell line (BALB/c *Prkdc* allele) showed a significant elevation in telomere-DSB fusion frequencies over the C57BL/6 controls ($p > 0.05$) (Figure 3). The increased background frequency in the B6.C cell line is remarkably similar to that observed in the BALB/c cell line background. In addition, 1 Gy γ-ray irradiation resulted in a significant increase in the frequency of telomere-DBS fusions over non-irradiated B6.C cells ($p < 0.001$). Interestingly, the frequency of telomere-DSB fusions in irradiated B6.C did not

significantly differ from that observed in irradiated BALB/c cells (Figure 3). Cumulatively, we conclude the *Prkdc* alleles in the congenic cell lines are the determining factor for DNA-PKcs-dependent telomere stability, not mouse genetic the background in which they reside (Figure 3).

2.2.3 LEWES cell line efficiently repairs DSBs and maintains capped telomeres.

Next, we questioned whether both of the BALB/c SNPs are responsible for the DNA-PKcs-dependent genome instability phenotypes, or, there is a separation of function between each. The recently derived LEWES mouse (on a novel background) contains only the BALB/c SNP in the *Prkdc* PIKK domain (M3844V) and therefore, provided a practical means of addressing this question.

CO-FISH analysis of the LEWES mouse cell line (PIKK SNP (M3844V)) revealed a frequency of telomere-DSB fusions not significantly different from telomere-DSB fusion frequencies observed in the C57BL/6 cell line, possessing the wild-type *Prkdc* allele (with or without IR ($p > 0.05$)) (Figure 4 [23]). In addition, we observed no statistically significant difference in telomere-DSB fusions between LEWES cells treated with IR and those which were not ($p > 0.05$) (Figure 4 [23]). Based on these findings, we conclude that the *Prkdc* SNP within the PI3-K domain (M3844V) does not play a critical role in telomere end-capping, *suggesting* that the SNP residing within the leucine zipper domain of the *Prkdc* allele (R2140C) is critical to the appropriate function of DNA-PKcs at the telomere. However, we are not able to rule out the possibility that it is the combination of the PIKK and leucine zipper *Prkdc* SNPs that result in the telomere instability phenotypes observed in the BALB/c mouse.

2.3.0 Discussion:

2.3.1 The leucine zipper domain is essential for DNA-PKcs-dependent telomere end-capping

To investigate the role of DNA-PKcs in telomere end-capping and classical NHEJ-mediated DNA-repair, we analyzed multiple cell lines containing various *Prkdc* alleles crossed onto various mouse genomic backgrounds; distinguishing DNA-PKcs variants from the mouse cell background in telomere end-capping. Further, we determined the impact of specific SNPs within the *Prkdc* allele have on DNA-PKcs function in telomere end-capping. CO-FISH analysis facilitated accurate identification of telomere-DSB fusions in each cell line, both in non-irradiated populations and those exposed to 1 Gy γ-rays.

Over the course of our investigation, murine C57BL/6 cell line was used as the 'wild-type' regarding both the *Prkdc* allele and cell background for comparison. Other cell lines that were generated and/or selected contained a unique variant of the *Prkdc* allele, in order to improve understanding of the role played by each functional domain within the DNA-PKcs protein. The murine cell lines investigated included: SCID, which harbors an early stop codon at amino acid position 4045 in the *Prkdc* allele rendering a functionally null DNA-PKcs phenotype [15-17]; BALB/c, which has two SNPs within *Prkdc* (PIKK domain (M3844V)) and the leucine zipper domain (R2140C)) [18-20]; congenics C.B6 and B6.C with the common C57BL/6 *Prkdc* allele on a BALB/c background and the BALB/c *Prkdc* allele on the common C57BL/6 background respectively [3]; LEWES containing only one of the BALB/c *Prkdc* SNPs, M3844V in the PIKK domain [23].

Consistent with prior findings, SCID and BALB/c cell lines each maintained a high background level of chromosome aberrations, specifically telomere-based fusions. In addition to high background levels of telomere-telomere fusions following IR exposure [2], uncapped telomeres in SCID cells experienced inappropriate fusion with DSBs (telomere-DSB fusions) as observed in previous studies [2, 14]. For this reason, SCID cells serve as an appropriate positive control for DNA-PKcs protein deficiency phenotypes. Although BALB/c cells do not maintain a telomere instability phenotype as robust in nature as SCID, the dual SNPs within the BALB/c *Prkdc* allele produces reduced levels of a variant form of the DNA-PKcs protein (compared to the C57BL/6 control) resulting in significantly elevated telomere-DSB chromosome fusion frequencies compared to the common C57BL/6 *Prkdc* allele under irradiated and non-irradiated conditions. The purpose of the current study was to investigate individual domains of the *Prkdc* allele that are critical for the expression of a functional DNA-PKcs protein capable of effective telomere end-capping. The BALB/c mouse model provides a valuable resource in this regard, as it possesses two SNPs in *Prkdc*, in separate domains, which are necessary for accurate DNA-PKcs-mediated end-joining of DSBs and telomere end-capping; the PIKK domain (M3844V) and the leucine zipper domain (R2140C) [18-20].

To date, we have evidence that the *Prkdc* allele variant carried by BALB/c results in DNA-PKcs deficiency, contributing to the telomere uncapping and mis-joining of uncapped telomeres and DSBs generated by exposure to IR. To further support this supposition, we used two recently generated congenic mice: one containing the BALB/c *Prkdc* allele on the common C57BL/6 background (B6.C) in one case, or the converse, containing the common C57BL/6 allele on the BALB/c background (C.B6). CO-FISH

analysis of metaphase spreads in each case paralleled the trends for chromosome fusions observed in the cell line containing the respective *Prkdc* allele. This particular finding indicates it is in fact the *Prkdc* allele that impacts mammalian telomere stability, and therefore chromosome stability.

The LEWES mouse carries only one of the SNPs within the BALB/c variant *Prkdc* allele, residing in the PIKK domain (M3844V) [23]. Interestingly, cells derived from the LEWES mouse did not show significantly elevated levels of telomere-DSB fusions whether exposed to IR or not, indicating that telomeres are accurately capped. These results suggest the presence of a functional form of DNA-PKcs despite the single *Prkdc* SNP in the LEWES mouse. At the telomere end, only one molecule of DNA-PKcs is thought to be present and therefore, autophosphorylation in *trans*- as it likely occurs in NHEJ at DSB ends is not likely at the telomere [24].

SNPs and DNA-PKcs Autophosphorylation at Telomere

Importantly, our studies using the LEWES mouse demonstrates the SNP in the PIKK domain of the *Prkdc* allele (M3844V) alone is not responsible for the telomere end-capping dysfunction phenotypes observed in the BALB/c mouse. However, it is important to recognize the extent to which this particular SNP impacts the kinase function of the variant DNA-PKcs produced is unknown [18, 20, 25]; i.e. reduced DNA-PKcs kinase activity in BALB/c may be the consequence of the PIKK SNP (M3844V) and/or lower protein levels. It has been previously shown that DNA-PKcs autophosphorylation at residue Thr-2609 is necessary for accurate telomere capping [22]. We speculate that the SNP in the *Prkdc* PIKK domain (M3844V) does impact kinase activity, so autophosphoryation in *cis*- would be less frequent and result in less efficient

end-capping. However, previous studies have demonstrated DNA-PKcs phosphorylation at the Thr-2609 residue can be performed by ATM following irradiation [26]. In C57BL/6 and BALB/c mice, Atm protein levels and kinase activity are readily detectable; whereas cells derived from SCID show little Atm protein and activity [27]. Thus, the activity of Atm (not the intrinsic kinase activity of DNA-PKcs alone) may contribute to the phosphorylation of DNA-PKcs at Thr-2609 and enable DNA-PKcs end-capping function. This would explain the more severe telomere uncapping phenotypes observed in SCID mice compared to BALB/c despite reduced DNA-PKcs protein levels and kinase activity in each. However, it is doubtful that the SNP residing in the PIKK domain results in a kinase dead form of DNA-PKcs; if this were the case the LEWES cell line would be expected to resemble SCID phenotypes. We therefore suggest the leucine zipper domain plays a critical role in the ability for DNA-PKcs to interact with relevant telomere components, perhaps by inducing conformational changes that are necessary to maintain an essential degree of autophosphorylation capability in *cis*-.

At first glance, the generation of telomere-DSB fusions in cells derived from mice containing the *Prkdc* PIKK domain SNP (M3844V) seems counterintuitive, as it could inhibit DNA-PK-mediated NHEJ by influencing the kinase activity of DNA-PKcs and challenge the ability for the necessary autophosphorylation events to occur in *trans*- at the DSB synapse. To explain this phenomenon, we examined SCID cells that are effectively 'null' for DNA-PKcs and experience significantly high frequencies of telomere-based fusions (compared to the C57BL/6 wild-type) [22]. Telomere-DSB fusions have been identified by prior reports that observe telomere fusions arising from DNA-PKcs deficient, uncapped telomeres, which require DNA-ligase IV for NHEJ [28].

In addition, it is not known to what extent, if at all, the PIKK SNP impacts kinase function in the respective cell lines; lowered kinase activity may be the consequence of lower DNA-PKcs protein expression levels in the LEWES cell line (compared to C57BL/6) (data not shown). Previous reports show mRNA expression of the *Prkdc* allele does not significantly differ between BALB/c and C57BL/6 cell lines [18]. However, protein expression does differ significantly, indicating that either BALB/c *Prkdc* SNP (M3844V or R2140C) may lead to the destabilization of the DNA-PKcs protein following mRNA translation [18].

Ultimately, we find that with and without IR exposure, the congenic mouse cells reflect the telomere end-capping phenotypes associated with the respective *Prkdc* donor allele (C57BL/6 or BALB/c). Therefore, the telomere instability phenotype of BALB/c results from the variant DNA-PKcs, not the mouse genetic background. Further, cells derived from the LEWES mouse reveal telomere-DSB fusions at frequencies similar to those observed in the C57BL/6 line, containing the wild-type *Prkdc* allele. Based on these findings, we reason the BALB/c SNP within the PIKK domain does not affect DNA-PKcs-dependent telomere end-capping. Rather, the SNP within the leucine zipper domain (R2140C) contributes to the reactive, uncapped telomere phenotype observed in the BALB/c mouse, indicating that this domain is required for effective DNA-PKcs-mediated telomere end-capping.

We speculate that the SNP within the PIKK domain maintains a degree of functionality and thus does not resemble the DNA-PKcs null SCID phenotype. LEWES maintains a higher intracellular level of the DNA-PKcs protein and is more capable to perform telomere capping function as compared to BALB/c. Perhaps the combination of

the SNPs in BALB/c results in a reduced DNA-PKcs protein half life, stemming from an unknown biochemical mechanism. The increased level of DNA-PKcs protein in LEWES as compared to BALB/c argues the LEWES DNA-PKcs variant possessing only the PIKK *Prkdc* SNP (M3844V) is more stable than the BALB/c DNA-PKcs variant containing *Prkdc* SNPs in both the PIKK domain (M3844V) and the leucine zipper domain (R2140C). As the result of expressing a DNA-PKcs variant with a longer half-life, LEWES maintains a higher capacity for DNA-PKcs-mediated telomere end-capping, less chromosomal instability and thus, resembles the wild-type C57BL/6 mouse.

2.4.0 Materials and Methods

Irradiations

Irradiations were performed at a dose rate of 3.9Gy/min using a sealed-source Mark I ^{137}Cs γ-ray irradiator (J.L. Shepherd and Associates), located at Colorado State University, Department of Environmental and Radiological Health Sciences.

Generation of the C.B6 and B6.C congenic strains by marker-assisted "speed" congenics

Two novel strains of congenic mice were generated by a combination of conventional and marker-assisted backcrossing [29]. The *C.B6-Prkdc*^{B6} congenic strain (C.B6) possesses the common allele of *Prkdc* (*Prkdc*^{B6}), introgressed onto a BALB/c background. Conversely, the *B6.C-Prkdc*^{BALB} congenic strain (B6.C) contains the BALB/c allele (*Prkdc*^{BALB}) introgressed onto a resistant strain background (C57BL/6) [23].

Genotyping

The PCR restriction fragment length polymorphism (RFLP) method was used to genotype all mouse strains. The R2140C SNP located downstream of the leucine zipper

abolishes a *BsmB1* site while the M3844V SNP in the kinase domain creates a novel *Hph1* site. PCR primers were designed to flank the SNP loci and sequences amplified using *Taq* polymerase (Invitrogen; *Taq* DNA polymerase, Recombinant 10342-020). Primer sequences used are: exon48 (PKF-GCCTAAGGTAAGGTGCTGTA & PKR-GCCATGATCCTTAGCAAGTG) and exon81 (81F-ATGTTCTTTGCCATGCAGT AND 81R-TTCTTCCCTCCCTTCTCAGTA). The PCR products were digested with *BsmB1* or *Hph1* and were compared against known size samples by electrophoresis through a 2% or 3% agarose gel [23].

Sequencing

The entire coding region of the LEWES *Prkdc* gene was sequenced and compared to C57BL/6 and BALB/c sequences obtained from the Ensemble and NCBI databases (http://www.ensembl.org/index.html and http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nuccoreandid=124517705). A total of 19 PCR primer sets were designed using the Primer3 website (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (sequences to primers in supplementary methods). The amplified products were purified and sequenced [23].

Chromosome-Orientation Fluorescence in situ hybridization (CO-FISH)

Following irradiation, cell cultures were incubated for various times, trypinsized and sub-cultured into 5'-bromo-2'-deoxyuridine (BrdU) for one cell cycle; colcemid (0.1 µg/ml; Gibco) was added during the final 3-4 hours to accumulate mitotic figures, which were collected and processed for telomere CO-FISH as previously described with some modification [7, 30]. Briefly, samples were fixed in 3:1 methanol/acetic acid and dropped onto microscope slides, which were dried and treated with RNase A (100 µg/ml;

Sigma, 10 min at 37°C), rinsed in PBS, fixed in 1% formaldehyde (10 minutes at room temperature), rinsed in PBS, then dehydrated through a cold ethanol series (75%, 85% and 100%). Slides were air dried and stained with Hoechst 33258 (0.5ng/µl; Fischer) for 15 minutes and exposed to 365 nm UV light (Stratalinker 2400) for 25 minutes. Following UV exposure, BrdU incorporated stands were digested with Exonuclease III (2U/µl in provided reaction buffer; Promega) at room temperature for 10 minutes. Slides were rinsed and denatured briefly in 70% formamide at 75˚C for 1 minute and 15 seconds. Following an additional ethanol dehydration and air drying, a Cy-3 conjugated $(TTAGGG)_3$ PNA telomere probe (0.2µg/ml; Applied Biosystems) was hybridized at 37˚C for 1.5 hrs. Slides were rinsed in 70% formamide at 32˚C for 10 minutes and dehydrated in ethanol series before re-probing at 37˚C for two hours. Following the second hybridization, slides were rinsed with 70% formamide at 32˚C for 15 minutes followed by a 5 min rinse in PN Buffer. Chromosomes were counterstained with DAPI (4,6-Diamidine-2-phenylindole dihydrochloride; Vectashield with DAPI, Vector Laboratories).

Statistical Analysis

Metaphases were scored (n = 25) and statistical significance was determined via an unpaired t-test comparing to means to generate a p-value. Results were considered to be statistically different when $p \leq 0.05$ and not significantly different when $p > 0.05$.

LIST OF FIGURES

2.5.0 Figures

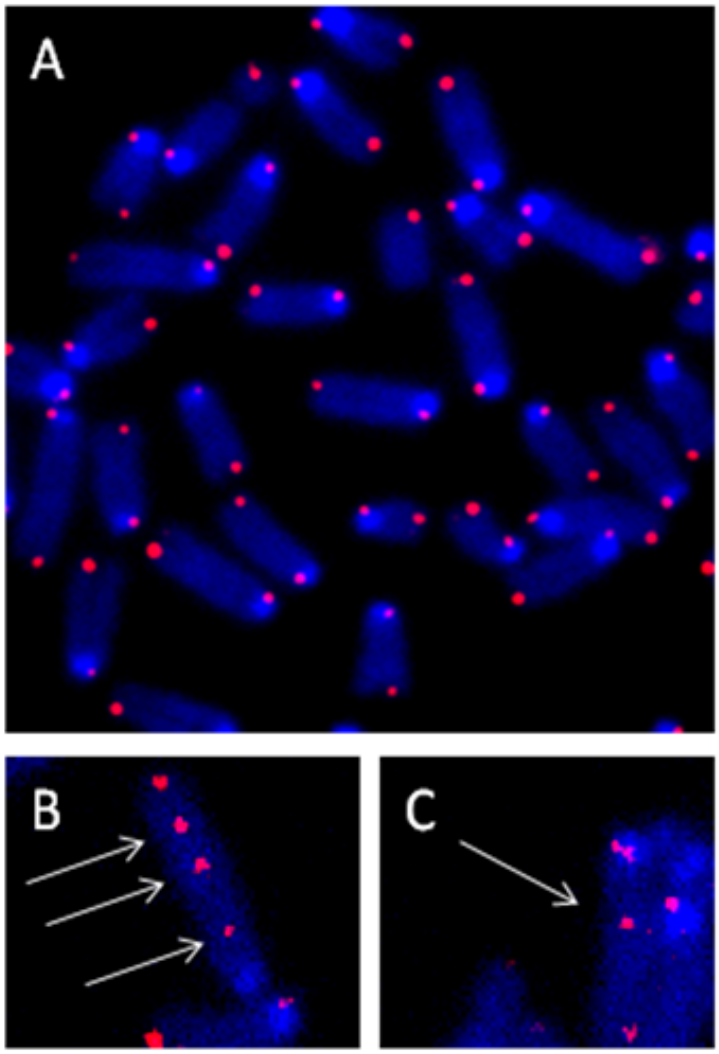


Figure 1. Metaphase spreads analyzed via CO-FISH following 1 Gy IR γ-rays (A) Control C57BL/6, no aberrations **(B)** Telomere-DSB fusions (arrows) in SCID **(C)** Telomere-DSB fusions (arrows) in BALB/c

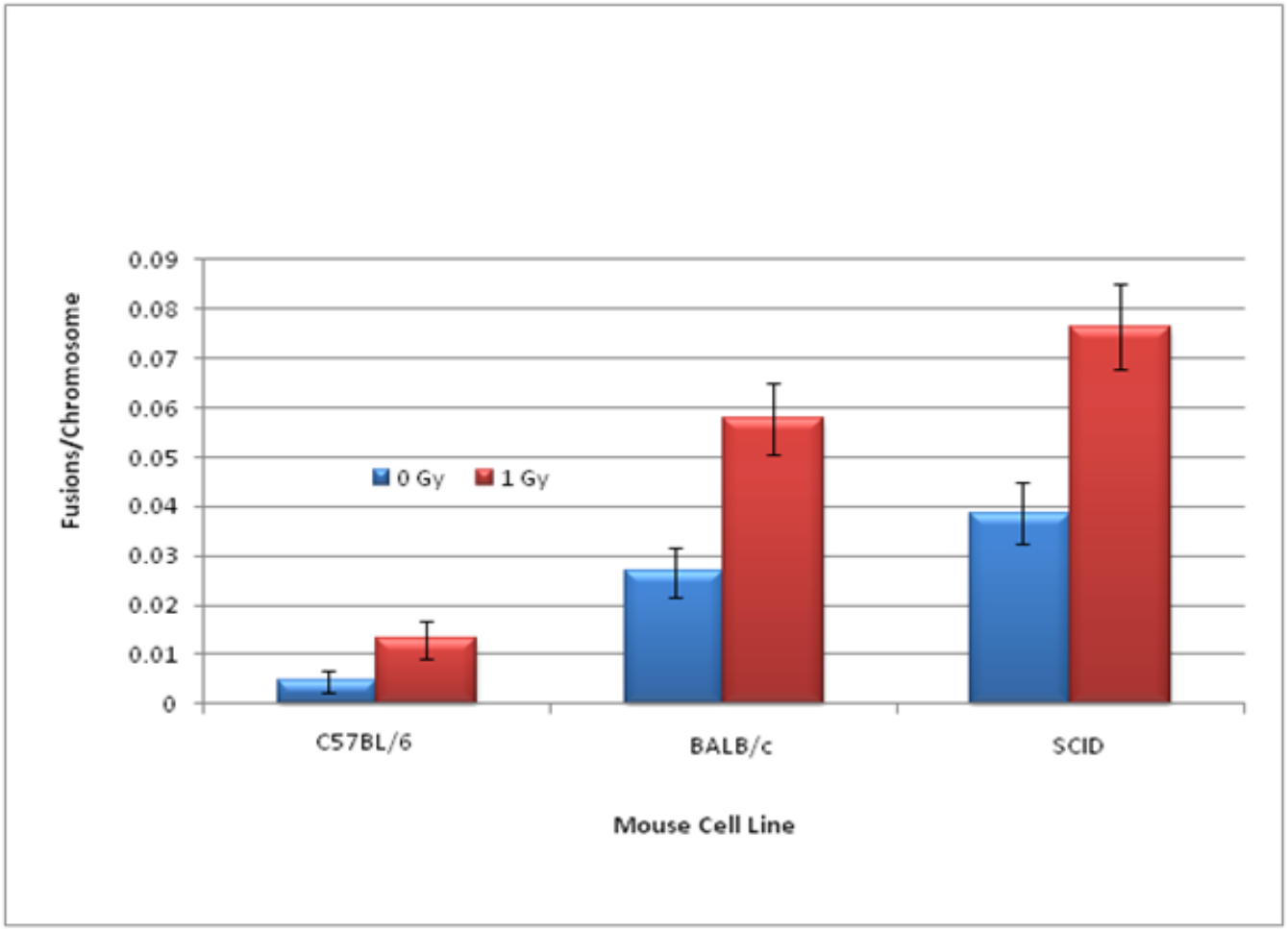


Figure 2. CO-FISH analysis of mouse cells for Telomere-DSB fusion frequencies. SCID and BALB/c cell lines contain null/variant *Prkdc* alleles which result in extensive telomere uncapping as well as deficient DNA-repair.

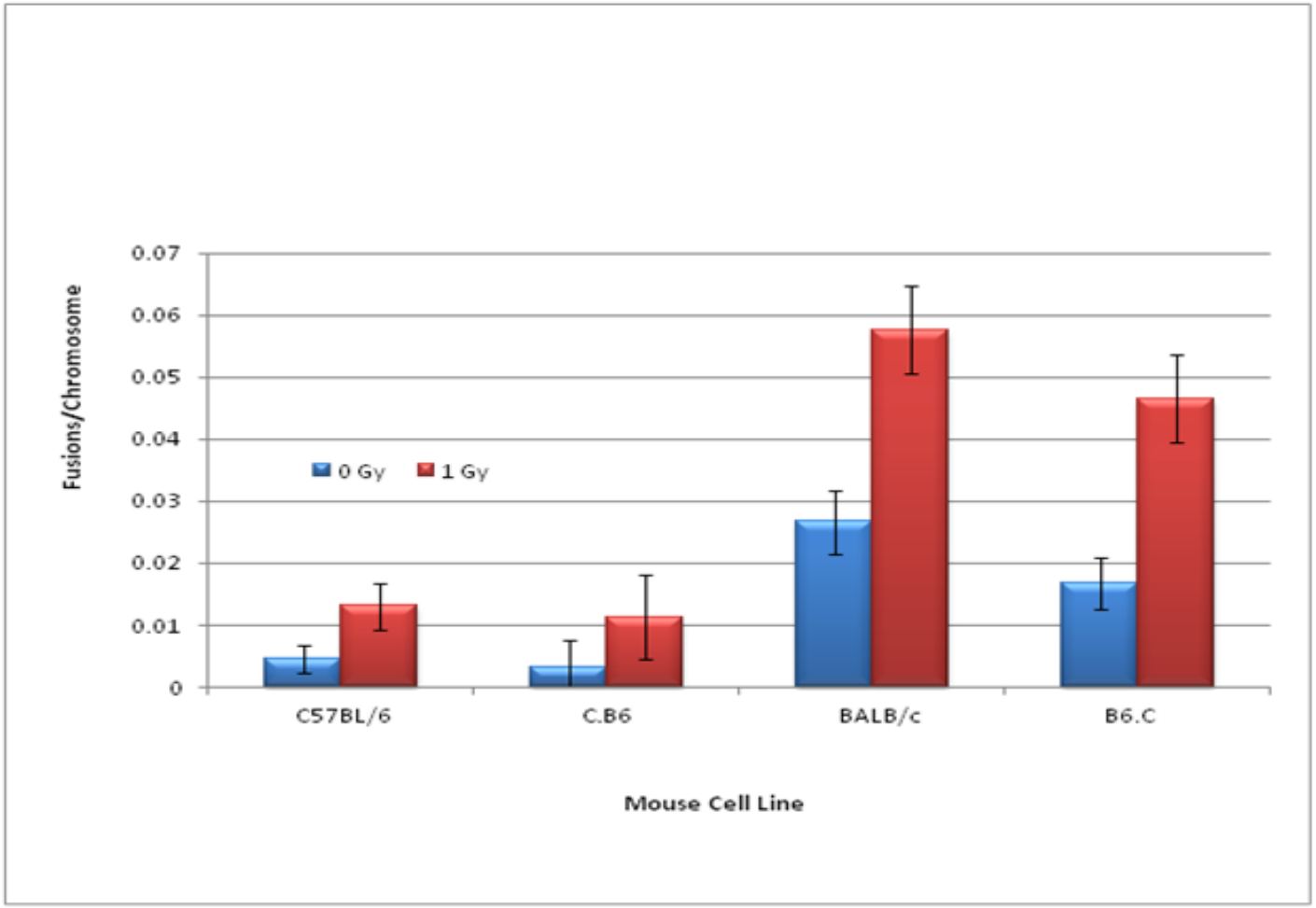


Figure 3. CO-FISH analysis of mouse cells for Telomere-DSB fusion frequencies. Congenic mouse cell lines (C.B6 (wild-type allele) and B6.C (BALB/c allele)) telomere-DSB fusion frequencies resemble the *Prkdc* allele donor cell line, C57BL/6 and BALB/c respectively.

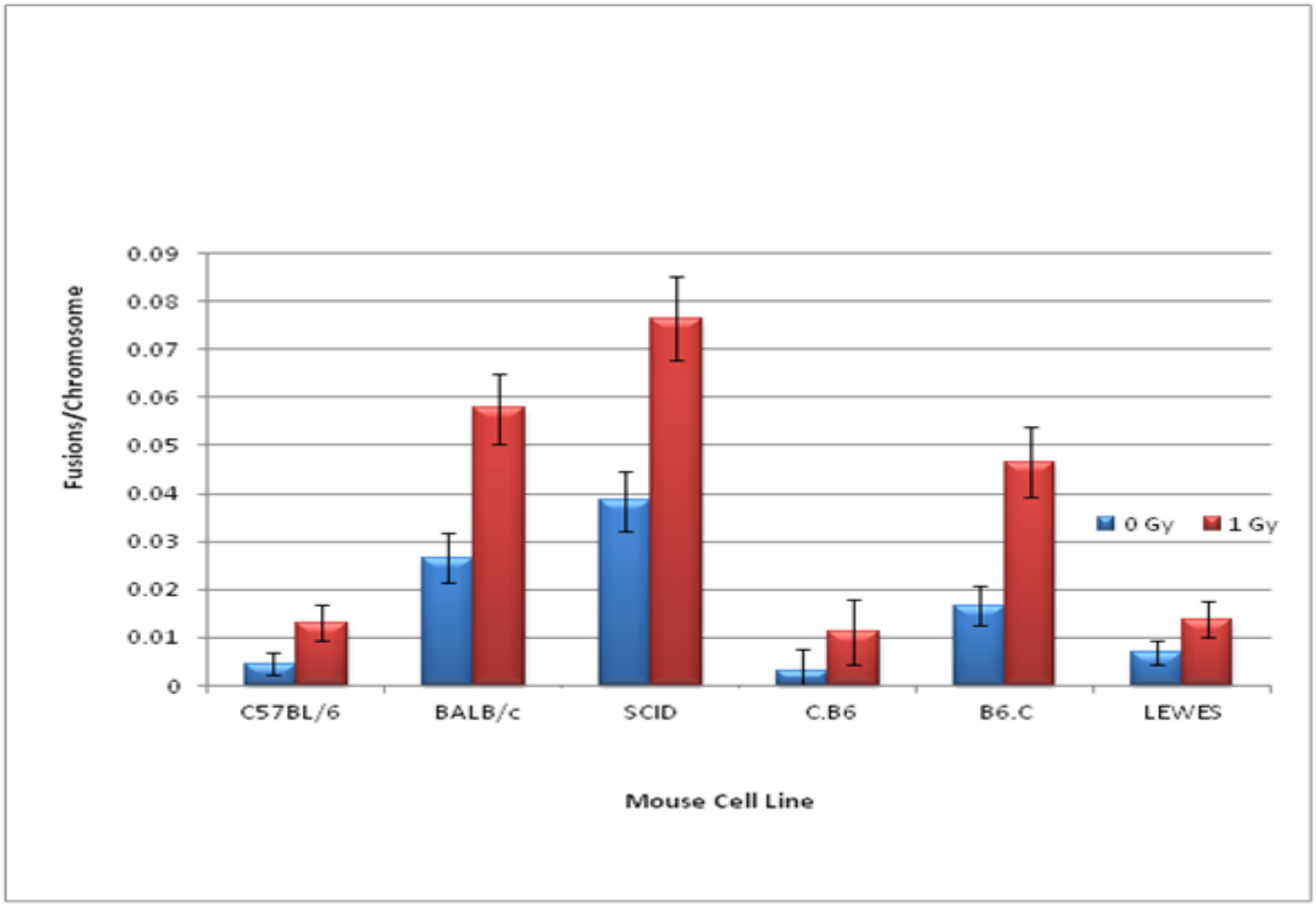


Figure 4. CO-FISH analysis of mouse cells for Telomere-DSB fusion frequencies. The Lewes mouse strain containing the *Prkdc* SNP in the PI3-kinase like-kinase domain (PIKK) (M3844V) displays telomere-DSB fusion frequencies similar to the control, wild-type *Prkdc* allele (C57BL/6).

REFERENCES

2.6.0 References

1. Bailey SM, Cornforth MN, Kurimasa A, Chen DJ, Goodwin EH: **Strand-specific postreplicative processing of mammalian telomeres**. *Science* 2001, **293**(5539):2462-2465.
2. Bailey SM, Meyne J, Chen DJ, Kurimasa A, Li GC, Lehnert BE, Goodwin EH: **DNA double-strand break repair proteins are required to cap the ends of mammalian chromosomes**. *Proc Natl Acad Sci U S A* 1999, **96**(26):14899-14904.
3. Hagelstrom RT, Askin KF, Williams AJ, Ramaiah L, Desaintes C, Goodwin EH, Ullrich RL, Bailey SM: **DNA-PKcs and ATM influence generation of ionizing radiation-induced bystander signals**. *Oncogene* 2008, **27**(53):6761-6769.
4. Hagelstrom RT, Blagoev KB, Niedernhofer LJ, Goodwin EH, Bailey SM: **Hyper telomere recombination accelerates replicative senescence and may promote premature aging**. *Proc Natl Acad Sci U S A* 2010, **107**(36):15768-15773.
5. Gilley D, Tanaka H, Hande MP, Kurimasa A, Li GC, Oshimura M, Chen DJ: **DNA-PKcs is critical for telomere capping**. *Proc Natl Acad Sci U S A* 2001, **98**(26):15084-15088.
6. Ullrich RL: **Tumor induction in BALB/c female mice after fission neutron or gamma irradiation**. *Radiat Res* 1983, **93**(3):506-515.
7. Bailey SM, Goodwin EH, Cornforth MN: **Strand-specific fluorescence in situ hybridization: the CO-FISH family**. *Cytogenet Genome Res* 2004, **107**(1-2):14-17.
8. Bailey SM, Goodwin EH, Meyne J, Cornforth MN: **CO-FISH reveals inversions associated with isochromosome formation**. *Mutagenesis* 1996, **11**(2):139-144.
9. Goodwin E, Meyne J: **Strand-specific FISH reveals orientation of chromosome 18 alphoid DNA**. *Cytogenet Cell Genet* 1993, **63**(2):126-127.
10. Henegariu O, Bray-Ward P, Artan S, Vance GH, Qumsyieh M, Ward DC: **Small marker chromosome identification in metaphase and interphase using centromeric multiplex fish (CM-FISH)**. *Lab Invest* 2001, **81**(4):475-481.
11. Nietzel A, Rocchi M, Starke H, Heller A, Fiedler W, Wlodarska I, Loncarevic IF, Beensen V, Claussen U, Liehr T: **A new multicolor-FISH approach for the characterization of marker chromosomes: centromere-specific multicolor-FISH (cenM-FISH)**. *Hum Genet* 2001, **108**(3):199-204.
12. Cornforth MN, Meyne J, Littlefield LG, Bailey SM, Moyzis RK: **Telomere staining of human chromosomes and the mechanism of radiation-induced dicentric formation**. *Radiat Res* 1989, **120**(2):205-212.

13. Bailey SM, Meyne J, Cornforth MN, McConnell TS, Goodwin EH: **A new method for detecting pericentric inversions using COD-FISH**. *Cytogenet Cell Genet* 1996, **75**(4):248-253.
14. Bailey SM, Cornforth MN, Ullrich RL, Goodwin EH: **Dysfunctional mammalian telomeres join with DNA double-strand breaks**. *DNA Repair (Amst)* 2004, **3**(4):349-357.
15. Araki R, Fujimori A, Hamatani K, Mita K, Saito T, Mori M, Fukumura R, Morimyo M, Muto M, Itoh M *et al*: **Nonsense mutation at Tyr-4046 in the DNA-dependent protein kinase catalytic subunit of severe combined immune deficiency mice**. *Proc Natl Acad Sci U S A* 1997, **94**(6):2438-2443.
16. Blunt T, Gell D, Fox M, Taccioli GE, Lehmann AR, Jackson SP, Jeggo PA: **Identification of a nonsense mutation in the carboxyl-terminal region of DNA-dependent protein kinase catalytic subunit in the scid mouse**. *Proc Natl Acad Sci U S A* 1996, **93**(19):10285-10290.
17. Danska JS, Holland DP, Mariathasan S, Williams KM, Guidos CJ: **Biochemical and genetic defects in the DNA-dependent protein kinase in murine scid lymphocytes**. *Mol Cell Biol* 1996, **16**(10):5507-5517.
18. Okayasu R, Suetomi K, Yu Y, Silver A, Bedford JS, Cox R, Ullrich RL: **A deficiency in DNA repair and DNA-PKcs expression in the radiosensitive BALB/c mouse**. *Cancer Res* 2000, **60**(16):4342-4345.
19. Ponnaiya B, Cornforth MN, Ullrich RL: **Radiation-induced chromosomal instability in BALB/c and C57BL/6 mice: the difference is as clear as black and white**. *Radiat Res* 1997, **147**(2):121-125.
20. Yu Y, Okayasu R, Weil MM, Silver A, McCarthy M, Zabriskie R, Long S, Cox R, Ullrich RL: **Elevated breast cancer risk in irradiated BALB/c mice associates with unique functional polymorphism of the Prkdc (DNA-dependent protein kinase catalytic subunit) gene**. *Cancer Res* 2001, **61**(5):1820-1824.
21. Struhl K: **Helix-turn-helix, zinc-finger, and leucine-zipper motifs for eukaryotic transcriptional regulatory proteins**. *Trends Biochem Sci* 1989, **14**(4):137-140.
22. Williams ES, Klingler R, Ponnaiya B, Hardt T, Schrock E, Lees-Miller SP, Meek K, Ullrich RL, Bailey SM: **Telomere dysfunction and DNA-PKcs deficiency: characterization and consequence**. *Cancer Res* 2009, **69**(5):2100-2107.
23. Fabre KM, Ramaiah L, Dregalla RC, Desaintes C, Weil MM, Bailey SM, Ullrich RL: **Murine Prkdc Polymorphisms Impact DNA-PKcs Function**. *Radiat Res* 2011.
24. Meek K, Douglas P, Cui X, Ding Q, Lees-Miller SP: **trans Autophosphorylation at DNA-dependent protein kinase's two major autophosphorylation site clusters facilitates end processing but not end joining**. *Mol Cell Biol* 2007, **27**(10):3881-3890.
25. Moll U, Lau R, Sypes MA, Gupta MM, Anderson CW: **DNA-PK, the DNA-activated protein kinase, is differentially expressed in normal and malignant human tissues**. *Oncogene* 1999, **18**(20):3114-3126.
26. Chen BP, Uematsu N, Kobayashi J, Lerenthal Y, Krempler A, Yajima H, Lobrich M, Shiloh Y, Chen DJ: **Ataxia telangiectasia mutated (ATM) is essential for**

DNA-PKcs phosphorylations at the Thr-2609 cluster upon DNA double strand break. *J Biol Chem* 2007, **282**(9):6582-6587.
27. Peng Y, Woods RG, Beamish H, Ye R, Lees-Miller SP, Lavin MF, Bedford JS: **Deficiency in the catalytic subunit of DNA-dependent protein kinase causes down-regulation of ATM**. *Cancer Res* 2005, **65**(5):1670-1677.
28. Smogorzewska A, Karlseder J, Holtgreve-Grez H, Jauch A, de Lange T: **DNA ligase IV-dependent NHEJ of deprotected mammalian telomeres in G1 and G2**. *Curr Biol* 2002, **12**(19):1635-1644.
29. Weil MM, Brown BW, Serachitopol DM: **Genotype selection to rapidly breed congenic strains**. *Genetics* 1997, **146**(3):1061-1069.
30. Meyne J, Goodwin EH, Moyzis RK: **Chromosome localization and orientation of the simple sequence repeat of human satellite I DNA**. *Chromosoma* 1994, **103**(2):99-103.

CHAPTER 3

The role of tankyrase 1 at telomeres and in DNA-repair

The following chapter has been published in:
Dregalla RC, Zhou J, Idate RR, Battaglia CL, Liber HL, Bailey SM: **Regulatory roles of tankyrase 1 at telomeres and in DNA repair: suppression of T-SCE and stabilization of DNA-PKcs**. *Aging (Albany NY)* 2010, **2**(10):691-708.
Figures contributed by the dissertation author include figures 5, 6 and 10.

3.1.0 Introduction:

3.1.1 Telomeres are regulated by the Shelterin complex

Mammalian telomere stability requires an impressive variety of proteins, the core members of which are referred to as 'Shelterin', and are involved in telomere length regulation and end-capping function (reviewed in [1]). The Shelterin complex consists of the telomere repeat binding factors 1 and 2 (TRF1 and TRF2), TRF1 and TRF2 Interacting Nuclear Protein 2 (TIN2), Repressor/Activator Protein 1 (Rap1 (human ortholog)), Tripeptidyl-peptidase 1 (TPP1) and Protection of Telomeres 1 POT1 [2].

TRF1 & TRF2 regulate telomere length and telomere stability respectively

TRF and TRF2 directly bind double-stranded telomeric sequences (TTAGGG) as homodimers and form complexes with additional proteins to create the TRF1 and TRF2 complexes [1]. Each telomere repeat factor complex (TRF1 and TRF2) has a particular role in preserving the function of the telomere in protecting the end of the chromosome. The primary role of TRF1 is in telomere length regulation, whereas TRF2 is critical for telomere end-capping [3-7]. Prior studies have provided evidence of TRF1 involvement

in regulation of telomerase access in telomerase positive cell lines. TRF1 dissociation from the telomere is linked to providing accessibility to telomerase for telomere elongation, therefore TRF1 is considered a negative regulator of telomere length [3]. TRF2 is required to maintain end-capping, as depletion of TRF2 results in uncapped telomeres capable of fusing to each other or to double-stranded breaks (DSBs). Such fusions are mediated by non-homologous end-joining (NHEJ) DNA-repair [7] and are cytogenetically visible as telomere-double strand break and telomere-telomere fusions [8, 9]. Cumulatively, chromosome (and chromatid) fusions are hallmarks of instability and carcinogenic potential, resulting from errors in end-joining repair processes and/or telomere uncapping (reviewed in [10]).

3.1.2 Tankyrase 1 negatively regulates TRF1

TRF1 is recognized as a key regulator of telomere length and so, TRF1 dysfunction results in the loss of appropriate telomere length regulation [5, 6]. TRF1 function relies on 'TRF1-associated' proteins as well, which are vital in the appropriate regulation of TRF1 in complex with the telomere. One of the 'TRF1-associated' shelterin accessory proteins is tankyrase 1, and more indirectly, tankyrase 2 [1, 11, 12]. Tankyrase 1 plays a crucial role in the regulation of TRF1 by mediating the ability of TRF1 to interact with telomeric-DNA. This is achieved by tankyrase 1-dependent poly(ADP-ribosyl)ation (pADPr/PAR) modification of TRF1, resulting in dissociation of the homodimer from the telomere [13, 14]. However, there are reports that telomere elongation can occur in the absence of fully functional tankyrase 1 and TRF1 PARsylation [15]. Disruption of tankyrase 1 dynamics following siRNA-mediated

depletion of tankyrase 1 was shown to influence telomere stability and functionality, resulting in sister chromatid telomere fusions [16].

In telomerase-positive cells, tankyrase 1-dependent release of TRF1 from the telomere facilitates telomerase access and telomere elongation [1, 17]. TRF1 dissociation occurs as the result of tankyrase 1-meidiated PARsylation of TRF1 at a RXXADG motif, a modification that causes the homodimer to dissociate from telomeric DNA [17, 18]. Following PARsylation and dissociation from the telomere, TRF1 is tagged by an E3 ligase for proteasome-mediated degradation, generally by the addition of ubiquitin[19]. However, there is also evidence of TRF1 degradation occuring as the result of sumoylation and F-box protein, Fbx4 modifications [14, 20, 21]. Regardless of the mechanism by which TFR1 is degraded, tankyrase 1 is specifically tasked with removal of TRF1 from the telomere. Thus, tankyrase 1 is regarded as a negative regulator of TRF1 regarding telomere interaction and so, is also recognized as a positive regulator of telomere length in telomerase positive cells. Contradictory to this dogma, studies have shown that in some cell lines, failure to dissociate TRF1 from the telomere does not lead to critically shortened telomeres as would be expected [15].

3.1.3 Tankyrase 1 dysfunction results in reactive telomeres

Although the role of tankyrase 1 in TRF1 regulation inevitably has an impact on accurate telomere function, studies seek to further investigate the function of tankyrase 1 at the telomere. siRNA studies targeting tankrase 1 have yielded a wealth of interesting findings. One such study found certain cell types, including HeLa, respond to the depletion of tankyrase 1 by arresting the cell cycle in mitosis and ultimately, cell death [22]. The conclusion was that cells fail to dissociate sister-chromatid telomeres in a

tankyrase 1-dependent fashion, either by failure to dissociate TRF1 or cohesion complexes. These were amongst the first findings to identify tankyrase 1 as a critical protein in cell viability and a critical asset to appropriate cell cycle regulation. Additional experiments have identified a potential explanation for the cell cycle arrest upon the loss of tankyrase 1 function. In an effort to observe telomere malfunction beginning in S-phase and persisting through G2 in tankyrase 1 knockdown cells, chromosomes were analyzed via fluorescent *in situ* Hybridization (FISH). The result was a significant increase in cytogenetically visible sister chromatid-fusions of two varieties: cohesion-based protein interactions and sister chromatid telomere-fusions ([16]). Cohesion complexes aid to reduce the need for persistent decatenation of the sister chromatids in S-phase, throughout G2 and into mitosis [23]. Further, cohesion protein Smc1 has been shown to be necessary in the downstream signaling of the intra-S phase cell cycle checkpoint [24]. These results suggest that tankyrase 1, by an as yet unknown mechanism, is required for the dissociation of the Smc1/Scc1/Scc3 cohesion complex (SA1) between the newly replicated chromatids in late S/early G2 phase [16[25]; telomeres remain in close proximity in a TRF1/TIN2/SA1-dependent fashion following S-phase and ensuing end-joining events results in sister chromatid fusions between the uncapped telomeres. Further, it is speculated tankyrase 1 cohesion release is necessary for exonuclease access for 3'-end resection and T-loop formation[16].

Such observations provide supporting evidence for tankyrase 1-dependent telomere function in S-phase. When lacking tankyrase 1, telomeres fail to become properly capped in G2 (following replication) and so serve as substrates for telomere-telomere fusions via a Ligase IV-dependent NHEJ event. The resulting anaphase bridges

are lethal in some cell lines (e.g., HeLa), whereas other cells progress through the cell cycle accumulating cytogenetically visible damage, possibly the result of anaphase bridge resolution by breakage. Therefore, loss of tankyrase 1 results in persistent chromatid association, and in telomere ends not being properly capped, resulting in NHEJ-mediated sister chromatid-fusions, subsequent anaphase bridges and ultimately, lethality [16].

Here we report the identification of five key aspects of intracellular tankyrase 1 function. First, we find tankyrase 1 to be critical in preventing telomeric recombination between sister-chromatids (T-SCEs). Second, tankyrase 1 depletion is associated with genomic instability phenotypes, resulting in increased cell killing and mutagenesis following ionizing radiation (IR) exposure. Third, tankyrase 1 depletion mirrors DNA-dependent Protein Kinase catalytic subunit (DNA-PKcs) deficiency phenotypes in that telomeres become uncapped and end-joining becomes impaired, significantly increasing the frequency of cytogenetically visible terminal deletions and telomere-DSB fusions following treatment with IR compared to controls. Fourth, increased mutation frequencies following IR exposure under conditions of DNA-PKcs inhibition and tankyrase 1 knockdown positively correlate with one another. Lastly, we demonstrated that DNA-PKcs protein stability is dependent on the tankyrase 1 protein. Taken together, we establish the requirement for tankyrase 1 for the regulation of telomere stability, as well as provide the first evidence for an indirect role of tankyrase 1 in maintaining cell wide genomic stability as a regulator of the DNA-repair protein, DNA-dependent Protein Kinase catalytic subunit (DNA-PKcs).

3.2.0 Results:

3.2.1 Tankyrase 1 regulates telomere stability

Tankyrase 1 depletion has been shown to result in dysfunctional, uncapped telomeres that are prone to sister chromatid-fusions [16]. We aim to determine the consequences of misregulated TRF1 under tankyrase 1 depleted conditions with regards to telomere stability during DNA replication. Using Chromosome Orientation *In Situ* Hybridization (CO-FISH), we distinguished telomeres synthesized by leading strand vs. lagging strand synthesis [26]. To rule out a role for telomerase under tankyrase 1 knockdown conditions, we selected cell lines with a telomerase negative background: Li Fraumani (ALT) and normal human fibroblasts (5C). T-SCE frequencies were determined and compared to those of the telomerase positive BJ-hTERT cell line. Using CO-FISH, we visualized telomeres in a strand specific manner. This approach allows for detection of recombination events within/between telomeric repeats, specifically, we monitored telomere-sister chromatid exchanges (T-SCE) [26, 27].

Tankyrase 1 siRNA knockdown was achieved in each cell line with multiple siRNA constructs. Of these, two siRNA constructs that effectively reduced tankyrase 1 levels to <1% relative to the mock transfected control (detected by horseradish peroxidase) were selected (Figure 1 [25]). All siRNA-mediated tankyrase 1 knockdown tankyrase 1 studies were performed using siRNA construct "siRNA1".

Tankyrase 1 depletion elevates T-SCE frequencies

Following lipofectamine siRNA transfection and successful tankyrase 1 knockdown, cells were incubated in bromodeoxyuridine (BrdU) for one cell cycle. Metaphases were collected from mitotic cells and analyzed by CO-FISH and scored for

T-SCEs. Consistently, T-SCEs were significantly elevated in the telomerase negative background of Li Fraumeni and 5c fibroblasts (background T-SCE levels vary between cell lines). However, the BJ-hTERT telomerase positive cell line did not show an increase in T-SCEs following tankyrase 1 knockdown (Figure 2 [28]). These findings are in agreement with those showing increased T-SCE frequencies as the result of stalled replication-forks within the telomere in WRN and BLM helicase deficient cells under siRNA-depleted telomerase conditions, where no effect was observed in telomerase positive cell lines [29].

Furthermore, tankyrase 1 knockdown did influence the frequency of genomic sister chromatid exchanges (G-SCE) compared to mock transfection controls, similar to the trend observed in WRN deficient, telomerase negative cells [29]. This finding further supports the notion of the critical and specific roles played by tankyrase 1 at the telomere, not in chromatin structure or modeling dynamics throughout the genome. As a regulator of the association of TRF1 with telomeric DNA, tankyrase 1 is necessary for replication fork progression through the telomere, as TRF1 (and TRF2) have been shown to block/stall replication forks [6, 7, 30-32].

3.2.2 Tankyrase 1 maintains genomic stability

Tankyrase 1 knockdown results in increased IR sensitivity & mutagenesis.

Given the prominent telomere instability phenotypes resulting from the depletion of tankyrase 1 (T-SCEs and sister chromatid telomere-fusions [16]), we next questioned whether tankyrase 1 plays a role in genomic stability. To investigate the influence of tankyrase 1 on genome stability, we examined the impact of tankyrase 1 knockdown on cell survival and mutagenesis following exposure to ionizing radiation (IR). Under

tankyrase 1 siRNA knockdown conditions, double-stranded breaks (DSBs) were induced using γ-ray irradiation with doses ranging from 0-8 Gy and cell survival was determined using clonogenic assays in human dermal fibroblasts (5C) and Li Fraumeni cell lines (Figure 3 [25]). Somewhat to our surprise, tankyrase 1 depletion did in fact reduce cell survival following IR treatment over the range of doses (Figure 3 [28]).

Human lymphoblasts (WTK1) were also treated with the broad-range PARP inhibitor 3-aminobenzamide (3-AB), tankyrase 1 siRNA or a combination of the treatments (3AB + siRNA) and irradiated with 1.5 Gy γ-ray IR at 18 hours post treatment. An increase in mutation frequencies (MF) (measured at the thymidine kinase locus) under either tankyrase 1 knockdown conditions or 3-AB treatment would provide additional evidence for the role of tankyrase 1 in genomic stability.

In non-irradiated cells, there was no significant impact on MF under conditions of tankyrase 1 knockdown ($p = 0.24$) (Figure 4 [28]). Cells depleted of tankyrase 1 and irradiated with 1.5 Gy γ-rays resulted in a MF 1.5 times higher than that observed in the mock transfected irradiated control ($p = .002$); 3-AB treatment increased the MF 2.5 times over the respective control ($p < 0.001$) (Figure 4 [28]). The combination treatment of tankyrase 1 siRNA-mediated knockdown and 3-AB reflected the mutation frequency observed in the 3-AB treatment alone ($p = 0.48$) (Figure 4 [28]). The increase in MFs as a consequence of tankyrase 1 knockdown suggests that the role of tankyrase 1 extends beyond the telomere and is perhaps fundamental to maintaining genomic stability. Interestingly, treatment with 3-AB resulted in a significant increase in MF ($p = 0.004$) in non-irradiated cells compared to the mock transfected cell population (Figure 4 [28]). This result suggests the possibility that tankyrase-specific PARP activity is a necessary

element contributing to genomic stability, not just the presence of the tankyrase 1 protein alone.

Tankyrase 1 knockdown results in DNA-PKcs deficiency signatures

Cytogenetic whole-chromosome analysis of human lymphoblasts (WTK1) with CO-FISH revealed a significant increase in terminal deletions in cells depleted of tankyrase 1 via siRNA-mediated knockdown and irradiated with 1 Gy low-linear energy transfer (LET) γ-rays or high-LET ^{56}Fe ions (1 GeV/n) (compared to mock transfected controls) ($p < 0.03$) (Figure 5 [25]). Interestingly, all terminal deletions were the product of chromosome-type aberrations, indicating the aberrations occurred as a result of unrepaired DSBs induced during G1 of the cell cycle.

Though tankyrase 1 catalytic activity has been identified as a critical factor for the appropriate function of substrate proteins such as NuMA [33], identification of a tankyrase 1-dependent DNA-repair deficiency is a novel observation. The nature of the chromosome aberration-type observed highlights several important points: each terminal deletion was the product of unrepaired DSBs induced in G1 of the cell cycle, replicated in S-phase and is the consequence of deficient NHEJ. Defective NHEJ processes during G1 of the cell cycle will produce chromosome-type aberrations such as those observed here. These findings suggest defective NHEJ mediated by DNA-dependent Protein Kinase, likely resulting in the initiation of ligase IV-dependent NHEJ [34] or activation of the slower, error-prone PARP-1-mediated alternative-NHEJ pathway [35].

The conclusions drawn from our CO-FISH analysis of tankyrase 1 depleted cells are suggestive of impaired NHEJ machinery. To investigate this possibility, we targeted the core component of DNA-PK-mediated NHEJ, the DNA-dependent Protein Kinase

catalytic subunit (DNA-PKcs). To achieve this, we blocked DNA-PKcs kinase function using the potent inhibitor Nu7026 (Figure 5, DNA-PKcs I [28]). In support of our speculation regarding DNA-PK-mediated NHEJ deficiency as a possible mechanism for the production of terminal deletions, Nu7026 inhibition coupled with 1 Gy γ-ray or 1 GeV/n ^{56}Fe ion irradiation produced chromosome-type terminal deletions; these frequencies did not significantly differ from those observed during tankyrase 1 knockdown conditions ($p>0.76$) (Figure 5 [25]). Further, the combination of Nu7026 and tankyrase 1 knockdown did not produce terminal deletion frequencies that significantly differed from tankyrase 1 knockdown alone ($p >0.18$) (Figure 5 [28]).

Cytogenetic analysis revealed tankyrase 1 knockdown is coupled with additional chromosome-type aberrations that are generally the result of telomere uncapping, telomere-DSB fusions. To date, tankyrase 1 is not known to have a role in telomere capping during G1 of the cell cycle. CO-FISH analysis determined there was no significant change in telomere-DSB fusions with tankyrase 1 knockdown alone. However, γ-ray irradiated cells revealed a 3-fold increase in telomere-DSB fusions over mock transfected and unirradiated controls; whereas HZE 1 GeV/n ^{56}Fe irradiation increased the frequency of telomere-DSB fusions 2 fold ($p = .023$ and $p = 0.14$ respectively) (Figure 6 [25]). Consistent with the terminal deletion data, the observed instability phenotypes are not typical of inadequate tankyrase 1 function at the telomere. Interestingly, both uncapped telomeres and chromosome-based aberrations resulting from ineffective NHEJ are each hallmarks of DNA-PKcs dysfunction [34, 36-40]. Supporting this supposition, treatment with the DNA-PK inhibitor NU7026 (DNA-PKcs I), resulted in telomere-DSB fusion frequencies that parallel those observed in tankyrase 1

knockdown, irradiated cells ($p = 0.023$). The combination of tankyrase 1 knockdown and Nu7026 inhibition yielded telomere-DSB fusion frequencies similar to those seen in the Nu7026 treatment alone ($p < 0.023$) (Figure 6 [25]).

The connection between tankyrase 1 deficiency and decreased cell survival, increased mutagenesis, combined with the cytogenetically visible phenotypes that resemble DNA-PKcs-deficiencies provides sufficient evidence to further investigate the tie between tankyrase 1 deficiency and mis-regulated end-joining DNA-repair phenotypes. To achieve this goal, we ask whether MFs under conditions in which DNA-PKcs is depleted or inhibited correlates with the MF during tankyrase 1 knockdown. Consistent with our prior studies, siRNA-mediated DNA-PKcs knockdown and DNA-PKcs inhibition (via Nu7026) resulted in MFs that positively correlate with those observed in tankyrase 1 knockdown (Figure 7 [28]). Having consistently established a positive correlation between tankyrase 1 deficiency and DNA-PKcs depletion across multiple cell lines and multiple genomic instability end-points, we speculate tankyrase 1 was not directly inducing genomic instability. Rather, tankyrase 1 may be indirectly involved in DNA-repair as a regulator of the key end-joining protein DNA-PKcs.

3.2.3 DNA-PKcs stability requires tankyrase 1

Cytogenetic evidence that both DNA-repair and telomere end-capping deficiencies are consequences of tankyrase 1 knockdown, suggests a role for tankyrase 1 in DNA-repair. We postulate tankyrase 1 is not directly involved in DNA-repair but rather, regulates the stability of the NHEJ proteins, perhaps DNA-PKcs.

Tankyrase 1 depletion is coupled with DNA-PKcs protein loss

To provide evidence for this supposition, we determined the relative level of DNA-PK holoenzyme proteins Ku80 and DNA-PKcs at 12, 24 and 48 hours time points following tankyrase 1 knockdown (Figure 8 [28]). Western blot analysis and quantification verified knockdown of the tankyrase 1 protein to < 1% of the mock transfected control (Figure 8 [28]). Interestingly, DNA-PKcs was reduced to < 10% relative to the mock treatment 12 hours following transfection with tankyrase 1 siRNA. Further, the time course of tankyrase 1 protein depletion was coupled with the significant reduction of the DNA-PKcs protein (Figure 8 [28]). Illustrating the intimate relationship, tankyrase 1 protein recovery was accompanied by the rapid rebound of DNA-PKcs protein levels (data not shown).

Reciprocal knockdown has no effect on tankyrase 1

In the interest of determining dynamics of the tankyrase 1-DNA-PKcs protein relationship, we preformed the reciprocal knockdown to determine if tankyrase 1 protein stability requires the DNA-PKcs protein. Supporting prior reports [41], DNA-PKcs siRNA-mediated knockdown does not result in a reduction of DNA-PKcs protein levels until approximately 48 hours (with maximal effect at 72 hours) post siRNA induction (Figure 9 [41]). This finding in itself suggests tankyrase 1 is playing a regulatory role in DNA-PKcs protein stability; tankyrase 1 knockdown depletes DNA-PKcs protein levels several fold faster than DNA-PKcs targeted siRNA knockdown. Furthermore, over the course of 122 hours following DNA-PKcs siRNA transfection, no negative impact on tankyrase 1 protein levels was observed (Figure 9 [28]). Therefore, we conclude that tankyrase 1 regulates DNA-PKcs protein stability by an unknown mechanism.

DNA-PKcs related PI3-kinase superfamily members are not impacted by tankyrase 1 depletion

Destabilization of phosphatidylinositol 3-kinase-related protein (PIKK) has been shown to negatively impact the level of related proteins on a transcriptional level [41]. A prime example is the relationship between the PIKK DNA-PKcs and ataxia telangiectasia mutated (ATM). Here, siRNA knockdown of the DNA-PKcs protein was coupled with ATM protein depletion. Further investigation of this relationship demonstrated that siRNA-mediated knockdown of DNA-PKcs transcript and subsequent DNA-PKcs protein depletion was coupled with down-regulation of ATM gene transcription [41]. Hence, the level of the ATM protein was dependent upon the stability of the DNA-PKcs protein which appears to regulate the rate of ATM transcription [41]. Due to this relationship, we were obligated to investigate the relative mRNA levels of DNA-PKcs following tankyrase 1 siRNA-mediated knockdown. The relative levels of DNA-PKcs and tankyrase 1 mRNA were determined via qRT-PCR at various time courses following transfection of tankyrase 1 siRNA.

Concurring with our initial explanation of a posttranslational relationship, qRT-PCR revealed normal and in some cases, elevated levels of DNA-PKcs mRNA levels in response to tankyrase 1 knockdown (Figure 10 [28]). In addition, we used qRT-PCR analysis to validate the specificity of the tankyrase 1 siRNA construct by quantifying the relative mRNA levels of the closely related PARP, tankyrase 2; no significant reduction of tankyrase 2 mRNA was observed over 48 hours of tankyrase 1 knockdown (Figure 10 [28]). Supporting the exclusive nature of the tankyrase 1 and DNA-PKcs protein

relationship, ATM protein levels *were not* affected by tankyrase 1 knockdown and concurrent DNA-PKcs protein depletion over a 48 hour time period (Figure 11 [28]).

Cumulatively these results support a post-translational relationship between tankyrase 1 protein levels and DNA-PKcs protein stability. In an attempt to demonstrate a physical and perhaps stable protein-protein interaction, we performed a protein complex immunoprecipitation assay (Co-IP) against the DNA-PKcs protein. Pending a successful pull-down of the DNA-PKcs protein in complex with tankyrase 1, this method would provide definitive evidence to identify tankyrase 1 as a DNA-PKcs binding partner. However, multiple Co-IP attempts failed to demonstrate the existence of a stable, physical DNA-PKcs-tankyrase 1 protein complex, suggesting a lack of physical interaction between the two.

3.3.0 Discussion

3.3.1 Tankyrase 1 depletion increases T-SCE frequencies

TRF1 remains on telomeres & stalls replication

Elevated T-SCE frequencies are representative of telomere instability and premature aging phenotypes (reviewed in [27]). In our studies, tankyrase 1 knockdown in human cells was characterized by elevated T-SCE frequencies with no significant effect on the frequency of G-SCEs, indicating tankyrase 1 serves to regulate recombination specifically within telomeres but does not regulate global genomic recombination events between sister chromatids. Telomere recombination in response to tankyrase 1 depletion is likely a result of inappropriate regulation of TRF1 association with the telomere. TRF1 in complex with the telomere is required for telomere stability, length regulation and accurate formation of the shelterin complex [1, 42]. However, the

appropriate regulation of TRF1, including its release from the telomere, is required for appropriate function. We speculate that the inability to dissociate TRF1 from the telomere during S-phase in order to facilitate replication, leads to the stalling of the replication fork [6, 32]. We propose the telomere engages a recombination mechanism between sister chromatids to 'bypass' this obstacle, allowing for recombination-dependent advancement of the replication fork. Consistent with this model, we find these sister chromatid-recombination events occur exclusively within the telomere and not within genomic regions of the chromosome (G-SCEs).

Human lymphoblasts (WTK1) contain a heterogeneous thymidine kinase (TK) locus, allowing for a sensitive method for determining the mutation frequencies at the TK loci by the addition of aminopterin and trifluorothymidine to cell culture [43, 44]. Due to the fact that IR induced mutations are random and characterized by deletions (the product of two DSB events in close proximity), the probability of a mutation/deletion at any one location throughout the genome is equal. Therefore, determining the mutation frequency of a specific gene is *representative* of the expected mutation frequency throughout the genome. Carcinogenic potential increases with the accumulation of mutations in tumor suppressor genes and proto-oncogenes (reviewed in [45]), primary roles of which are to regulate the cell cycle and cellular proliferation. We find that deficiency of the telomeric PARP family member tankyrase 1 increases IR-induced mutation frequencies, suggesting that variant forms of tankyrase 1 experiencing a loss of function would pose an increased risk for carcinogenesis.

siRNA-mediated knockdown demonstrated that tankyrase 1 is required for accurate DNA-repair by DNA-PK-mediated NHEJ. Inhibitor studies using the PARP-

domain inhibitor 3AB mirrored the phenotypic consequences of tankyrase 1 siRNA knockdown, suggesting it is not simply the presence of tankyrase 1 that is necessary for genomic stability but rather, the requirement for tankyrase 1 catalytic activity specifically. Furthermore, we observed an increase in IR sensitivity and cell killing in cells experiencing tankyrase 1 knockdown. Taken together, our initial findings demonstrate that tankyrase 1 knockdown results in telomeres that are prone to hyper-recombination, as well as increased mutation frequencies genome-wide and IR sensitivity. These novel observations indicated that tankyrase 1 plays roles beyond the telomere, which appear to correlate with deficient DNA-repair phenotypes.

3.3.2 Tankyrase 1 depletion or inhibition results in DNA-repair deficient phenotypes

Complementing our assays that suggest tankyrase 1 is necessary for preserving genomic stability, cytogenetic analysis of Li Fraumeni and human fibroblasts (5C) via CO-FISH following 1 Gy γ-ray or HZE ^{56}Fe revealed two instability phenotypes: elevated terminal deletions and telomere-DSB fusions. Terminal deletions are the result of failed DNA-repair/end-joining and because the deletion is a chromosome type aberration, the break occurred in G1. Telomere-DSB fusions result from successful end-joining of a DSB, revealing that telomeres have become uncapped and are substrates for end-joining processes. Therefore, the telomere is recognized as a DSB and a substrate for end-joining. Having identified tankyrase 1 as a regulator of DNA-PKcs protein stability, we attributed the uncapped telomere phenotypes as a consequence of DNA-PKcs protein deficiency. Likewise, the increase in terminal deletion frequencies resulting from irradiation during tankyrase 1 knockdown (lacking in the mock transfected irradiated controls) is likely the consequence of DNA-PKcs protein depletion and the inability to

perform optimal end-joining mediated by DNA-PK. However, other methods of end-joining, including DNA-Ligase IV/XRCC4-mediated NHEJ and PARP-1-mediated 'alternative NHEJ' are independent of DNA-PKcs and are capable of DNA-repair, resulting in the fusions observed between telomere and DSB.

Additionally, we observed that the quality of IR, i.e., high verses low linear energy transfer (LET), following tankyrase 1 knockdown impacted the frequency of terminal deletions & telomere-DSB fusions per metaphase. Cells treated with high mass, charged, high energy (HZE) ^{56}Fe ions following tankyrase 1 knockdown increased terminal deletion frequencies well above that observed in cells treated with low LET γ-ray IR (Figure 5). Conversly, exposure to low-LET IR (γ-rays) following tankyrase 1 knockdown produced telomere-DSB fusions at a frequency noticeably higher than those observed in cells irradiated with HZE ^{56}Fe ions (Figure 6).

Though each IR type poses a DNA-repair challenge by the induction of DSBs, HZE IR specifically results in complex DNA-damage that requires longer periods of time for repair. In many instances, the damage imparted by HZE IR is irreparable and results in cell death. These characteristics of high-LET IR combined with the loss of DNA-PK-mediated end-joining capability (DNA-PKcs depletion during tankyrase 1 knockdown) provides a plausible explanation for the high frequency of deletions observed following HZE ^{56}Fe ion irradiation (during tankyrase 1 knockdown), as well as the less frequent telomere-DSB fusions seen in HZE ^{56}Fe ion irradiated cells compared to γ-ray irradiated cells (during tankyrase 1 knockdown).

Likewise, the reduced frequency of terminal deletions and increased telomere-DSB fusions in tankyrase 1 knockdown, γ-ray irradiated cells compared to HZE tankyrase 1 knockdown cells can be explained based on differences in radiation quality; i.e., low-LET γ-ray irradiation induces DSBs that are less complex in nature compared to those produced by HZE IR. Though quantitatively the number of DSBs created by each IR type is approximately equal, increased frequencies of telomere-DSBs observed in low-LET irradiated cells suggested increased end-joining repair capability compared to the HZE ^{56}Fe ion irradiated cells. We postulate this finding is due to the high degree of complexity of the damage induced by HZE IR, which resulted in reduced repair kinetics in tankyrase 1 knockdown, DNA-PKcs depleted cells. Therefore, low-LET samples contain telomere-fusions generated by end-joining DNA-repair at a higher frequency compared to HZE irradiated cells, with fewer unrepaired terminal deletions.

We found it intriguing that dysfunctional end-capping and end-joining phenotypes are hallmarks of classic DNA-PKcs deficiency. We suspected tankyrase 1 perhaps may have an indirect role in regulating protein components of a DNA-repair pathway, as tankyrase 1 is not known to interact directly with DNA, nor recognize any chemical signature of damage. Terminal deletions following IR (high- or low-LET) are chromosome-type aberrations produced during G1 phase of the cell cycle, where NHEJ is the primary DSB-break repair mechanism and DNA-PKcs is a critical component for accurate, efficient end-joining. Therefore, we reasoned that tankyrase 1 was not directly responsible for the DNA-repair deficiencies and telomere uncapping but rather, played a role in regulating a NHEJ-repair protein, possibly DNA-PKcs.

Supporting this supposition, we investigated of multiple genome instability end-points that positively correlated between tankyrase 1 & DNA-PKcs deficiencies. Cells treated with the DNA-PKcs inhibitor Nu7026 resulted in outcomes similar to those observed in the tankyrase 1 knockdown conditions for cell survival (IR sensitivity), mutation frequencies, telomere deletions and telomere-DSB fusions end-points. Combination of tankyrase 1 knockdown and Nu7026 DNA-PKcs inhibition resulted in a cumulative effect, further elevating the frequencies of instability end-points such as DSB-telomere fusions and terminal deletions following high- & low-LET irradiation types. This observation supports the idea that DNA-PKcs loss following tankyrase 1 knockdown does not result in absolute abolishment of the protein, and so DNA-PKcs levels are not completely null. Likewise, Nu7026 is not capable of 100% inhibition of DNA-PKcs activity (inhibitor studies are accepted to be effective to a threshold 90%). Therefore, it is not surprising that the combination of the two treatments amplified the frequencies of observed DNA-PKcs deficient phenotypes.

The consistent positive correlation between tankyrase 1 knockdown and Nu7026 end-points was critical in furthering our supposition that in each case, the end-points were not arising through independent mechanisms. Rather, we suspect that tankyrase 1 plays a regulatory role for DNA-PKcs that when disrupted, results in the rapid loss of DNA-PKcs, which would be expected to have phenotypes mirroring DNA-PKcs kinase inhibition.

3.3.3 Tankyrase 1 protein regulates DNA-PKcs protein stability

DNA-PK Ku heterodimer is not impacted by tankyrase 1 knockdown

To validate our speculation for an indirect role of tankyrase 1 in regulating DNA-repair by regulating the stability of NHEJ protein components, specifically DNA-PKcs, we investigated DNA-PKcs protein levels at multiple time points following tankyrase 1 siRNA-mediated knockdown. We observed a *significant* reduction in DNA-PKcs protein levels 12 hours post-tankyrase 1 siRNA transfection. However, the Ku80 component of the DNA-PK holoenzyme remained unaffected by tankyrase-1 knockdown. These results indicate that the role of tankyrase 1 in the regulation of NHEJ components is specific for the stability of DNA-PKcs. Thus, we validated our postulation that the genomic instability phenotypes observed as a result of tankyrase 1 protein depletion are the consequence of DNA-PKcs protein destabilization and loss.

DNA-PKcs protein depletion does not impact tankyrase 1 protein stability

Our investigation of the tankyrase 1-DNA-PKcs protein relationship repeatedly revealed that tankyrase 1-targeted siRNA transfection resulted in the significant and rapid reduction of DNA-PKcs protein levels as early as 12 hours after siRNA transfection. Of relevance were studies that reported siRNA-mediated knockdown of DNA-PKcs requires approximately 48 hours post siRNA transfection before significant reduction in the DNA-PKcs protein becomes evident [41]. Taken together, these studies suggest DNA-PKcs protein is in a stable, protected conformation in the presence of tankyrase 1 and hence, the extended time required for DNA-PKcs targeted siRNA to reduce DNA-PKcs protein levels. Complementing this important finding, tankyrase 1 siRNA knockdown is coupled with the immediate reduction of DNA-PKcs with little, if any 'lag' time; indicating

DNA-PKcs protein stability is dependent upon the presence of the tankyrase 1 protein. These observations suggest that tankyrase 1 plays a role in regulation of DNA-PKcs protein stability. In support of this notion, the reciprocal knockdown of DNA-PKcs does not reduce the levels of tankyrase 1, indicating there is no co-dependency in this model; tankyrase 1 is the 'master regulator' in this protein-protein relationship and tankyrase 1 does not rely on DNA-PKcs protein for its own stability.

Tankyrase 1 is required for DNA-PKcs protein stability on a posttranslational level

Following tankyrase 1 siRNA knockdown, analysis of tankyrase 1, tankyrase 2 and DNA-PKcs mRNA levels were determined via quantitative Real-Time PCR. Analysis revealed that only tankyrase 1 mRNA levels were depleted, indicating the efficiency and specificity of the siRNA construct to the respective target. These results provide strong evidence that tankyrase 1, not the combined actions of the closely related tankyrases (1 & 2), is responsible for DNA-PKcs protein stability. Furthermore, qRT-PCR analysis demonstrates the depletion of the DNA-PKcs protein is not the result of an off target effect of the tankyrase 1 siRNA construct, nor is DNA-PKcs transcription impacted by the loss of the tankyrase 1 protein. These findings are essential in supporting the notion that the DNA-PKcs-tankyrase 1 relationship is on the posttranslational level.

3.3.4 The role of tankyrase 1 in DNA-PKcs stability is not the result of a stable protein complex.

Multiple attempts at protein complex immunoprecipitation (Co-IP) of DNA-PKcs to identify tankyrase 1 as an interacting protein failed to produce evidence of a stable physical interaction between the two proteins. The implication of this finding was that

the nature of this particular protein-protein relationship is not characterized by a persistent interaction. Rather, the physical interaction between DNA-PKcs & tankyrase 1 is likely transient in nature. From a biochemical perspective, transient interactions are often used to facilitate posttranslational modification of a specific target protein to induce a change in protein function and/or conformation; e.g. phosphorylation. In this case, it would be logical to speculate that the DNA-PKcs protein is dependent on tankyrase 1-specific pADPr modification. Thus, the 'failure' of the Co-IP is informative in determining the direction of additional studies investigating the relationship between our proteins of interest, as it is likely transient and serves a functional purpose in DNA-PKcs protein stabilization.

3.4.0 Materials and Methods

Cell lines

Characterization of telomerase activity during spontaneous immortalization of Li-Fraumeni syndrome skin fibroblasts [MDAH087 (087) telomerase negative (ALT) and MDAH041 (041) telomerase positive] has been described previously [46]. The mutant p53 status of these cell lines favored evaluation of telomere dysfunction and MDAH087 provided an ALT background. Telomerase negative (not ALT), normal neonatal 5C human dermal fibroblasts (HDFn; Cascade Biologics) were used at low passage, maintained in α-MEM medium (Hyclone) supplemented with 10% fetal calf serum (Sigma Aldrich) and 1% pen-strep (Hyclone), and incubated at 37^{o}C in an atmosphere of 95% air and 5% carbon dioxide. A telomerase positive background was evaluated in the hTERT-immortalized human foreskin fibroblast cell line BJ-5ta (ATCC), which was sustained similarly [25].

WTK1 human lymphoblastoid cells have a stable karyotype (47, X, Y 13+, 14q+) and were derived from the WI-L2 line [59]. WTK1 cells were used for mutation analyses as they are heterozygous at the thymidine kinase locus; they also have a single amino acid substitution in codon 237 at TP53. WTK1 cells were maintained in RPMI1640 medium (Hyclone) supplemented with 10% heat-inactivated horse serum (Sigma-Aldrich) and 1% pen-strep (Hyclone).

Tankyrase 1 siRNA knockdown

The following siRNA sequences were used for the targeted silencing of tankyrase 1 (Dharmacon Research) and DNA-PKcs (Qiagen): tankyrase 1 siRNA1: 5' AGG AAG GAG ACA CAG AUA UdTdT 3'; tankyrase 1 siRNA2: 5' CCU GGA AGU AGC UGA AUA UdTdT 3'; DNA-PKcs siRNA: 5'-GAUCGCACCUUACUCUGUUdTdT-3'. WTK1 lymphoblasts were seeded in RPMI 1640 medium with 5% horse serum (no antibiotics), at a concentration of $5x10^5$ cell/ml, 20 hr prior to transfection. The 5C human dermal fibroblasts were seeded at 50-60% confluency in α-MEM medium with 10% fetal bovine serum (no antibiotics), one day prior to transfection. Cells were transfected with tankyrase 1 or DNA-PKcs siRNA (20nM) using Lipofectamine 2000 and OptiMEM (Invitrogen) serum free media; in some cases, a second transfection was done 24 hr later to maintain knockdown. The mock sample included in every experiment, contained only Lipofectamine 2000 with OptiMEM and no siRNA. Cells were harvested at various times post siRNA transfection and processed for Western blot analysis, or used in experiments to assess radiation-induced effects [25].

Western blot analysis

Western blot analysis was always performed to confirm successful knockdown of target protein level before proceeding with evaluation of endpoints (representative blots shown in S1). Cells were harvested, centrifuged and resuspended in cold PBS (without Mg+ Ca+) twice, then immersed in 1x RIPA buffer (1x TBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide) and protease inhibitor cocktail (Santa Cruz Biotechnology), incubated on ice for 5-10 min, then passed through a 25 gauge syringe needle and centrifuged for 10 min at 140,000x g at 4°C. Protein in the supernatant was quantified using a BSA protein assay. Thirty five to 50μg of the supernatant proteins were fractionated by SDS-PAGE (Bio-Rad) and transferred to Immobilon-FL PVDF membranes (Millipore). Blots were blocked in 5% skim milk or 5% BSA in TBS containing 0.1% Tween 20 and incubated overnight at 4°C with the following primary antibodies: rabbit polyclonal anti-tankyrase 1 (200 μg/ml; Santa Cruz Biotechnology); mouse monoclonal anti-actin (200 μg/ml; Santa Cruz Biotechnology); mouse monoclonal anti-PKcs Ab-4 (200 μg/ml; Neomarker); rabbit polyclonal anti-ATM (1mg/ml; Abcam). The blots were washed three times with TBS containing 0.1% Tween 20 and incubated with secondary antibody 680IRDye-conjugated goat polyclonal anti-rabbit IgG or IRDye 800CW-conjugated goat polyclonal anti-mouse IgG (1:15,000; LI-COR Biosciences). Bound antibodies were detected and using an Odyssey fluorescent imaging system (LI-COR Biosciences); blots were quantified according to manufacturers instructions and normalized to independent actin loading controls [25].

Quantification of some blots was accomplished by importing images into Photoshop CS3 and analyzing as per a protocol adapted from the National Institutes of

Health (http:/rsb.info.nih.gov/ij/index.html). Analysis involved first, multiplying the mean measured value by the number of pixels to obtain an "absolute intensity" value, an integrated measure of intensity and size of bands. Next, the relative intensity for each sample band was calculated by dividing the absolute intensity of each band by the absolute intensity of the standard (the mock transfection sample) [25].

Chemical inhibition

Nu7026 (Sigma-Aldrich), a competitive and highly selective inhibitor of DNA-PKcs kinase activity, was added to WTK1 cultures after siRNA transfection at a final concentration of 9 □M [47, 48], and remained on samples until collected for mutagenesis or cytogenetic analyses. We have consistently found that this concentration of Nu7026 yields similar results for these end points as does siRNA knockdown of DNA-PKcs.

3-aminobenzamide (3-AB; Sigma-Aldrich) was used to inhibit global PARP activity at final concentrations ranging from 10 and 100 μM, to 10 and 20 mM. 3-AB was added to WTK1 cultures 24 hr prior to irradiation (or sham), which were then collected for mutation or western blot analyses [25].

Co-immunoprecipitation (Co-IP)

A Thermo Scientific Pierce Co-IP kit was used according to manufactures instructions to isolate native protein complexes from cell lysates by directly immobilizing purified antibody onto an agarose support. The following primary antibodies were used; rabbit polyclonal anti-tankyrase 1 (Santa Cruz Biotechnology) and mouse monoclonal anti-PKcs Ab-1 (Neomarker/Thermo Scientific) [25].

Irradiations

WTK1 lymphoblasts or 5C dermal fibroblasts were exposed to various doses of ^{137}Cs γ-rays in a Mark I irradiator (J.L. Shepherd) located at Colorado State University, or to 1 GeV/n ^{56}Fe (high Z high energy; HZE) particles at the NASA Space Radiation Laboratory at Brookhaven National Laboratory (NSRL/BNL) [25].

Mutation assay

WTK1 lymphoblasts were treated with CHAT (10^{-5} M 2'-deoxycytidine, 2 x 10^{-4} M hypoxanthine, 2 x 10^{-7} M aminopterin, 1.75 x 10^{-5} M thymidine; Sigma) for two days and CHT (CHAT without aminopterin) for one day to eliminate pre-existing TK$^-$ mutants. Following CHAT treatment, cells were transfected with tankyrase 1 siRNA and/or treated with Nu7026 or 3-AB. Three days later, cells were irradiated with γ-rays or HZE particles. Two days after irradiation, when phenotypic expression of newly induced mutants was complete, the mutant fractions (MF) were determined by plating in 96 well dishes. For plating efficiency, one cell/well was seeded, or for scoring mutants, 2000 cells/well were seeded in the presence of 2μg/ml trifluorothymidine (TFT; Sigma-Aldrich). Fresh TFT was added 11 days after plating, and plates were scored for positive or negative wells after 20 days. The MFs were calculated using the Poisson distribution [49] and statistical analyses were done by t-tests using Sigma Stat 3.5 (Systat Software) [25].

Surviving fraction assay

Two hours before exposure, exponentially growing cells were seeded into 60 mm dishes at various densities depending on the radiation dose to be delivered. After irradiation, plates were incubated at 37°C for 14-20 days in normal growth medium to

allow for colony formation. Plates were rinsed, fixed with methanol, and stained with methylene blue. Colonies with >50 cells were counted and absolute plating efficiencies calculated for each dose. Surviving fractions represent the plating efficiency for the treated culture divided by the untreated control [25].

Cytogenetic analyses

Chromosome-Orientation Fluorescence in situ hybridization (CO-FISH) was performed as previously described [26, 50] with some modification. Following irradiation, cell cultures were incubated for various times, trypsinized and sub-cultured into medium containing the thymidine analog 5-bromo-2-deoxyuridine (BrdU, 10μM; Sigma-Aldrich) for one cell cycle. Slides were air dried and stained with Hoechst 33258 (0.50ng/μl; Sigma-Aldrich) for 15 minutes and exposed to 365 nm UV light (Stratalinker 2400) for 25 minutes. Following UV exposure, BrdU incorporated strands were digested with Exonuclease III (3U/μl in provided reaction buffer; Promega) at room temperature for 10 minutes. A Cy-3 conjuated $(TTAGGG)_3$ PNA telomere probe (0.2μg/ml; Applied Biosystems) was hybridized at 37˚C for 1.5 hr. Slides were rinsed in 70% formamide at 32˚C for 10 min and dehydrated in another ethanol series before re-probing at 37˚C for two hr. Following the second hybridization, slides were rinsed with 70% formamide at 32˚C for 15 min followed by 5 min rinse in PN Buffer. Chromosomes were counterstained with DAPI (4,6-Diamidine-2-phenylindole dihydrochloride; Vectashield, Vector Laboratories). Preparations were examined and images captured and analyzed using a Zeiss Axioskop2 Plus microscope equipped with a Photometrics Coolsnap ES2 camera and Metavue 7.1 software [25].

Scoring Criteria

T-SCE were scored as a CO-FISH telomere signal split between the two chromatids of a metaphase chromosome, which were often of unequal intensity due to unequal SCE [51]. G-SCE were scored on cells that had progressed through two rounds of replication in the presence of the BrdU; characteristic FPG harlequin staining was visualized using a mouse monoclonal anti-BrdU conjugated to Alexafluor 488 (FITC; Invitrogen) after CO-FISH treatment [25].

Telomere fusion necessitates that telomeres of adjoining chromosomes/chromatids fuse into a single CO-FISH signal and the DAPI signal remain continuous [52]. Telomere-DSB fusion appears as single-sided (on only one chromatid of a mitotic chromosome) interstitial blocks of CO-FISH telomere signal [34, 36]. Chromosome aberration frequencies (dicentrics, rings, terminal deletions, etc) were scored according to standard and accepted practice. Statistical analyses by Chi-square or Fisher's exact test (Sigma Stat 3.5; Systat Software) was done to determine significance [25].

Quantitative Real-Time PCR (RT-PCR)

Alpha-MEM media (no antibiotics; Hyclone) was added to 5C human dermal fibroblasts (~50% confluent) 24 hrs prior to transfection of tankyrase 1 siRNA1 with Lipofectamine RNAiMAX Reagent (Invitrogen). Following transfection, □-MEM (no FBS, no antibiotic) was added to the flasks. Cells were harvested at 4, 8, 12, 18, 24 and 48 hours post transfection, and RNA was extracted using the RNeasy kit (Qiagen) with the optional on-column DNase treatment (Qiagen). RNA was subjected to electrophoresis to affirm integrity and assure no genomic DNA contamination. A mock transfection (lipofectamine, no siRNA) was done for each time point [25].

Quantitative RT-PCR analysis was used to evaluate mRNA transcript levels of tankyrase 1, tankyrase 2, and DNA-PKcs, relative to the housekeeping gene transferrin receptor C (TFRC). Total RNA extracted for each time point was used for reverse transcription reactions using the Verso cDNA kit (Abgene). The RT-PCR was performed using ABsolute SYBR Green Fluorescein mix (Abgene) with a total cDNA concentration of 54ng/reaction. The primers used to detect specific gene transcripts were as follows:

tankyrase 1 forward, 5'-TTGCTCTTTCCAACACAAGC-3';

tankyrase 1 reverse, 5'-TACAGAACCACACGCTCCTC-3';

tankyrase 2 forward, 5'-TCTTCAGGTCCATCTAGCCC-3';

tankyrase 2 reverse, 5'-AAGCACCCTCTGTTCCACTT-3';

DNA-PKcs forward, 5'-AGCAAATGCACCGTTGTGGT-3';

DNA-PKcs reverse, 5'-TCCTTCTTCAGGAGCTTCCA-3';

TFRC forward, 5'-CGCTGGTCAGTTCGTGATTA-3';

TFRC reverse, 5'-GCATTCCCGAAATCTGTTGT-3'.

Each sample was analyzed in triplicate for each transcript evaluated. Relative transcript analyses were done using the delta-delta Ct method where expression is determined relative to the controls at each time point [53]. Three independent RT-PCR runs were evaluated for statistical significance via the SAS System MEANS Procedure to generate means, standard deviations and standard error of the means for comparisons of each gene at each time point. Statistical analyses were performed using GraphPad Prism software. Figures containing three or more means were analyzed using ANOVA. When means differed significantly ($p<0.05$), Tukey's *post hoc* test was employed [25].

LIST OF FIGURES

3.5.0 Figures

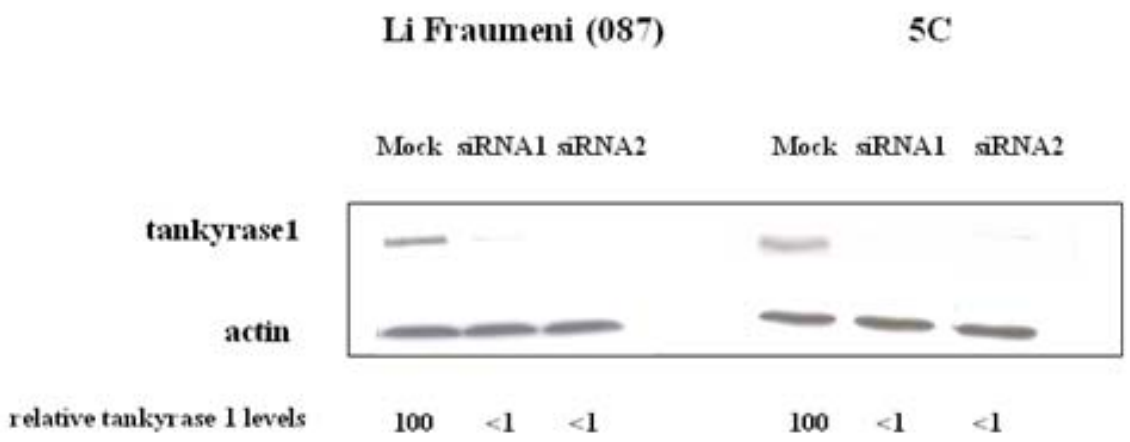


Figure 1. Effective knockdown of tankyrase 1 protein levels with two different siRNAs. Western blot analyses of tankyrase 1 in Li-Fraumeni 087 (ALT) and 5C primary human fibroblasts (telomerase negative) 18 hr after siRNA transfection. Upper bands probed with tankyrase 1 antibody; lower bands with β-actin. Percentages of protein remaining are shown below; all values were normalized to β-actin and the mock transfection.

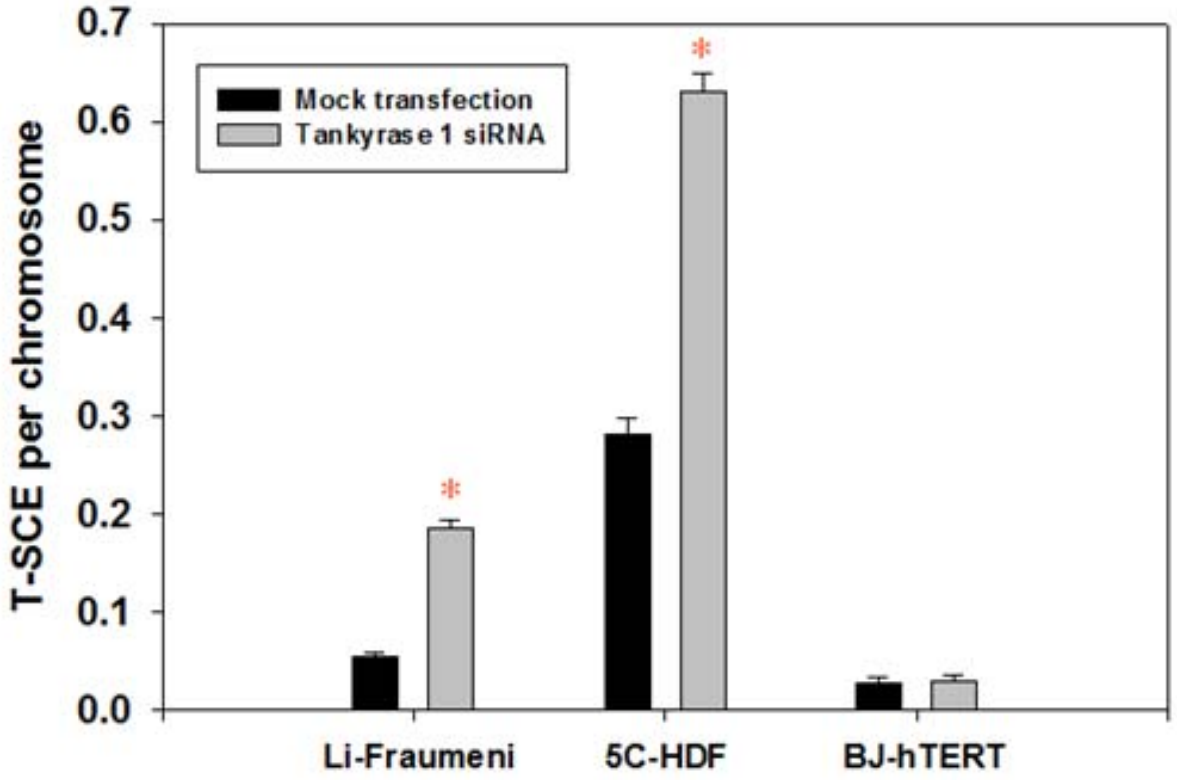


Figure 2. T-SCE frequencies are significantly elevated upon depletion of tankyrase 1 in telomerase negative backgrounds. T-SCE/chromosome levels were determined in three human fibroblast cell lines with various telomerase statuses; Li-Fraumeni 087 (ALT), 5C-normal human dermal (telomerase negative) and BJ-hTERT (5ta; telomerase positive). (*) is $p<0.05$.

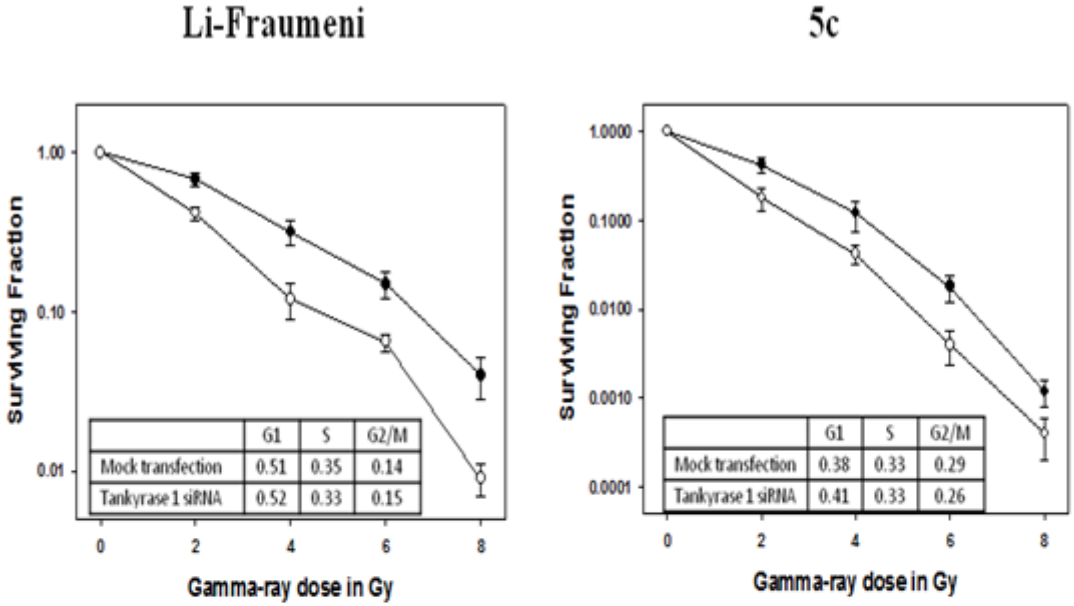


Figure 3. Increased radiation-induced cell killing (reduced survival) with reduced levels of tankyrase 1. Li-Fraumeni and 5C fibroblasts were treated with tankyrase 1 siRNA, then were exposed to γ-rays and the surviving fractions determined by clonogenic assay. Points are averages of three experiments; error bars are standard deviations. Mock transfection (•), tankyrase 1 siRNA transfection (o). Cell cycle distributions were assessed by flow cytometry and found to be unaffected by tankyrase 1 depletion. Cell cycle distributions were determined as follows: eighteen hours after transfection with tankyrase 1 siRNA, or mock-transfection with Lipofectamine 2000 reagent only, cells were trypsinized and resuspended in two ml cold PBS. Two ml cold 100% ethanol was added dropwise while vortexing the cells vigorously. Three ml cold 100% ethanol was added to bring the final ethanol concentration to 70%. Fixed samples were refrigerated for a minimum of 20 minutes before staining. Ethanol was aspirated, and cell pellets resuspended in 1 ml of propidium iodide (PI, 50ug/ml in PBS; Invitrogen) with RNAse (40 KU/ml; Sigma-Aldrich) added. All cell samples were analyzed with the EPICS IV Flow Cytometer (DakoCytomation, Inc., Fort Collins, CO) using a 488 nm laser.

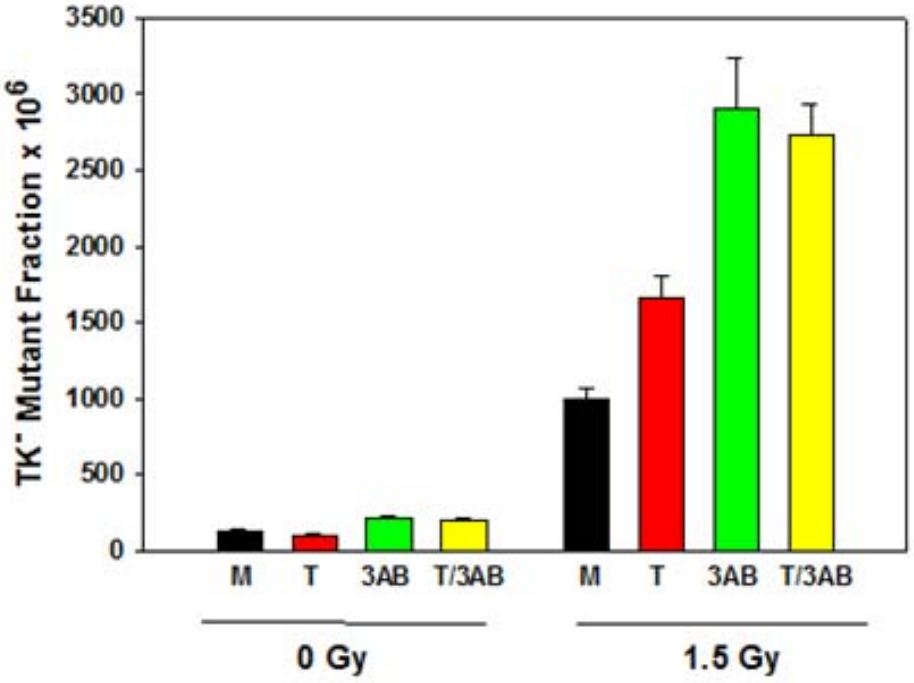


Figure 4. Increased γ-ray mutagenicity in WTK1 lymphoblasts upon tankyrase 1 siRNA depletion and/or inhibition of PARP with 3-AB. Cells were treated (representative knockdown, Figure S3), then irradiated the next day. Data are the average of three independent determinations; error bars are standard deviations. (M) mock transfection, (T) tankyrase 1 siRNA, (3-AB) inhibitor, and (T/3-AB) two combined.

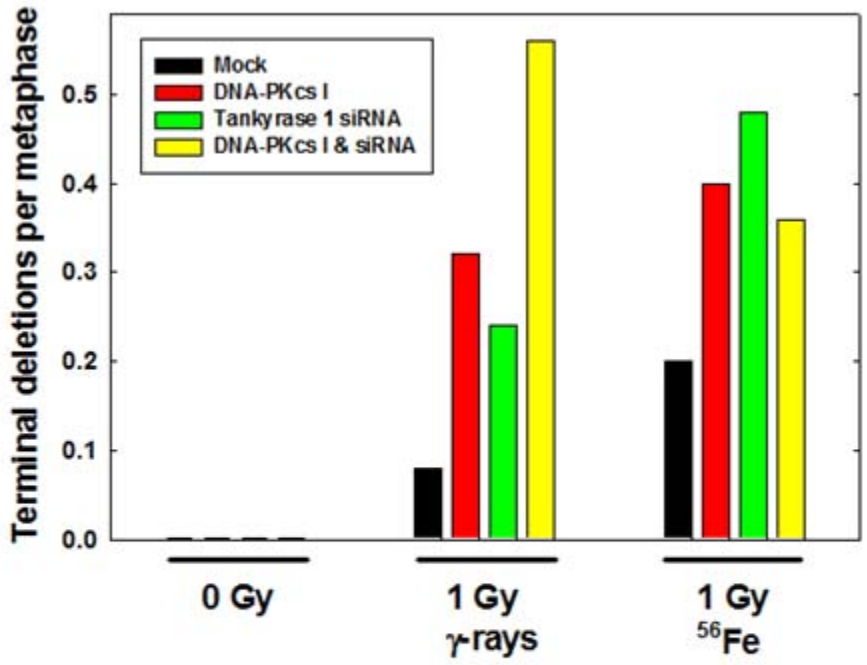


Figure 5. IR-induced chromosomal terminal deletions are increased with tankyrase 1 siRNA knockdown. WTK1 lymphoblasts were treated on successive days with tankyrase 1 siRNA, or with the DNA-PKcs inhibitor (I), or with both, and irradiated (γ-rays or 1GeV 56Fe) 48 hr after the second transfection. Frequencies of terminal deletion, hallmarks of defective NHEJ, following IR exposure were elevated with either DNA-PKcs inhibition or depletion of tankyrase 1.

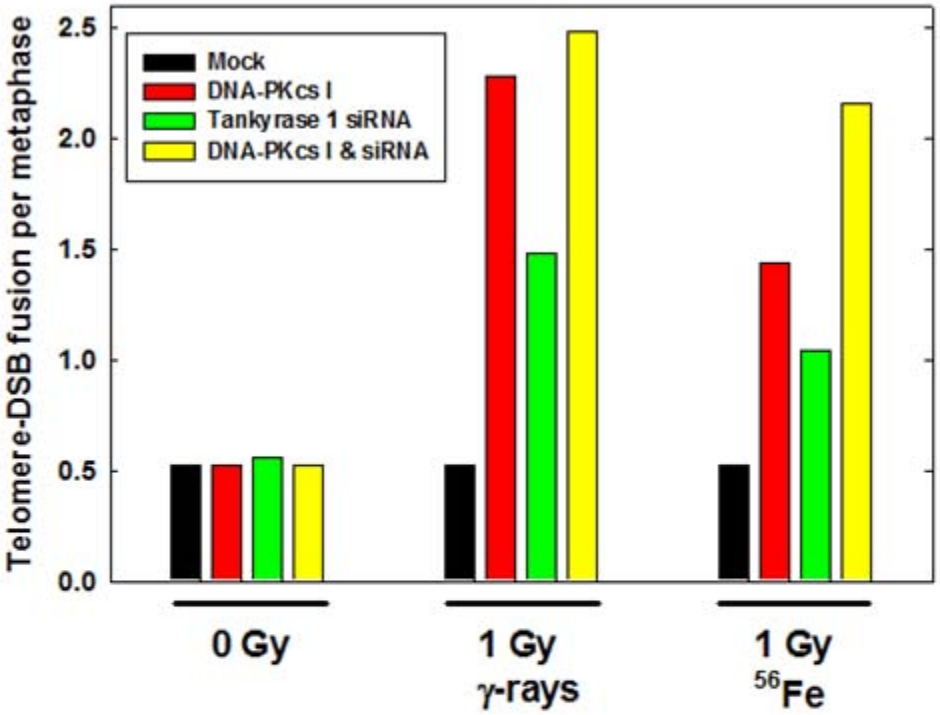


Figure 6. IR-induced telomere-DSB fusions are increased with tankyrase 1 siRNA knockdown. WTK1 lymphoblasts were treated on successive days with tankyrase 1 siRNA, or with the DNA-PKcs inhibitor (I), or with both, and irradiated (γ-rays or 1GeV 56Fe) 48 hr after the second transfection. Frequencies of IR-induced telomere-DSB fusions, events characteristic of telomere uncapping, were elevate with inhibition of DNA-PKcs, and also with depletion of tankyrase 1. These data represent single experiments.

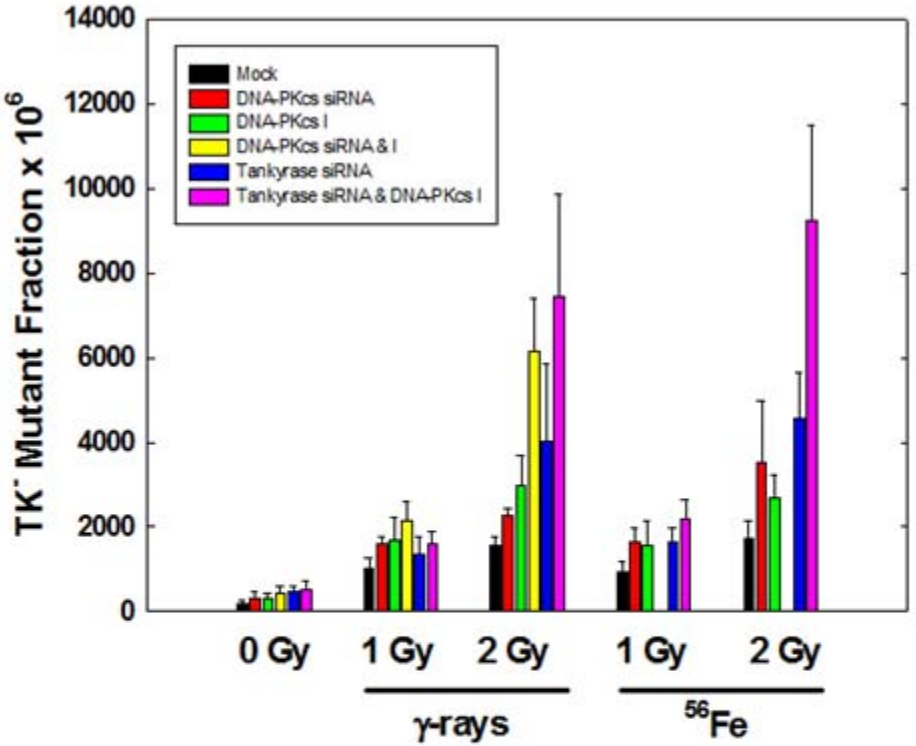
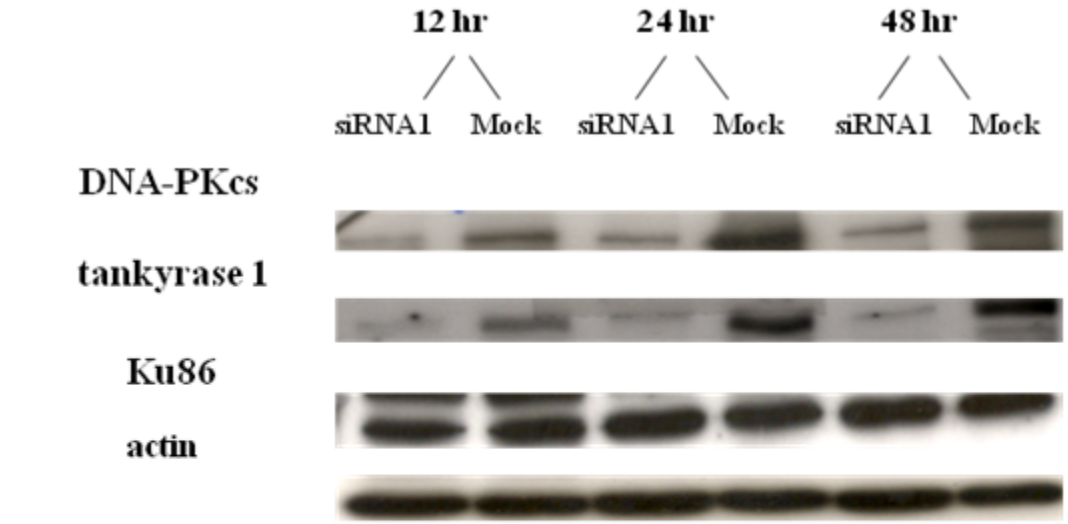


Figure 7. Spontaneous and IR-induced mutagenesis after depletion or inhibition of DNA-PKcs and/or tankyrase 1. WTK1 lymphoblasts were mock transfected (M) or treated with DNA-PKcs siRNA (P_S), DNA-PKcs inhibitor Nu7026 (P_N), tankyrase 1 siRNA (T), or combinations thereof i.e., DNA-PKcs siRNA plus DNA-PKcs inhibitor (P_S/P_N), or tankyrase 1 siRNA plus DNA-PKcs inhibitor (T/P_N). Cells were irradiated with γ-rays or ^{56}Fe ions and the MFs determined three days later. Data are means of at least three independent determinations, and error bars are standard deviations.



RE %				
	DNA-PKcs	8	11	14
	tankyrase 1	6	8	13
	Ku86	98	100	104

Figure 8. Tankyrase 1 depletion rapidly *reduces DNA-PKcs protein levels, while Ku86 levels remain* unchanged. Li-Fraumeni fibroblasts were transfected with tankyrase 1 siRNA or were mock transfected. Relative protein expression levels (RE %) of DNA-PKcs, tankyrase 1 and Ku86 were determined by Western blot 12, 24 or 48 hr after transfection. Percentages of protein remaining were normalized to β-actin and the mock transfection.

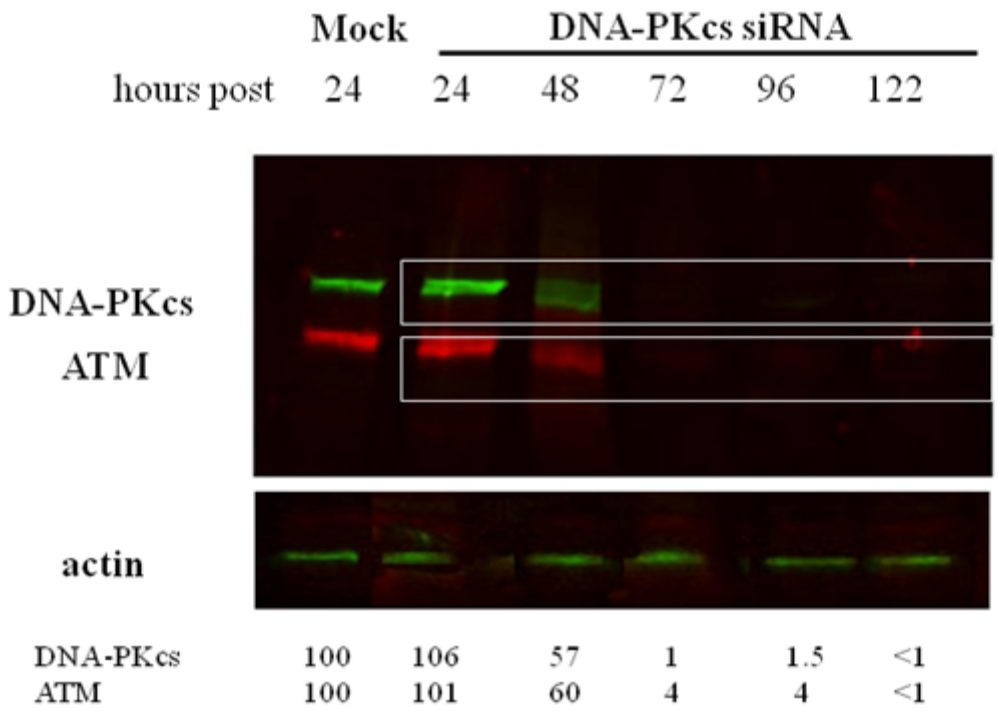


Figure 9. DNA-PKcs siRNA knockdown resulted in reduction of ATM protein levels. Samples from Figure S5 were also used to evaluate the levels of DNA-PKcs, ATM and β-actin by western blot at the indicated times after transfection. Percentages of protein remaining are shown below; all values were normalized to β-actin and the mock transfection.

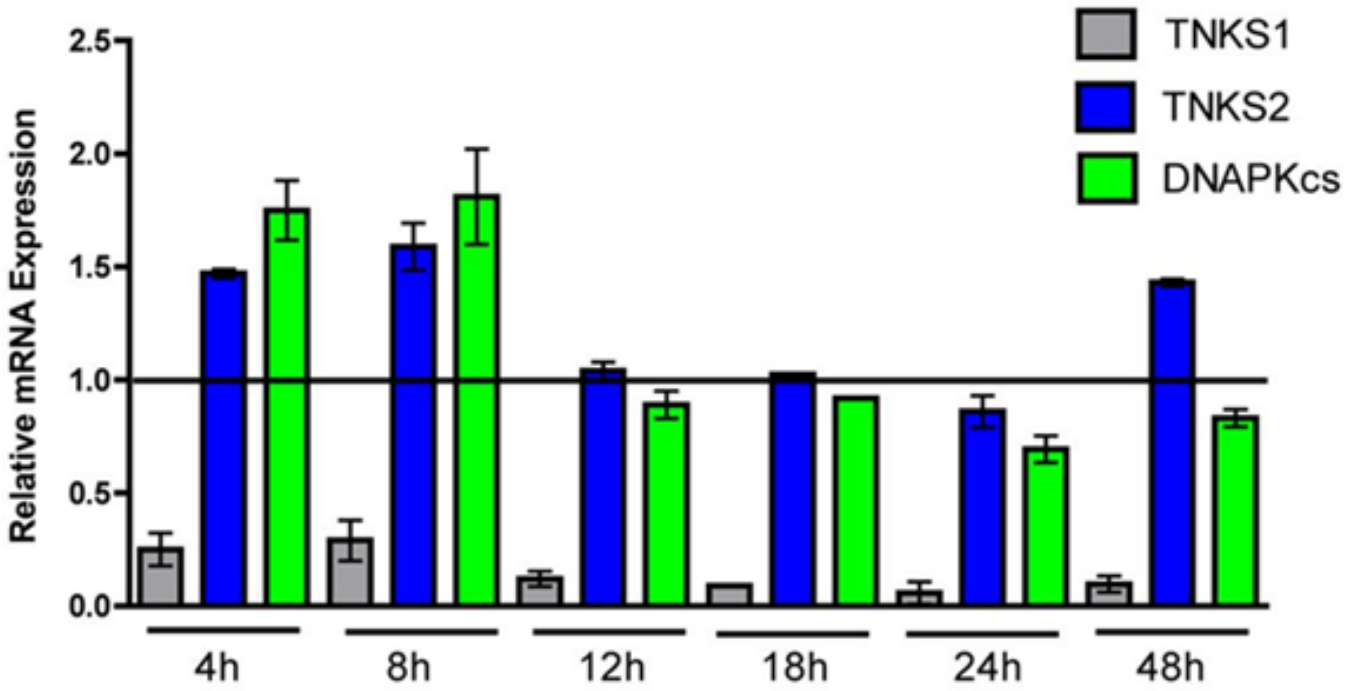


Figure 10. Time course of tankyrase 1 (TNKS1), tankyrase 2 (TNKS2) and DNA-PKcs relative mRNA expression following tankyrase 1 siRNA depletion. Quantitative RT-PCR of mRNA at 4, 8, 12, 18, 24 and 48 hr (compared to the relative 100% (1.0)) demonstrates dramatic reduction of tankyrase 1 mRNA (confirming efficiency of knockdown), as well as no significant reduction of tankyrase 2 (confirming specificity of knockdown) or DNA-PKcs (all $p > 0.05$).

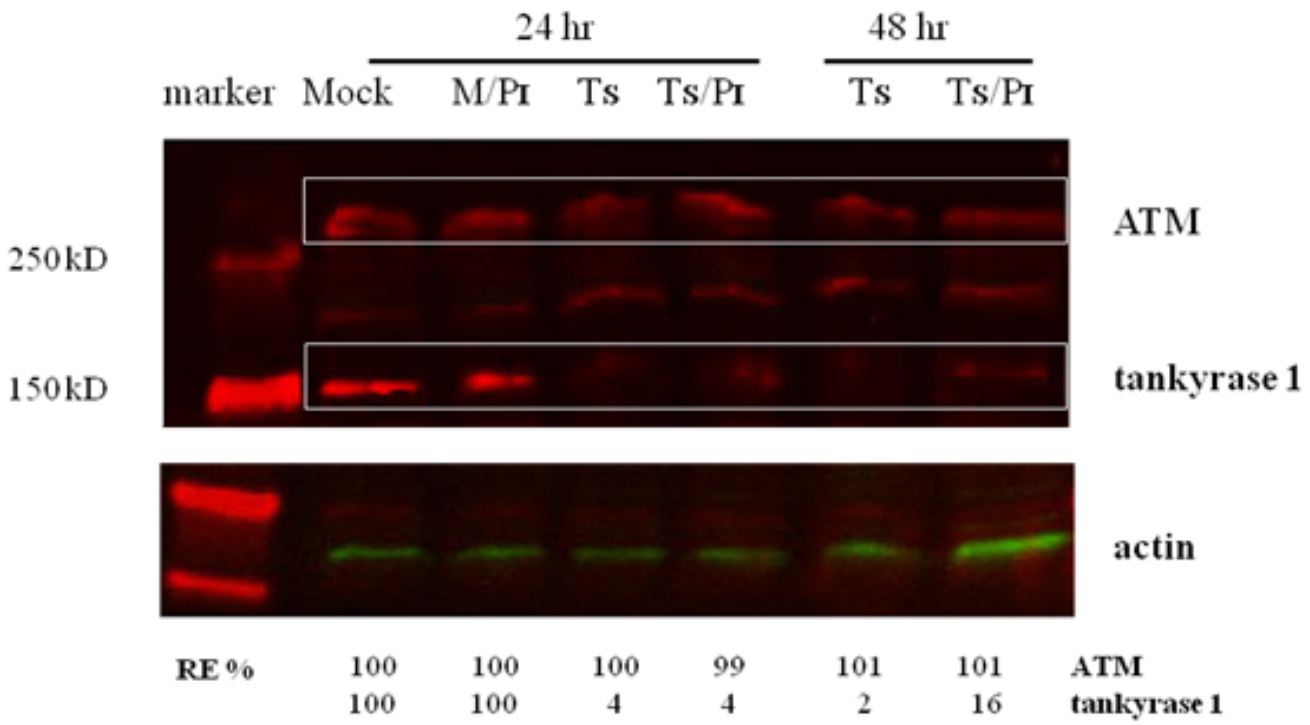


Figure 11. ATM protein levels are not affected by tankyrase 1 siRNA knockdown. WTK1 lymphoblasts were treated with tankyrase 1 siRNA (TS), and/or the DNA-PKcs inhibitor (PI), or the two combined (TS/PI). Western blot analysis for ATM, tankyrase 1, or β-actin at 24 and 48 hr post transfection is shown. Percentages of protein remaining (RE %) are shown below; all values were normalized to β-actin and the mock transfection.

REFERENCES

3.6.0 References

1. de Lange T: **Shelterin: the protein complex that shapes and safeguards human telomeres**. *Genes Dev* 2005, **19**(18):2100-2110.
2. Chen Y, Yang Y, van Overbeek M, Donigian JR, Baciu P, de Lange T, Lei M: **A shared docking motif in TRF1 and TRF2 used for differential recruitment of telomeric proteins**. *Science* 2008, **319**(5866):1092-1096.
3. Blackburn EH: **Telomere states and cell fates**. *Nature* 2000, **408**(6808):53-56.
4. Karlseder J, Broccoli D, Dai Y, Hardy S, de Lange T: **p53- and ATM-dependent apoptosis induced by telomeres lacking TRF2**. *Science* 1999, **283**(5406):1321-1325.
5. Smogorzewska A, van Steensel B, Bianchi A, Oelmann S, Schaefer MR, Schnapp G, de Lange T: **Control of human telomere length by TRF1 and TRF2**. *Mol Cell Biol* 2000, **20**(5):1659-1668.
6. van Steensel B, de Lange T: **Control of telomere length by the human telomeric protein TRF1**. *Nature* 1997, **385**(6618):740-743.
7. van Steensel B, Smogorzewska A, de Lange T: **TRF2 protects human telomeres from end-to-end fusions**. *Cell* 1998, **92**(3):401-413.
8. Smogorzewska A, de Lange T: **Different telomere damage signaling pathways in human and mouse cells**. *Embo Journal* 2002, **21**(16):4338-4348.
9. Chan SS, Chang S: **Defending the end zone: studying the players involved in protecting chromosome ends**. *FEBS Lett* 2010, **584**(17):3773-3778.
10. De Boeck G, Forsyth RG, Praet M, Hogendoorn PC: **Telomere-associated proteins: cross-talk between telomere maintenance and telomere-lengthening mechanisms**. *J Pathol* 2009, **217**(3):327-344.
11. Smith S: **The world according to PARP**. *Trends Biochem Sci* 2001, **26**(3):174-179.
12. Ame JC, Spenlehauer C, de Murcia G: **The PARP superfamily**. *Bioessays* 2004, **26**(8):882-893.
13. Smith S, de Lange T: **Tankyrase promotes telomere elongation in human cells**. *Curr Biol* 2000, **10**(20):1299-1302.
14. Smith S, Giriat I, Schmitt A, de Lange T: **Tankyrase, a poly(ADP-ribose) polymerase at human telomeres**. *Science* 1998, **282**(5393):1484-1487.
15. Muramatsu Y, Tahara H, Ono T, Tsuruo T, Seimiya H: **Telomere elongation by a mutant tankyrase 1 without TRF1 poly(ADP-ribosyl)ation**. *Exp Cell Res* 2008, **314**(5):1115-1124.
16. Hsiao SJ, Smith S: **Sister telomeres rendered dysfunctional by persistent cohesion are fused by NHEJ**. *J Cell Biol* 2009, **184**(4):515-526.

17. Donigian JR, de Lange T: **The role of the poly(ADP-ribose) polymerase tankyrase1 in telomere length control by the TRF1 component of the shelterin complex**. *J Biol Chem* 2007, **282**(31):22662-22667.
18. Sbodio JI, Chi NW: **Identification of a tankyrase-binding motif shared by IRAP, TAB182, and human TRF1 but not mouse TRF1. NuMA contains this RXXPDG motif and is a novel tankyrase partner**. *J Biol Chem* 2002, **277**(35):31887-31892.
19. Chang W, Dynek JN, Smith S: **TRF1 is degraded by ubiquitin-mediated proteolysis after release from telomeres**. *Genes Dev* 2003, **17**(11):1328-1333.
20. Lee TH, Perrem K, Harper JW, Lu KP, Zhou XZ: **The F-box protein FBX4 targets PIN2/TRF1 for ubiquitin-mediated degradation and regulates telomere maintenance**. *J Biol Chem* 2006, **281**(2):759-768.
21. Potts PR, Yu H: **The SMC5/6 complex maintains telomere length in ALT cancer cells through SUMOylation of telomere-binding proteins**. *Nat Struct Mol Biol* 2007, **14**(7):581-590.
22. Dynek JN, Smith S: **Resolution of sister telomere association is required for progression through mitosis**. *Science* 2004, **304**(5667):97-100.
23. Germe T, Miller K, Cooper JP: **A non-canonical function of topoisomerase II in disentangling dysfunctional telomeres**. *EMBO J* 2009, **28**(18):2803-2811.
24. Lehmann AR: **The role of SMC proteins in the responses to DNA damage**. *DNA Repair (Amst)* 2005, **4**(3):309-314.
25. Losada A, Hirano T: **Dynamic molecular linkers of the genome: the first decade of SMC proteins**. *Genes Dev* 2005, **19**(11):1269-1287.
26. Bailey SM, Goodwin EH, Cornforth MN: **Strand-specific fluorescence in situ hybridization: the CO-FISH family**. *Cytogenet Genome Res* 2004, **107**(1-2):14-17.
27. Blagoev KB, Goodwin EH, Bailey SM: **Telomere sister chromatid exchange and the process of aging**. *Aging (Albany NY)* 2010, **2**(10):727-730.
28. Dregalla RC, Zhou J, Idate RR, Battaglia CL, Liber HL, Bailey SM: **Regulatory roles of tankyrase 1 at telomeres and in DNA repair: suppression of T-SCE and stabilization of DNA-PKcs**. *Aging (Albany NY)* 2010, **2**(10):691-708.
29. Hagelstrom RT, Blagoev KB, Niedernhofer LJ, Goodwin EH, Bailey SM: **Hyper telomere recombination accelerates replicative senescence and may promote premature aging**. *Proc Natl Acad Sci U S A* 2010, **107**(36):15768-15773.
30. Laud PR, Multani AS, Bailey SM, Wu L, Ma J, Kingsley C, Lebel M, Pathak S, DePinho RA, Chang S: **Elevated telomere-telomere recombination in WRN-deficient, telomere dysfunctional cells promotes escape from senescence and engagement of the ALT pathway**. *Genes Dev* 2005, **19**(21):2560-2570.
31. Opresko PL, von Kobbe C, Laine JP, Harrigan J, Hickson ID, Bohr VA: **Telomere-binding protein TRF2 binds to and stimulates the Werner and Bloom syndrome helicases**. *J Biol Chem* 2002, **277**(43):41110-41119.
32. Opresko PL, Otterlei M, Graakjaer J, Bruheim P, Dawut L, Kolvraa S, May A, Seidman MM, Bohr VA: **The Werner syndrome helicase and exonuclease cooperate to resolve telomeric D loops in a manner regulated by TRF1 and TRF2**. *Mol Cell* 2004, **14**(6):763-774.

33. Chang P, Coughlin M, Mitchison TJ: **Interaction between Poly(ADP-ribose) and NuMA Contributes to Mitotic Spindle Pole Assembly**. *Mol Biol Cell* 2009, **20**(21):4575-4585.
34. Williams ES, Klingler R, Ponnaiya B, Hardt T, Schrock E, Lees-Miller SP, Meek K, Ullrich RL, Bailey SM: **Telomere dysfunction and DNA-PKcs deficiency: characterization and consequence**. *Cancer Res* 2009, **69**(5):2100-2107.
35. Mansour WY, Rhein T, Dahm-Daphi J: **The alternative end-joining pathway for repair of DNA double-strand breaks requires PARP1 but is not dependent upon microhomologies**. *Nucleic Acids Research* 2010, **38**(18):6065-6077.
36. Bailey SM, Cornforth MN, Ullrich RL, Goodwin EH: **Dysfunctional mammalian telomeres join with DNA double-strand breaks**. *DNA Repair (Amst)* 2004, **3**(4):349-357.
37. Bailey SM, Meyne J, Chen DJ, Kurimasa A, Li GC, Lehnert BE, Goodwin EH: **DNA double-strand break repair proteins are required to cap the ends of mammalian chromosomes**. *Proc Natl Acad Sci U S A* 1999, **96**(26):14899-14904.
38. Okayasu R, Suetomi K, Yu Y, Silver A, Bedford JS, Cox R, Ullrich RL: **A deficiency in DNA repair and DNA-PKcs expression in the radiosensitive BALB/c mouse**. *Cancer Res* 2000, **60**(16):4342-4345.
39. Ponnaiya B, Cornforth MN, Ullrich RL: **Radiation-induced chromosomal instability in BALB/c and C57BL/6 mice: the difference is as clear as black and white**. *Radiat Res* 1997, **147**(2):121-125.
40. Yu Y, Okayasu R, Weil MM, Silver A, McCarthy M, Zabriskie R, Long S, Cox R, Ullrich RL: **Elevated breast cancer risk in irradiated BALB/c mice associates with unique functional polymorphism of the Prkdc (DNA-dependent protein kinase catalytic subunit) gene**. *Cancer Res* 2001, **61**(5):1820-1824.
41. Peng Y, Woods RG, Beamish H, Ye R, Lees-Miller SP, Lavin MF, Bedford JS: **Deficiency in the catalytic subunit of DNA-dependent protein kinase causes down-regulation of ATM**. *Cancer Res* 2005, **65**(5):1670-1677.
42. de Lange T: **Protection of mammalian telomeres**. *Oncogene* 2002, **21**(4):532-540.
43. Chuang YY, Chen Q, Brown JP, Sedivy JM, Liber HL: **Radiation-induced mutations at the autosomal thymidine kinase locus are not elevated in p53-null cells**. *Cancer Res* 1999, **59**(13):3073-3076.
44. Zhang Y, Lim CU, Williams ES, Zhou J, Zhang Q, Fox MH, Bailey SM, Liber HL: **NBS1 knockdown by small interfering RNA increases ionizing radiation mutagenesis and telomere association in human cells**. *Cancer Res* 2005, **65**(13):5544-5553.
45. Lee EY, Muller WJ: **Oncogenes and tumor suppressor genes**. *Cold Spring Harb Perspect Biol* 2010, **2**(10):a003236.
46. Gollahon LS, Kraus E, Wu TA, Yim SO, Strong LC, Shay JW, Tainsky MA: **Telomerase activity during spontaneous immortalization of Li-Fraumeni syndrome skin fibroblasts**. *Oncogene* 1998, **17**(6):709-717.

47. Veuger SJ, Curtin NJ, Richardson CJ, Smith GC, Durkacz BW: **Radiosensitization and DNA repair inhibition by the combined use of novel inhibitors of DNA-dependent protein kinase and poly(ADP-ribose) polymerase-1**. *Cancer Res* 2003, **63**(18):6008-6015.
48. Veuger SJ, Curtin NJ, Smith GC, Durkacz BW: **Effects of novel inhibitors of poly(ADP-ribose) polymerase-1 and the DNA-dependent protein kinase on enzyme activities and DNA repair**. *Oncogene* 2004, **23**(44):7322-7329.
49. Furth EE, Thilly WG, Penman BW, Liber HL, Rand WM: **Quantitative assay for mutation in diploid human lymphoblasts using microtiter plates**. *Anal Biochem* 1981, **110**(1):1-8.
50. Zhang Q, Williams ES, Askin KF, Peng Y, Bedford JS, Liber HL, Bailey SM: **Suppression of DNA-PK by RNAi has different quantitative effects on telomere dysfunction and mutagenesis in human lymphoblasts treated with gamma rays or HZE particles**. *Radiat Res* 2005, **164**(4 Pt 2):497-504.
51. Bailey SM, Brenneman MA, Goodwin EH: **Frequent recombination in telomeric DNA may extend the proliferative life of telomerase-negative cells**. *Nucleic Acids Res* 2004, **32**(12):3743-3751.
52. Bailey SM, Meyne J, Chen DJ, Kurimasa A, Li GC, Lehnert BE, Goodwin EH: **DNA double-strand break repair proteins are required to cap the ends of mammalian chromosomes**. *Proceedings of the National Academy of Sciences of the United States of America* 1999, **96**(26):14899-14904.
53. Livak KJ, Schmittgen TD: **Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method**. *Methods* 2001, **25**(4):402-408.

CHAPTER 4

DNA-PKcs protein stability is regulated by tankyrase 1-specific PARsylation activity.

The following has been published in:
Dregalla RC, Zhou J, Idate RR, Battaglia CL, Liber HL, Bailey SM: **Regulatory roles of tankyrase 1 at telomeres and in DNA repair: suppression of T-SCE and stabilization of DNA-PKcs**. *Aging (Albany NY)* 2010, **2**(10):691-708.
The dissertation author contributed all figures with the exception of Figure 1.

4.1.0 Introduction

Our identification of genomic instability phenotypes that suggest deficient DNA-repair in response to tankyrase 1 knockdown provided the first evidence for involvement of tankyrase 1 in DNA-repair [1]. Further investigation provided evidence that the instability phenotypes observed were the consequence of DNA-dependent Protein Kinase catalytic subunit (DNA-PKcs) protein destabilization as a consequence of tankyrase 1 knockdown, suggesting tankyrase 1 is not directly involved in DNA-repair [1]. We speculated that tankyrase 1 may play a regulatory role in DNA-repair by stabilizing the DNA-PKcs protein, likely though a transient interaction. Here, we investigate the mechanism by which tankyrase 1 stabilizes the DNA-PKcs protein.

We found that the stability of the DNA-PKcs protein is dependent on tankyrase 1 at the posttranscriptional level; tankyrase 2 and DNA-PKcs mRNA levels were not negatively influenced by siRNA-mediated tankyrase 1 knockdown. Considering the converse siRNA knockdown of DNA-PKcs had no impact on tankyrase 1 protein levels,

we speculated that the stability of DNA-PKcs is dependent on the catalytic activity of tankyrase 1. The pADPr modification of protein substrates has been shown to play a role in the negative regulation of proteins (TRF1) [2, 3], non-covalent scaffolding (NuMA) [4, 5], protein recruitment (PARP-1-mediated single-stranded break-repair) [6, 7] and catalytic activation [8]. We aimed to uncover the mechanism underlying tankyrase 1-dependent DNA-PKcs protein stability in order to provide valuable insight to the influences of poly(ADP-ribosyl)ation (pADPr/PARsylating) protein modification.

4.1.1 Tankyrase-dependent protein regulation by poly(ADP-ribosyl)ation

Tankyrase-dependent PARsylation of a substrate protein is coupled with tankyrase auto-pADPr modification in order to dissociate tankyrase monomers from the multimerized tankyrase complex formed via sterile-alpha motif (SAM) interactions [9, 10]. Tankyrase multimerization (including tankyrases 1 & 2) is thought to optimize tankyrase catalytic addition of ADP-ribose [9]; longer pADPr additions prolong the half-life of the modification before being removed by Poly(ADP-ribose) Glycohyrdolase (PARG) activity [9]. Tankyrase auto-modification disrupts SAM motif-dependent tankyrase complexes [9, 10]. Once auto-PARsylated, tankyrases become substrates for E3 Ub-ligase and subsequent proteasome degradation if the pADPr chain is not cleaved via PARG catalytic activity [11]. Thus, the tankyrases are auto-regulated by pADPr modification, which enhances their vulnerability to degradation.

Poly(ADP-Ribose) Glycohydrolase (PARG) is the PARP counter enzyme that performs de-PARsylating activity at an extremely rapid rate; biochemical studies suggest that the cessation of all cellular PARP activity would result in the de-PARsylation of every acceptor protein within 60 seconds via PARG [12-14]. The random cleavages

between pADPr groups by PARG allows for tankyrase-dependent formation of a heavily branched pADPr chain that serve as an effective method to extend the receptor proteins half-life in a PARsylated form [15-18]. Appropriate regulation of tankyrase-dependent pADPr protein modification requires tankyrase multimerization in order to achieve rapid extension of branched pADPr chains and subsequent PARG activity.

4.1.2 Tankyrase 1 modifies a broad spectrum of proteins with various effects

Tankyrase 1 modification of pADPr-acceptor proteins has been shown to occur with a growing number of substrate proteins [19]. In addition to TRF1 regulation [2, 3], tankyrase 1-dependent pADPr protein modification is essential for accurate GLUT4 storage vesicle (GSV) trafficking by the insulin responsive amino peptidase (IRAP) [11, 20]; stabilization of the spindle-pole apparatus via modification of the nuclear mitotic apparatus complex (NuMA) [4, 5]; negative -regulation of the master beta-catenin sequestering protein, Axin [21] and autoregulation of tankyrase 1 itself [11]. In each of these cases, pADPr modification of the acceptor protein alters the modified proteins function, ability to form protein complexes, stabilization, or affinity for additional modifications; e.g. ubiquitination (Ub).

GSVs containing the glucose transporter GLUT4 and the hormone protease IRAP are usually translocated from the golgi apparatus to the plasma membrane for glucose uptake. Tankyrase 1 is an IRAP binding partner and tankyrase 1 knockdown results in ineffective, unorganized GSV trafficking. Inhibition of tankyrase 1 activity has also been shown to interfere with appropriate GSV trafficking and function [20, 22]. These studies demonstrate the role of tankyrase 1 pADPr-modification of IRAP, necessary for the appropriate function of IRAP in GSV orientation and trafficking to the

plasma membrane; the precise mechanism of this pADPr-dependent model is still under investigation.

Tankyrase 1-specific pADPr additions have been shown to play a critical role in protein complex stability, forming a lattice-like scaffolding network for non-covalent protein-protein interactions [9, 10] as in the case of NuMA and spindle pole assembly [4, 23]. The net negative charge of each ADP-ribose group provides an effective platform for stable, ionic-based protein-protein interactions, creating a temporary scaffolding structure between proteins [6, 7]. The integrity of the spindle pole apparatus during mitosis is dependent upon tankyrse 1 pAPDr-modification of NuMA monomers [4, 23]; pADPr-modified NuMA proteins associate non-covalently with one another (and tankyrases) to orient the microtubules in the spindle-pole apparatus assembly [4, 23-25]. Tankyrase 1 deficiency results in the loss of pADPr-dependent scaffolding of the spindle pole apparatus and defective microtubule function (during mitosis) [4, 23]. Tankyrase 1 dysfunction results in the random anchoring of microtubules in the cytoplasm, uncoordinated microtubule dynamics (extention and retraction) and failure to complete the mitotic process [4, 19, 23].

Tankyrase 1 has also recently been identified as a positive regulator of the β-catenin transcription factor by negatively regulating the master sequestering protein, Axin [21]. Activation of β-catenin is initiated via the Wnt signaling pathway [26]. When the appropriate cell signal is lacking, β-catenin remains in a sequestered, inactive state and eventually, becomes phosphorylated by casein kinase (CK1) or glycogen synthase kinase 3B (GSK3b), ubiquitinated and degraded [27-30]. The CK1 and GSK3b kinases are kept in proximity with β-catenin by the formation of the Axin-dependent ‘β-catenin

destruction complex'. Tankyrase 1 has been identified as the negative regulator of Axin, in which pADPr modification results in the degradation of Axin, disassembly of the 'β-catenin destruction complex' and subsequent activation of β-catenin [21].

4.1.3 The NHEJ protein DNA-PKcs as a substrate for tankyrase 1 PARsylation

We have found that the stability of DNA-PKcs is dependent on tankyrase 1 posttranslationally. However, it is not known whether DNA-PKcs protein stability requires the physical presence of the tankyrase 1 protein or its catalytic activity (PARsylation). Initial attempts to demonstrate tankyrase 1 and DNA-PKcs in complex via protein complex-immunoprecipitation (Co-IP) failed, suggesting that the interaction is transient in nature. Most transient interactions are characterized by enzymatic modification of a target protein to alter the modified proteins function and/or conformation; e.g. phosphorylation.

DNA-PKcs has been shown to be a pADPr-accepting protein and existing in a PARsylated state *in vivo* [31, 32]. However, the protein/s responsible for DNA-PKcs PARsylation and the functional implications of pADPr-modified DNA-PKcs have not been characterized. *In vitro* studies with DNA-PKcs and PARP-1 demonstrate that DNA-PKcs is capable of accepting the pADPr-modification in the presence of ^{32}P-adenylated NAD^+ [8]. Additionally, PARsylated forms of DNA-PKcs have been reported to have up-regulated kinase activity, approximately 7-fold higher than unmodified forms of DNA-PKcs [8]. Collectively, these findings indicate tankyrase 1-dependent pADPr modification of DNA-PKcs occurs *in vivo* and is a potential mechanism for catalytic activation of DNA-PKcs.

Evidence supporting a biochemical role for pADPr modified DNA-PKcs *in vivo* recently emerged, as DNA-PKcs was identified as a PARsylated member of the proteome in a pADPr immunoprecipatation assay. However, the mechanism and function of DNA-PKcs PARsylation was not determined [31]. This proteome-wide pADPr pull-down suggests that DNA-PKcs is covalently modified by a PARP family member, as opposed to a 'pADPr interacting' protein where pADPr is used as a scaffold to stabilize protein-protein interactions [32]. These *in vitro* and *in vivo* studies demonstrate that DNA-PKcs is a substrate for one, or possibly several PARP family members, as PARsylated forms of DNA-PKcs have functional implications regarding activation of its kinase activity [8, 31, 32].

Here, we sought to uncover the mechanism by which tankyrase 1 contributes to DNA-PKcs protein stability. Suspecting a transient interaction enzymatic in nature, we investigated tankyrase 1-dependent DNA-PKcs stability by inhibiting the catalytic activity of several proteins/complexes that participate in pADPr regulation. We targeted broad-range PARP activity with 3-aminobenzamide (3-AB) [33, 34], tankyrase catalytic activity with XAV939 [21], proteasome-mediated degradation with MG132 [35] and PARG activity with ADP-Hydroxymethyl Pyrrolidinediol (ADP-HPD) [36]. Ultimately, we show that DNA-PKcs protein stabilization is acheived by tankyrase 1-specific pADPr-modification with no redundant function by other PARP family members. When the tankyrase 1-dependent pADPr modification of DNA-PKcs is abrogated, DNA-PKcs is targeted for proteasome-mediated degradation.

4.2.0 Results

4.2.1 Tankyrase PARP activity is required for DNA-PKcs protein stability

To investigate a possible protein-protein interaction between tankyrase 1 and DNA-PKcs, multiple protein complex immuneprecipitation (Co-IP) experiments were performed, but failed to demonstrate tankyrase 1 and DNA-PKcs in complex. A negative result via this assay does not rule out the possibility of the formation of a transient DNA-PKcs-tankyrase-1 complex. However, it does suggest that the interaction does not persist as a stable complex. Often, transient protein interactions are enzymatic in nature and serve a regulatory function for a finite period of time [37].

Initial support for pADPr modification of DNA-PKcs was provided by treatment with the general PARP inhibitor 3-AB, which demonstrated that PARP inhibition increases radiation-induced mutation frequencies [1]. Next, we question whether 3-AB treatment alone challenged the integrity of the DNA-PKcs protein. Treatment with high concentrations of 3-AB (10 and 20 mM) were sufficient to induce an observable reduction of DNA-PKcs protein levels (Figure 1 [1]), suggesting that *PARP catalytic activity is required* for DNA-PKcs protein stability. However, 3-AB has a higher affinity for PARP-1 and PARP-2 catalytic domains than for tankyrase 1; treatment with 3-AB at low concentrations inhibits the catalytic activity of PARP-1 and PARP-2, not tankyase 1 or 2. This is confounding in that PARP-1 has been shown to PARsylate DNA-PKcs *in vitro* [8] and thus, we cannot contribute the reduction in DNA-PKcs protein levels to tankyrase 1 PARP activity specifically.

4.2.2 Catalytic activity of tankyrase 1 stabilizes DNA-PKcs

To determine the importance of tankyrase 1 catalytic activity in DNA-PKcs protein stability, we treated human lymphoblasts (WTK1) with the recently available small molecule inhibitor XAV939, a tankyrase-specific PARP domain inhibitor [21]. XAV939 treatment (0.5 and 1.0 μM) rapidly and *dramatically* reduced DNA-PKcs protein levels (Figure 2 [1]). This observation confirmed that DNA-PKcs protein stability is dependent on tankyrase-specific poly(ADP-ribosyl)ating activity, as XAV939 does not bind the relevant interaction domains (i.e. ankyrin repeats, SAM).

With respect to XAV939 concentrations used in culture, the catalytic activity tankyrase 1 & 2 are inhibited specifically, indicating the activity of no PARP family member (other than tankyrases) are responsible for DNA-PKcs protein stability [21]. Biochemical characterization of XAV939 revealed that the inhibitor has a higher affinity for the tankyrase 2 PARP domain compared to tankyrse 1 (IC50 values for tankyrase 1 and tankyrase 2 are 0.011 μM and 0.004 μM respectively) [21]. Therefore, we can assume that under conditions of XAV939-dependent inhibition of tankyrase 1, tankyrase 2 will be inhibited as well. However, following siRNA-mediated knockdown of tankyrase 1, qRT-PCR analysis of tankyrase 1 & tankyrase 2 mRNA levels showed the siRNA construct targeted tankyrase 1 mRNA specifically; tankyrase 2 was not affected. Therefore, DNA-PKcs stability is dependent on tankyrase 1 depletion specifically, excluding the involvement of tankyrase 2. Building on this conclusion, we attribute the depletion of DNA-PKcs protein levels in the presence of XAV939 to tankyrase 1 PARP domain inhibition, not tankyrase 2.

Examination of various time points (2, 5, 8, 12, 18, 24 & 48 hours) and XAV939 concentrations (0.1, 0.5 or 1.0μM) revealed a significant reduction of DNA-PKcs protein levels to ~50% (relative to the normal resting level) by 8 hours with both 0.5 μM or 1.0 μM XAV939 (Figure 2 [1]). The greatest reduction of DNA-PKcs protein levels (< 25% relative expression compared to DMSO treated controls) occurred at 12 hr with 1.0 μM XAV939 exposure. Later time points (24 hr) showed recovery of DNA-PKcs protein levels relative to DMSO treated controls, likely due to loss of potency of the inhibitor in culture (data not shown). Cumulatively, these results support tankyrase 1-specific PARsylating activity as a critical contributing factor to stabilization of the DNA-PKcs protein and provide preliminary evidence that DNA-PKcs protein stability relies on tankyrase 1 catalytic activity, as opposed to the physical presence of tankyrase 1 itself.

4.2.3 Tankyrase 1 protein levels increase in response to tankyrase PARP inhibition

Inhibition of the tankyrase PARP domain with XAV939 treatment did not diminish tankyrase 1 protein levels. To the contrary, treatment of human lymphoblasts with 1.0 μM XAV939 resulted in a significant increase of tankyrase 1 levels to ~150% relative to the DMSO treated controls (Figure 3 [1]). This finding correlates with the the mechanism for the autoregulation of tankyrases through PARsylation [9-11].

During PARsylation events, tankyrases "multimerize" for maximum catalytic efficiency [9, 10]. Tankyrase autoPARsylation allows tankyrase monomers to dissociate from multimerized complex [9]. PARsylated tankyrase is a potential substrate for E3-Ligase dependent ubiquitination and proteasome-mediated degradation if the pADPr modification is not removed by PARG [11]. Therefore, XAV939 inhibition of tankyrase catalytic activity not only blocks tankyrase-dependent pADPr modification of substrate

proteins, but also blocks the ability of tankyrase 1 to auto-PARsylate and thereby shields tankyrase 1 from potential ubiquitination and subsequent degradation. These findings further illustrate that DNA-PKcs stability is dependent on tankyrase 1-specific PARP activity, but also demonstrate that the physical presence of tankyrase 1 *per se* is not responsible for the integrity of the DNA-PKcs protein. We speculated that tankyrase 1 directly PARsylates DNA-PKcs, resulting in a dynamic, yet consistent pool of PARsylated DNA-PKcs.

4.2.4 Tankyrase 1 stabilizes DNA-PKcs by protecting it from proteolytic degradation

Our earlier studies showed that DNA-PKcs stability requires tankyrase 1 at a posttranslational level (qRT-PCR studies, Chapter 3). To investigate the mechanism of DNA-PKcs protein depletion following tankyrase 1 inhibition or knockdown, we partially inhibited the proteasome by the addition of the chymotrypsin inhibitor MG132 [38]. At various times following tankyrase 1 siRNA transfection (8, 12, and 24 hr), during the time that both tankyrase 1 and DNA-PKcs protein levels are depleted, cells were treated with MG132 for two hour time windows prior to cell culture harvest. Similarly, 12 hour treatments with XAV939 were combined with MG132 treatment for the final two hours and cells harvested.

Although only a partial proteasome inhibitor (i.e. chymotrypsin-like activity, not trypsin-like or caspase-like activity), treatment with MG132 for 2 hours in combination with a 12 hour XAV939 treatment resulted in a detectable recovery of DNA-PKcs protein levels to ~10-15% above the steady-state DNA-PKcs levels in XAV939 treated cultures (Figure 4 [1]). This result demonstrated that inhibition of proteasome-mediated protein degradation allowed cells to accumulate DNA-PKcs protein and so, provide support for

the notion that tankyrase 1 protects DNA-PKcs from proteolytic degradation. This observation is also consistent with our qRT-PCR results demonstrating sufficient levels of DNA-PKcs mRNA following tankyrase 1 knockdown; i.e., ample DNA-PKcs message is available for translation. That DNA-PKcs protein levels were only minimally restored upon proteasome inhibition, may reflect the short time window allowed for recovery, that MG132 does not completely inhibit the proteasome, and/or that it takes time to synthesize such a large and abundant protein.

Taken together, the MG132 studies demonstrated that loss of DNA-PKcs occurs at the protein level and is degraded in a proteasome-mediated fashion. Proteasome inhibition under tankyrase 1 siRNA knockdown conditions showed that loss of DNA-PKcs via proteasome-mediated degradation is dependent upon the presence of the tankyrase 1 protein. Furthermore, XAV939 results complement the MG132 studies in combination with siRNA knockdown, showing a ~15% increase of the DNA-PKcs protein over conditions lacking a 2 hour MG132 treatment. From these observations we conclude that the PARsylating activity of tankyrase 1 protects DNA-PKcs from proteasome-mediated degradation, as opposed to functioning stoichiometrically at the protein level.

4.2.5 DNA-PKcs protein levels decrease in response to PARG inhibition

Hydrolysis and removal of ADP-ribose polymers from modified proteins is rapidly catalysed by poly(ADP-ribose) glycohydrolase (PARG) [39]. To further explore tankyrase-dependent PARsylation of DNA-PKcs, we utilized the potent PARG inhibitor adenosine diphosphate (hydroxymethyl)pyrrolidinediol (ADP-HPD) [36]. We anticipated that inhibition of PARG activity without tankyrase inhibition would result in

increased DNA-PKcs protein levels, as DNA-PKcs would become stably and irreversibly PARsylated in a proteasome-resistant conformation.

Unexpectedly, DNA-PKcs protein levels were rapidly and dramatically diminished under conditions in which lymphoblasts were treated with the ADP-HPD PARG inhibitor alone (1.2 μM), and in combination with XAV939 (1.0 μM). Due to the unstable nature of hydrated ADP-HPD (from lyophilized form), we questioned whether the inhibitor was sustaining its structural integrity and function over the time course of the treatment when in combination with XAV939. Therefore, we initiated addition of ADP-HPD (1.2 μM) to culture every 2.5 hours, resulting in a final concentration of 4.8 μM (assuming each molecule remains active). Consistent with 1.2 μM ADP-HPD treatments, treatment with 4.8 μM ADP-HPD alone and in combination with 1.0 μM XAV939 over a 10 hour time course resulted in the significant reduction of the DNA-PKcs protein to ~55% and 40% expression respectively, relative to the DMSO treated control (Figure 5 [1]).

4.2.6 PARG inhibition depletes tankyrase 1 protein levels

The lysate from cells treated with ADP-HPD was also probed for the tankyrase 1 protein by Western blot analysis, revealing that tankyrase 1 was reduced to an undetectable level in cells treated with ADP-HPD (Figure 6 [1]). It became evident that PARG inhibition was interfering with the regulatory dynamics of the tankyrase 1 protein in some way. To dissociate from the multimerized tankyrase complex that forms during substrate protein PARsylation, tankyrases autoPARsylate, becoming a substrate for either E3 ubiquitin ligase or PARG. We suspect that treatment with ADP-HPD

directly interferes with the ability of the autoPARsylated tankyrase 1 to become dePARsylated, as PARG activity is blocked.

ADP-HPD treatment in combination with XAV939 resulted in *increased* levels of tankyrase 1 (Figure 6 [1]). We explain this as the consequence of treatment with XAV939, which alone, increases tankyrase 1 protein levels (relative to the DMSO treated controls). Therefore, in combined treatments, the activity of the tankyrase inhibitor is epistatic, blocking autoPARsylation of tankyrase 1 and shielding tankyrase 1 from becoming a substrate for PARG and/or E3 ubiquitin ligase activity, resulting in increased tankyrase 1 protein levels.

4.2.7 PARG inhibition depletes tankyrase 1 and destabilizes DNA-PKcs

Treatment with the PARG inhibitor ADP-HPD (alone) parallels tankyrase 1 siRNA knockdown in respect to the method of DNA-PKcs protein depletion. In each case, tankyrase 1 protein is depleted (by protein destabilization verses transcript manipulation) and so it is not available to perform its catalytic function, consequentially depleting DNA-PKcs protein levels. The ADP-HPD/XAV939 combined treatment resulted in increased levels of tankyrase 1, suggesting that the inability to auto-PARsylate predominates; i.e., there is little available to de-PARsylate. Considering the evidence and implications provided by both PARG inhibition studies and tankyrase 1-specific PARP domain inhibition studies, we verified that tankyrase 1-specific PARsylation is required for DNA-PKcs protein stability.

4.2.8 Electrophoretic separation of PARsylated DNA-PKcs from unmodified pools of DNA-PKcs via SDS-PAGE

DNA-PKcs has been shown to be covalently modified by addition of pADPr via PARP1 *in vitro,* resulting in a significant increase in DNA-PKcs kinase activity, suggesting a functional effect for DNA-PKcs PARsylation beyond protein stability [40]. Considering that our inhibitor studies cumulatively suggested covalent modification of DNA-PKcs via tankyrase 1-dependent PARsylation as the mechanism of DNA-PKcs protein stability, we sought evidence of a high molecular weight pool of DNA-PKcs dependent upon tankyrase 1 catalytic PARP activity.

Initially, we sought to demonstrate that a pool of DNA-PKcs exists with a tankyrase-dependent pADPr modification. Prior reports have demonstrated that DNA-PKcs is capable of accepting pADPr groups *in vitro,* and that it exists as such to some extent intracellularly [8, 31]. We investigated whether the pADPr modification of DNA-PKcs resulted from tankyrase 1-specific PARP activity. Detection of pADPr at the molecular weight corresponding to DNA-PKcs failed despite multiple attempts (data not shown). Due to the high variability in the number of pADPr groups that can exist on DNA-PKcs and the question of the effectiveness/sensitivity of the antibody, we moved toward a more progressive method for the detection of tankyrase 1-dependent PARsylated forms of DNA-PKcs.

Gel electrophoresis facilitated visualization (upon over exposure) of a high molecular weight pool of DNA-PKcs present in DMSO treated controls, much of which resided in the loaded well (Figure 7B [1]). Further, treatment with XAV939, ADP-HPD and XAV939/ADP-HPD combined resulted in deterioration of this high molecular weight

pool of DNA-PKcs, as well as a corresponding increase in DNA-PKcs degradation products compared to the DMSO treated controls (Figure 7B [1]). Due to the fact that the high molecular weight pool of DNA-PKcs was dependent on catalytically active tankyrase (the only experimental variable), the high molecular weight pool represented PARsylated forms of DNA-PKcs.

To further support this supposition, DMSO untreated controls and XAV939 treated samples (8 hr) were independently loaded every 2 hours in individual wells of a gradient gel (4-20%) over 6 hours (2, 4, and 6 hr total run times). Here, our aim was to separate high molecular weight forms of DNA-PKcs from unmodified pools with time. A significant reduction of the primary DNA-PKcs protein band was observed between the 2 and 4 hour run times (not over exposed); at 4 hours, DNA-PKcs levels in the DMSO treated control were reduced to ~50% relative to the DNA-PKcs levels detected in the 2 hour run; the 4 hour and 6 hours run times did not differ significantly in the levels of detected DNA-PKcs (Figure 7A [1]). In contrast, the XAV939 treated samples lost little DNA-PKcs over the range of run times, indicating that a pool of tankyrase-dependent modified DNA-PKcs exists under normal conditions, which is not present under conditions of tankyrase PARP inhibition. This result, in conjunction with detection of a high molecular weight pool of DNA-PKcs in DMSO treated controls, which is absent in XAV939 treated samples, supports the presence of a heterogeneous population of DNA-PKcs spanning a wide range of molecular weights, consistent with various levels of tankyrase 1-dependent PARsylated DNA-PKcs.

4.3.0 Discussion

4.3.1 DNA-PKcs protein stability requires tankyrase 1 specific PARP catalytic activity

Our tankyrase 1 siRNA studies first demonstrated the dependence of DNA-PKcs protein stability on tankyrase 1 specifically. Evidence also indicated that the DNA-PKcs-tankyrase 1 relationship occurred at the protein level, so we sought a mechanistic explanation. Attempts to demonstrate that DNA-PKcs and tankyrase 1 physically associate by Co-IP revealed no such stable complex between the two proteins, suggesting the interaction is transient in nature.

Transient interactions are often enzymatic and serve regulatory functions [37, 41]. Fitting the description of pADPr modification, we addressed the role of tankyrase 1 catalytic activity in the stability of DNA-PKcs. Treatment with the broad-range PARP domain inhibitor 3-aminobenzamide (3-AB) [33, 34, 42] produced a prominent reduction in levels of the DNA-PKcs protein, indicating that PARsylating activity might be a key component of DNA-PKcs stability. However, the non-specific nature of 3-AB does not provide solid evidence as to which of the PARP family member(s) specifically are involved in DNA-PKcs regulation via PARsylating activity. Though our results suggested tankyrase 1 as a prime candidate, others have identified PARP-1 as a binding partner and potential modifier of DNA-PKcs [8, 43].

4.3.2 DNA-PKcs protein stability requires tankyrase-specific catalytic activity

The recent characterization of tankyrase-specific inhibition with the novel small molecule PARP inhibitor XAV939 [21] provided a valuable tool to investigate the role of tankyrase PARP activity in DNA-PKcs protein stability. Use of XAV939 has also aided in the characterization of the role of tankyrases in the stability of the β-catenin master

sequestering protein, Axin [21]. It is important to note that XAV939 has a higher affinity for the tankyrase 2 PARP domain and thus, inhibition of tankyrase 1 with XAV939 will inevitably be coupled with tankyrase 2 inhibition. Therefore, it could be argued that the reduction in DNA-PKcs protein levels is not consequence of tankyrase 1 inhibition alone. However, our earlier qRT-PCR studies revealed the loss of tankyrase 1 mRNA in tankyrase 1-siRNA mediated knockdown was not coupled with tankyrase 2 transcript depletion.

Using a concentration of XAV939 in culture consistent with previous studies [21], we observed maximum depletion of DNA-PKcs protein levels over a surprisingly short time course of 12 hours, half the time used in tankyrase 1 siRNA studies (Figure 8). We reason the difference in time courses results from the means of tankyrase 1 manipulation used in each case. siRNA-mediated depletion requires successful transfection, degradation of the tankyrase 1 transcript, ubiquitination and degradation of the tankyrase 1 protein before DNA-PKcs protein stability is influenced. Inhibition of tankyrase PARP activity/domain with XAV939 is nearly instantaneous.

Our results following tankyrase 1-specific catalytic inhibition address several important questions regarding the tankyrase 1-DNA-PKcs relationship. First, they indicate that the catalytic activity of tankyrase 1 specifically is required for DNA-PKcs stability, involving no redundant function by other PARP family members. Second, we find an explanation for the observation that DNA-PKcs siRNA knockdown, which requires 48 to 72 hours for maximum depletion [44], requires a longer time course compared to the siRNA knockdown of tankyrase 1, taking only 12-24 hours to deplete DNA-PKcs protein levels. siRNA-mediated depletion of DNA-PKcs operates at the

mRNA/transcript level and so does not influence the regulatory dynamics of tankyrase 1 in regards to DNA-PKcs protein stability, thus delaying the rate at which existing DNA-PKcs protein is degraded. Loss of tankyrase 1 via siRNA knockdown abrogates this regulatory role and therefore, results in the rapid depletion of DNA-PKcs.

4.3.3 DNA-PKcs protein stability requires more than the physical presence of tankyrase 1

Several lines of evidence suggested that it is the PARsylating activity of tankyrase 1 that is important to DNA-PKcs protein stability, not the ability of tankyrase 1 to facilitate protein-protein interactions. The ankyrin-like repeat domains in tankyrases allow tankyrases to interact with an array of target proteins, whereas the sterile alpha motif (SAM) is the functional domain responsible for tankyrase-based multimerization during PARsylation. XAV939 does not bind to either the ankyrin-like repeat domain nor the SAM and thus, normal protein-protein interactions are not interfered with during XAV939 treatment. This helps to rule out the formerly viable role for a stable DNA-PKcs-tankyrase 1 complex in DNA-PKcs stability, functioning stoichiometrically.

Interestingly, we also found that tankyrase 1 protein levels are increased with XAV939 treatment, supporting the mechanism of tankyrase 1 autoregulation proposed in prior studies [11] (Figure 9). Tankyrase 1 automodification is used for tankyrase dissociation from multimerized tankyrase complexes that are created during PARsyaltion of a substrate protein [9, 10], which results in tankyrase ubiquitination and degradation if not dePARsylated by PARG [11]. In our studies, XAV939 blocks tankyrase autoPARsylation and because tankyrase 1 is ubiquitously expressed [45], tankyrase 1 accumulates in an unPARsylated, proteasome resistant form (Figure 9). This finding demonstrates that the level of tankyrase 1 protein is not critical to the stability of DNA-

PKcs, as we find that even elevated levels of catalytically inhibited tankyrase 1 also results in the depletion of DNA-PKcs.

4.3.4 Tankyrase 1 protects DNA-PKcs from proteasome-mediated degradation

Depletion of DNA-PKcs protein levels was evident after both siRNA-mediated tankyrase 1 knockdown and XAV939-mediated catalytic inhibition of tankyrase 1. For proteasome-mediated protein degradation, multiple posttranslational-modifications serve as a signal for protein degradation by the proteasome, i.e. Ub and small ubiquitin like modifier (SUMO) [46, 47]. In an effort to determine in DNA-PKcs protein degradation is mediated by selective 'tagging' (e.g. Ub, SUMO) or by vesicle trafficking (proteasome vs. lysosomal), we employed the partial proteasome inhibitor MG132. Inhibition of the chymotrypsin proteolytic activity of the proteasome via MG132 under conditions optimized for DNA-PKcs depletion via tankyrase 1 siRNA knockdown and XAV939 inhibition showed some recovery of the DNA-PKcs protein within a 2 hour time window. Although not an astounding return, DNA-PKcs protein recovery to 10-15% above the levels of DNA-PKcs in cell populations treated with siRNA or XAV939 alone, providing supporting evidence that degradation of the DNA-PKcs protein is mediated by the proteasome.

4.3.5 Inhibition of PARG activity disrupts the dynamics of tankyrase autoregulation

We anticipated that inhibition of the PARP counter-enzyme, poly(ADP-ribose) glycohydrolase (PARG) would preserve poly(ADP-ribose) residues on pADPr modified proteins, and therefore, maintain normal levels of DNA-PKcs. However, inhibition of PARG activity with ADP-HPD alone and in combination with XAV939 resulted in the rapid, significant reduction of DNA-PKcs protein levels, supporting the notion that

PARG inhibition results in DNA-PKcs protein destabilization and degradation. Acknowledging the fact that ADP-HDP is extremely unstable once hydrated [48, 49] and considering the impact of cell culturing conditions on the potency of the inhibitor are poorly characterized, we speculated perhaps this observation was the result of ineffective ADP-HPD function. Treatment with ADP-HPD also resulted in a dramatic reduction in tankyrase 1 protein levels, to such an extent that western blot analysis failed to detect any quantifiable tankyrase 1 protein. We conclude that PARG inhibition disrupted the autoregulatory dynamics of the tankyrase 1 protein by inhibiting pADPr removal from tankyrase 1 following automodification, resulting in the ubiquitination and degradation of tankyrase 1. ADP-HPD-mediated depletion of tankyrase 1 therefore, resulted in DNA-PKcs depletion, similar to the mechanism by which siRNA knockdown of tankyrase 1 depletes DNA-PKcs (by the depletion of tankyrase 1) (Figure 10).

Our results from combining XAV939/ADP-HPD treatments reflected XAV939 treatment alone (DNA-PKcs depleted). Since XAV939 inhibits tankyrase 1 PARyslating activity, including autoPARsylation, tankyrase 1 did not accumulate in a pADPr-modified state and therefore, did not become ubiquitinated & degraded. Further, DNA-PKcs cannot be PARsylated by tankyrase 1 in the combined treatment and so, PARG inhibition is irrelevant. Hence, the reduction of DNA-PKcs protein levels in the combined XAV939/ADP-HPD treatment occurred by the same mechanism as treatment with XAV939 alone. Supporting this explanation, we found that combined treatment also resulted in elevated levels of the tankyrase 1 protein compared to the DMSO treated controls.

4.3.6 DNA-PKcs exists in a tankyrase-dependent, high molecular weight, PARsylated form

Having determined the DNA-PKcs protein is dependent on the catalytic activity and regulatory dynamics of tankyrase 1, we sought to determine if DNA-PKcs is pADPr-modified by tankyrase 1 or, if an intermediate effector protein might be involved in pADPr-dependent DNA-PKcs protein stability. We were encouraged by recent studies that identified DNA-PKcs as a pADPr-modified member of the proteaome [31]. Further, *in vitro* studies had demonstrated DNA-PKcs is capable of accepting pADPr residues with functional implications in upregulating DNA-PKcs kinase activity (approximately 7 fold over unmodified forms) [8]. It is important to recognize the highly variable nature of the pADPr posttranslational modification; pADPr groups can vary in length from 2 groups through 200 [6, 7, 17, 50]. Therefore, pADPr modified forms of DNA-PKcs would be of various high-molecular weight sizes and smeared throughout a gel above the protein band (470kD).

In an effort to determine if PARsylated forms of DNA-PKcs protein are in fact tankyrase 1 dependent, we sought to identify a high molecular weight group of DNA-PKcs (above 470 kDa). Extended periods of incubation with the primary DNA-PKcs antibody coupled with overexposed development of the western blot (fluorescent detection) successfully detected a high molecular weight pool of DNA-PKcs, most of which failed to migrate far beyond the loaded well and represented hyper-PARsylated forms of DNA-PKcs. Importantly, cell lysates from XAV939, ADP-HPD or combined XAV939/ADP-HPD treatments lacked the prominent high molecular weight pool of DNA-PKcs observed in the DMSO treated control. Furthermore, a considerable level of

DNA-PKcs "degradation product" was noted below the DNA-PKcs band, a feature not present in the DMSO treated control. Cumulatively, these observations demonstrate a high molecular weight pool of DNA-PKcs that is entirely dependent on tankyase 1catalytic activity, and when disrupted, DNA-PKcs becomes dePARsyalted via PARG and targeted for proteasome degradation. These findings further support the concept of a critical dynamic between pADPr-addition and removal that when challenged, resulted in destabilization of the DNA-PKcs protein.

We speculate there may be a significant proportion of pADPr-modified DNA-PKcs that contains few pADPr units (hypoPARsylated) that migrate with the DNA-PKcs band. To investigate this possibility, we utilized gel electrophoretic filtration using extended run times (on a 4-20% gradient gel) to 'filter out' pADPr-modified, high molecular weight forms of DNA-PKcs (>470kD) from unmodified forms (470kD). Extended run-times through the gradient gel allows for greater resolution between the forms of DNA-PKcs that are 'truly' 470kD (no modification) and those that are modified (e.g. various numbers of pADPr residues and/or Ub/SUMO).

In the DMSO treated controls, we observed a considerable reduction in the quantity of DNA-PKcs protein in the migrating band over longer run times. Conversely, XAV939 treated samples showed only a modest reduction in the quantity of DNA-PKcs in the migrating band across all time points. These findings demonstrate that the control sample possesses a considerable quantity of modified DNA-PKcs that is not present (to the same extent) in the XAV939 treated samples. We suspect that in XAV939 inhibited samples, a small portion of the protein may contain pADPr residues added by incompletely inhibited tankyrase 1. However, some proportion of DNA-PKcs that did

not migrate with unmodified forms of DNA-PKcs is likely to possess degradation signal residues (i.e., Ub or SUMO). Interestingly, at the end of the time course, DMSO controls and XAV939 treated samples resulted in the same quantity of 'unmodified' DNA-PKcs relative to the 100% control (~36%).

Taken together, our results demonstrate that DNA-PKcs exists in a wide-range of high molecular weight pools of both hypo- and hyperPARsylated forms that are dependent on tankyrase 1 cataytic activity. Inhibition of tankyrase 1 PARP activity abolishes these variant high molecular weight forms (exceeding 470kD) of DNA-PKcs.

4.3.7 DNA-PKcs is stabilized in a tankyrase-pADPr-dependent, proteasome-resistant form

We have demonstrated that the DNA-PKcs protein is directly PARsylated by the catalytic activity of tankyrase 1 specifically, resulting in a proteasome resistant form of DNA-PKcs. Further, we found that the extent of DNA-PKcs pADPr-modification has a considerable range. Conditions that challenge the ability of tankyrase 1 to perform its catalytic PARP activity results in the dePARsylation of DNA-PKcs, proteasome-mediated degradation and the rapid reduction of intracellular DNA-PKcs protein levels. Our results stress the importance of pADPr dynamics in the appropriate regulation of the modified target protein. Taken together, our findings support a mechanistic model of tankyrase 1-dependent DNA-PKcs protein stabilization (Figure 11 [1]).

We now speculate that the role of pADPr-modified DNA-PKcs goes beyond DNA-PKcs protein stability alone, possibly relevant in the function of DNA-PKcs in NHEJ DNA-repair. Consistent with the findings of previous DNA-PKcs-pADPr kinase studies *in vitro* [8], we speculate that tankyrase 1-dependent pADPr-modification

enhances the kinase activity of DNA-PKcs *in vivo* [8]; perhaps a critical component in activation of the DNA-PK holoenzyme at double-stranded breaks. In addition, pADPr may be necessary for DNA-PKcs recruitment to the DSB and scaffolding with the Ku heterodimer; Ku80 and DNA-PKcs have been shown to accept pADPr *in vivo* [31], whereas Ku70 and DNA-PKcs each possess pADPr interacting motifs (non-covalent interactions) [32].

4.4.0 Materials and Methods

Cell lines

WTK1 human lymphoblastoid cells have a stable karyotype (47, X, Y 13+, 14q+) and were derived from the WI-L2 line [51]. WTK1 cells are heterozygous at the thymidine kinase locus; they also have a single amino acid substitution in codon 237 at TP53. WTK1 cells were maintained in RPMI1640 medium (Hyclone) supplemented with 10% heat-inactivated horse serum (Sigma-Aldrich) and 1% pen-strep (Hyclone. [25].

Western blot analysis

Western blot analysis was always performed to confirm successful knockdown of target protein level before proceeding with evaluation of endpoints. Cells were harvested, centrifuged and resuspended in cold PBS (without Mg+ Ca+) twice, then immersed in 1x RIPA buffer (1x TBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide) and protease inhibitor cocktail (Santa Cruz Biotechnology), incubated on ice for 5-10 min, then passed through a 25 gauge syringe needle and centrifuged for 10 min at 140,000x g at 4°C. Protein in the supernatant was quantified using a BSA protein assay. Thirty five to 50µg of the supernatant proteins

were fractionated by SDS-PAGE (Bio-Rad) and transferred to Immobilon-FL PVDF membranes (Millipore). Blots were blocked in 5% skim milk or 5% BSA in TBS containing 0.1% Tween 20 and incubated overnight at 4°C with the following primary antibodies: rabbit polyclonal anti-tankyrase 1 (200 μg/ml; Santa Cruz Biotechnology); mouse monoclonal anti-actin (200 μg/ml; Santa Cruz Biotechnology); mouse monoclonal anti-PKcs Ab-4 (200 μg/ml; Neomarker); rabbit polyclonal anti-ATM (1mg/ml; Abcam). The blots were washed three times with TBS containing 0.1% Tween 20 and incubated with secondary antibody 680IRDye-conjugated goat polyclonal anti-rabbit IgG or IRDye 800CW-conjugated goat polyclonal anti-mouse IgG (1:15,000; LI-COR Biosciences). Bound antibodies were detected and using an Odyssey fluorescent imaging system (LI-COR Biosciences); blots were quantified according to manufacturers' instructions and normalized to independent actin loading controls [25].

Quantification of some blots was accomplished by importing images into Photoshop CS3 and analyzing as per a protocol adapted from the National Institutes of Health (http:/rsb.info.nih.gov/ij/index.html). Analysis involved first, multiplying the mean measured value by the number of pixels to obtain an "absolute intensity" value, an integrated measure of intensity and size of bands. Next, the relative intensity for each sample band was calculated by dividing the absolute intensity of each band by the absolute intensity of the standard (the mock transfection sample) [25].

Chemical inhibition

Nu7026 (Sigma-Aldrich), a competitive and highly selective inhibitor of DNA-PKcs kinase activity, was added to WTK1 cultures after siRNA transfection at a final concentration of 9 μM [52, 53], and remained on samples until collected for mutagenesis

or cytogenetic analyses. We have consistently found that this concentration of Nu7026 yields similar results for these end points as does siRNA knockdown of DNA-PKcs [25].

3-aminobenzamide (3-AB; Sigma-Aldrich) was used to inhibit global PARP activity at final concentrations ranging from 10 and 100 μM, to 10 and 20 mM. 3-AB was added to WTK1 cultures 24 hr prior to irradiation (or sham), which were then collected for mutation or western blot analyses [25].

XAV939, the recently identified small molecule shown to specifically inhibit PARP activity of tankyrase 1 (and tankyrase 2 at higher concentrations) [21], was used here at much lower concentrations than 3-AB. The tankyrase specific inhibitor XAV939 (Tocris) was solubilized in DMSO at 55 ˚C to a stock concentration of 10mM, which was diluted to a working concentration of 100μM; final concentrations of 0.5μM or 1μM were well within the concentration parameters suggested for cell culture experiments to inhibit tankyrase specifically. Cultures were maintained under these conditions for the duration of the designated time course. Controls were exposed to DMSO alone [25].

MG132. WTK1 lymphoblasts were transfected with tankyrase 1 siRNA, or treated with 1.0 μM XAV939, then incubated with the proteosome inhibitor MG-132 (12.5 μM; Sigma-Aldrich) for 2 hr time windows starting at 8, 10, 12 or 24 hours after transfection [54]. Cell samples were harvested 4 hours after treatment for western blot analysis [25].

ADP-HPD. WTK1 lymphoblasts were treated with the PARG inhibitor ADP-HPD [36] at 1.2 μM (EMD Chemicals) every 2.5 hours for a period of 10 hours, either alone or concurrently with XAV939 (1.0 μM final), at a final concentration of 4.8 μM

ADP-HPD. Samples were harvested at 10 hours following the respective treatment and lysates were prepared for western blot analysis [25].

Electrophoretic separation of high molecular weight DNA-PKcs.

WTK1 lymphoblasts treated with either DMSO or 1.0 μM XAV939 for 8 hours were loaded into independent wells of a 4-20% gradient SDS-PAGE every 2 hours over the course of 6 hours. At each time point, DMSO and XAV939 samples were loaded into wells immediately adjacent to the prior time point. The corresponding load times at 0, 2 and 4 hours resulted in total run times of 2, 4 and 6 hours respectively. Following completion of the final run time, the gel was analyzed via western blot for DNA-PKcs and actin loading controls, then quantified 25].

4.5.0 Figures

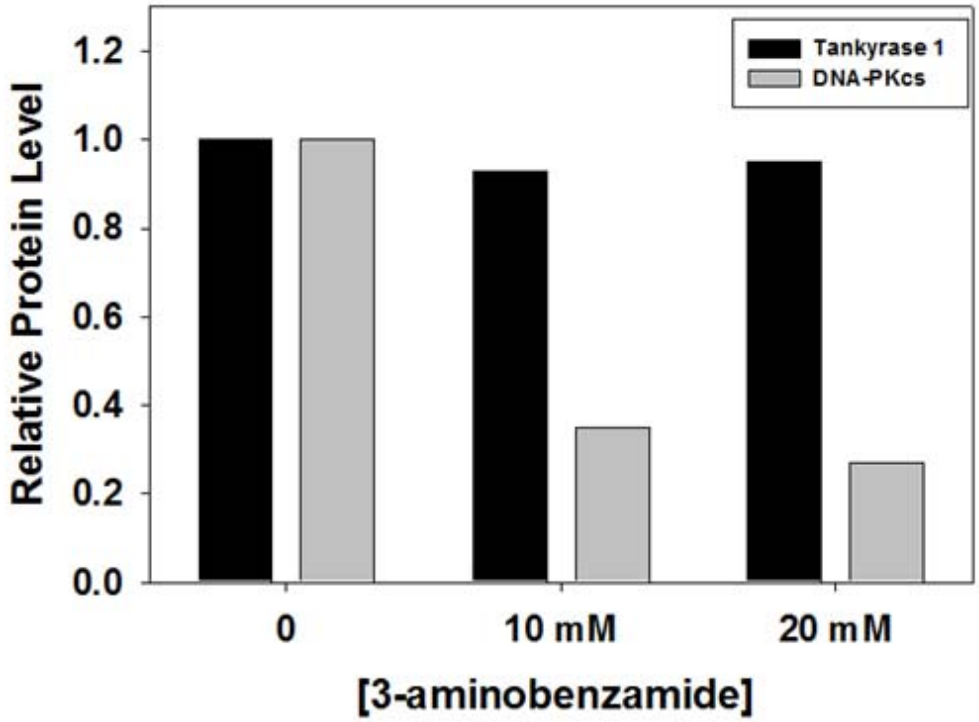


Figure 1. PARP inhibition results in DNA-PKcs depletion. Treatment with high concentrations of the general PARP inhibitor 3-AB (10 and 20 mM) resulted in lower levels of DNA-PKcs protein as compared to untreated controls; tankyrase 1 protein levels were not affected.

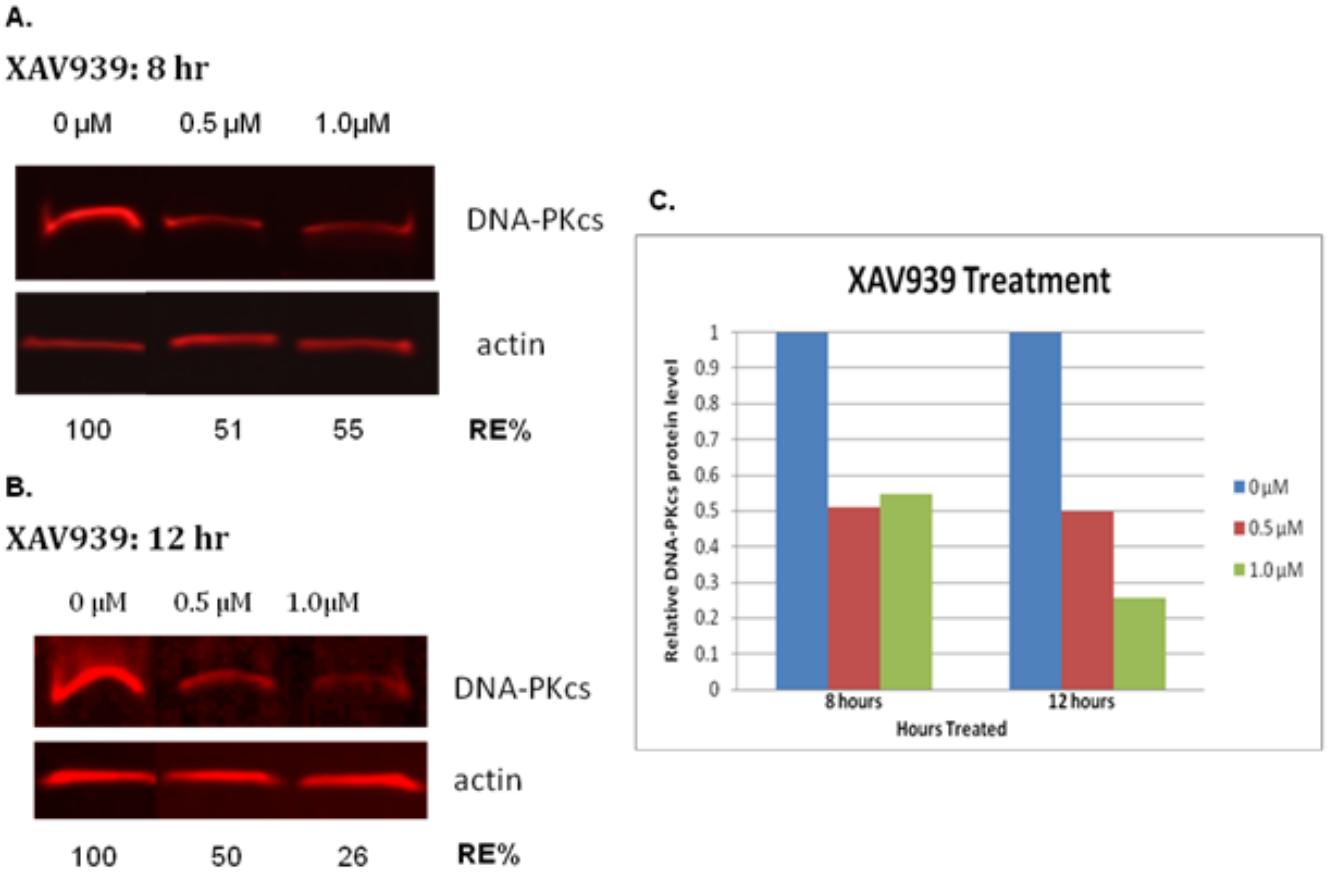


Figure 2. Inhibition of tankyrase PARP activity *decreases* DNA-PKcs protein levels. (A) Western blot. Treatment of human lymphoblasts with the tankyrase PARP domain inhibitor XAV939 (0.5 or 1.0 µM) for 8 hr reduced DNA-PKcs protein levels to ~ 50% of the relative DMSO control (RE%). **(B) Western blot** XAV939 treatment (1.0 µM) for 12 hr reduced DNA-PKcs levels to approximately 25% of the relative DMSO control. **(C)** Graphical depiction of the respective western blots, graph does not depict multiple averages (no SEM).

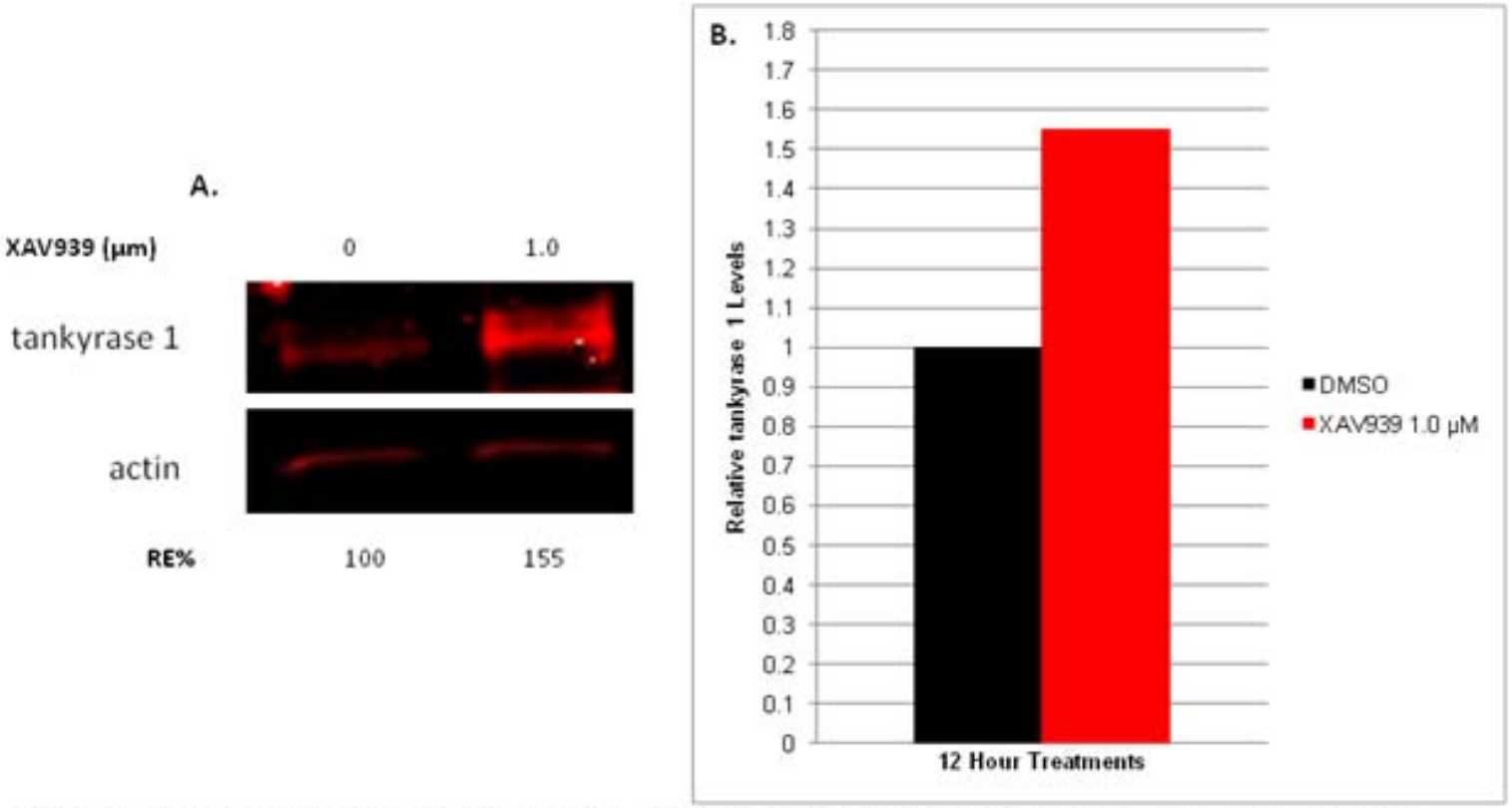


Figure 3. Inhibition of tankyrase PARP activity *increases* tankyrase 1 protein levels. (A) Tankyrase 1 levels increased over DMSO controls in cells treated with XAV939, providing evidence that DNA-PKcs protein stability relies on the catalytic function of tankyrase 1. (RE) Relative Expression. **(B)** Graphical depiction of the Western blot (A), does not represent multiple averages (no SEM).

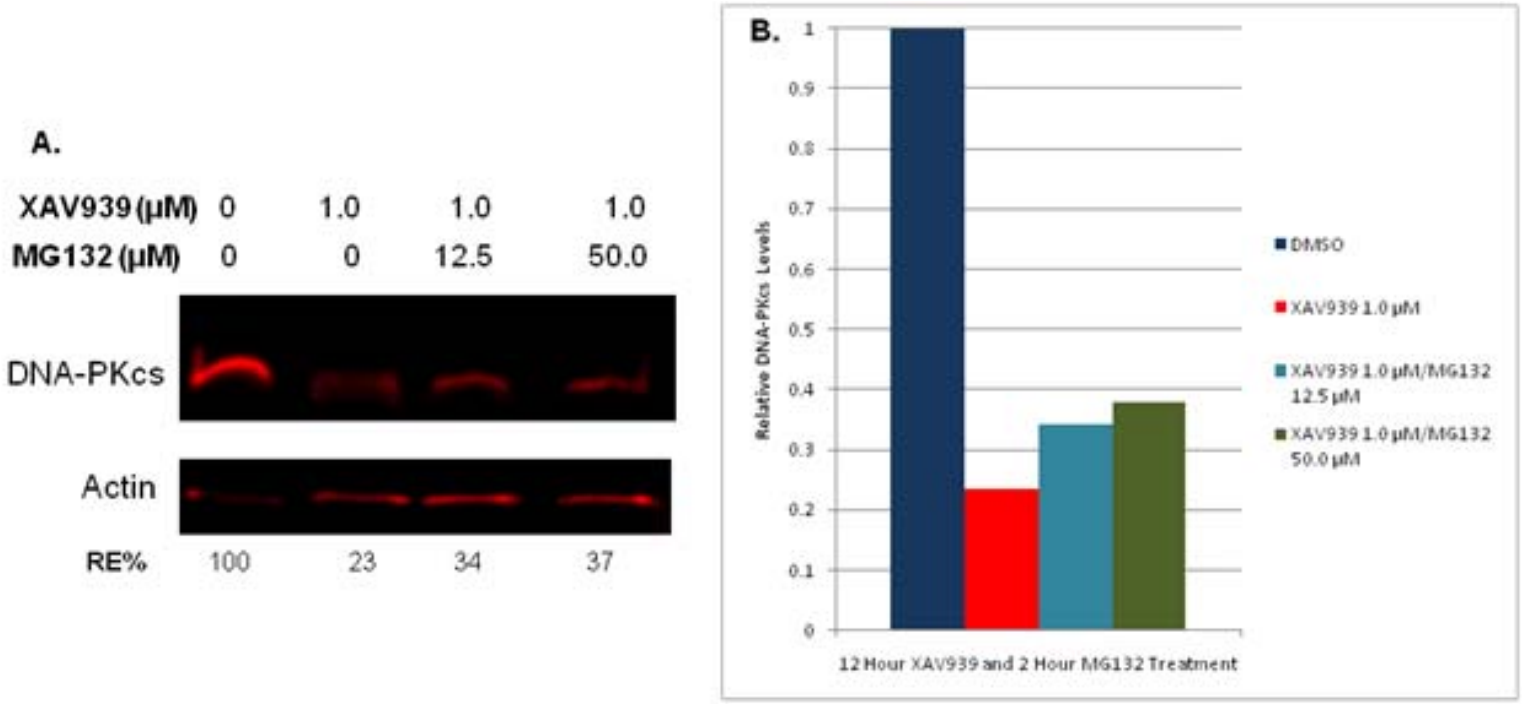


Figure 4. Proteosome inhibition facilitates DNA-PKcs protein recovery. (A) Western blot. Comparison of 12 hour XAV939 treatment alone to XAV939/MG132 combined treatments (12 hour and 2 hour respectively) reveals recovery of DNA-PKcs protein, suggesting tankyrase 1 prevents DNA-PKcs proteolytic degradation (RE% - Relative Expression). **(B)** Graphical depiction of the Western blot (A), does not represent multiple averages (no SEM).

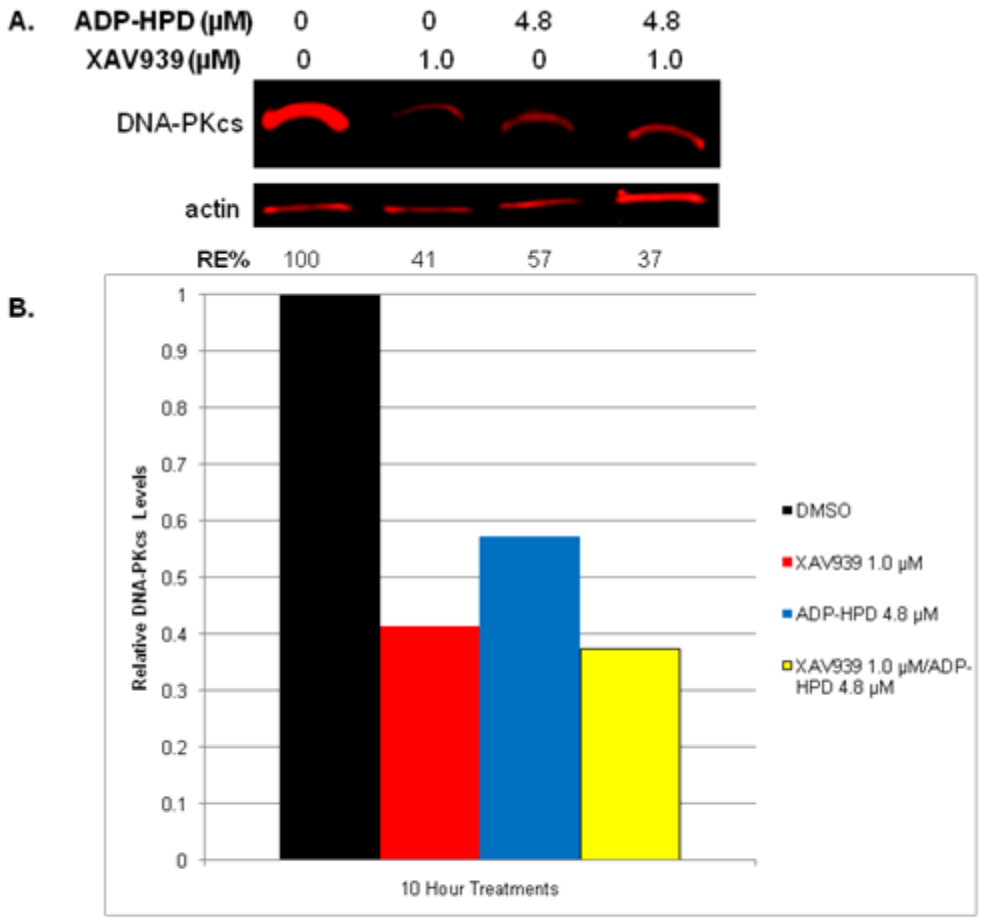


Figure 5. Inhibition of PARG activity reduces DNA-PKcs and protein levels. (A) Western blot. Treatment with the PARG inhibitor ADP-HPD for 10 hours prevents removal of pADPr from PARP modified proteins and resulted in reduction of DNA-PKcs levels relative to the DMSO treated control. The combination treatment of both ADP-HPD and XAV939 (10 hr) resulted in reduction of DNA-PKcs, similar to XAV939 treatment alone (Figure 3). **(B)** Graphical depiction of the Western blot (A), does not represent multiple averages (no SEM).

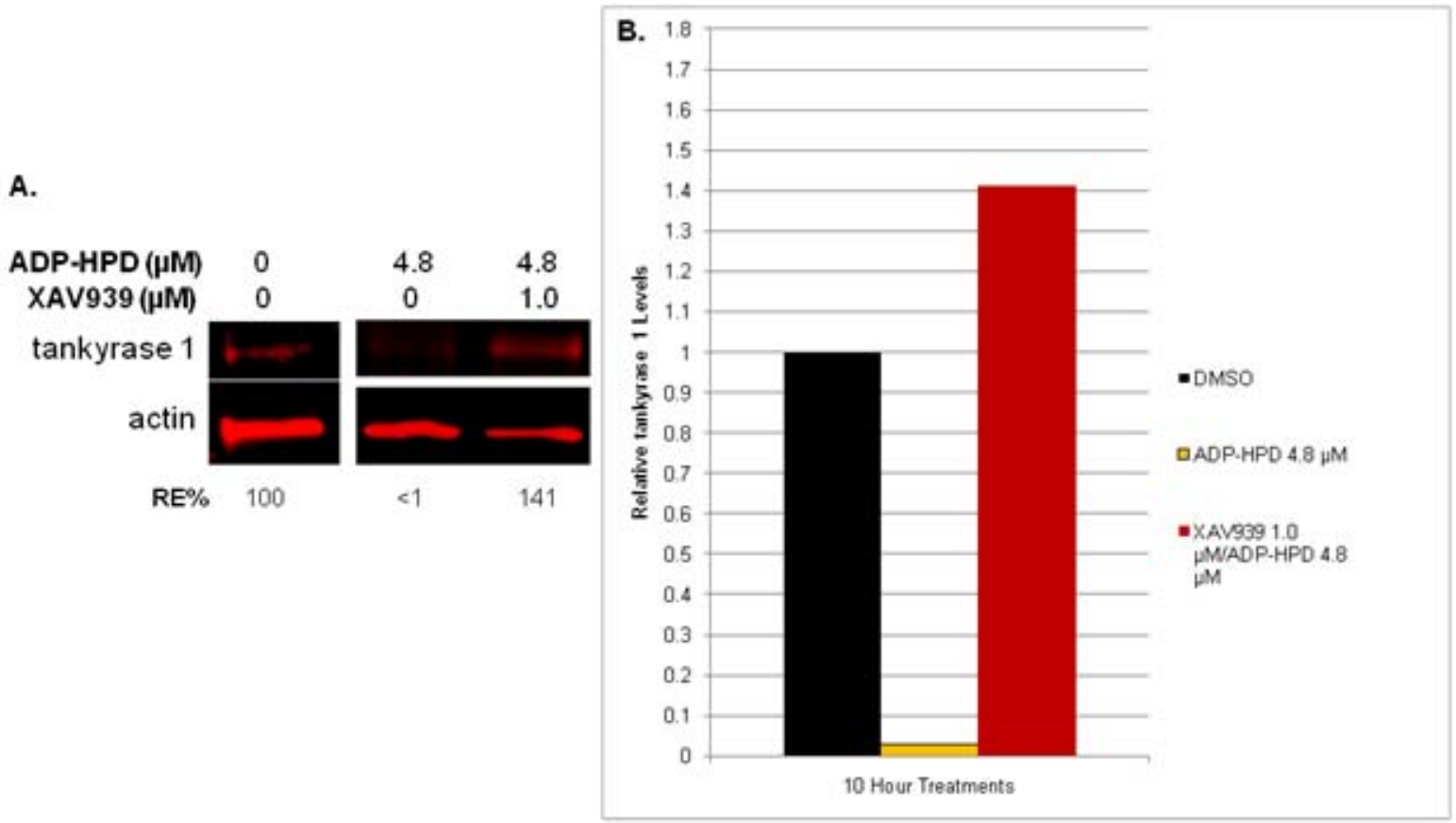


Figure 6. Inhibition of PARG activity reduces tankyrase 1 protein levels. (A) Western blot. PARG inhibitor administered at 4.8 μM also decreased protein levels of tankyrase 1 compared to the DMSO treated control. ADP-HPD/XAV939 combined treatment (4.8 μM and 1.0 μM respectively) resulted in elevated tankyrase 1 levels, similar to XAV939 treatment (1.0 μM) alone (Figure 3) (RE% - Relative Expression). **(B)** Graphical depiction of Western blot, does not represent multiple averages (no SEM).

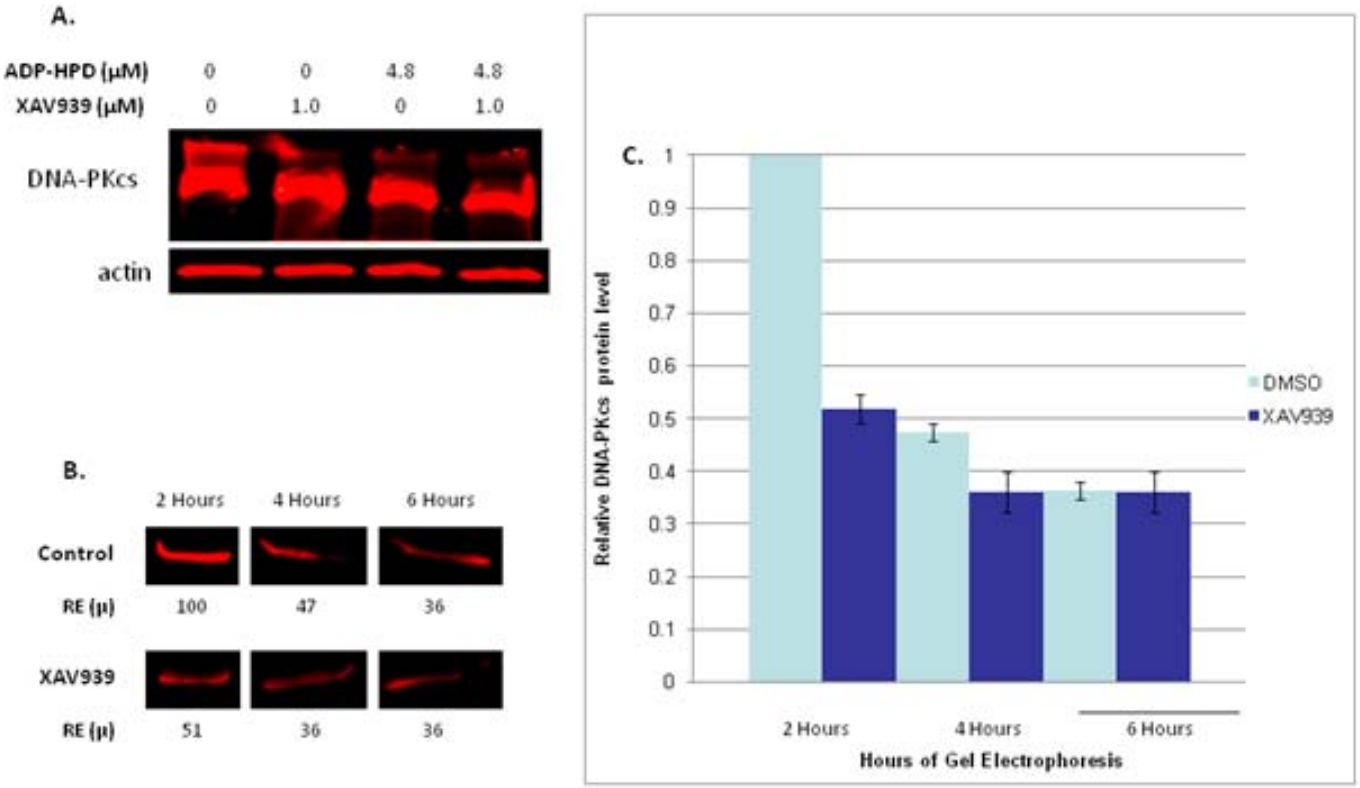


Figure 7. Identification of tankyrase-dependent high molecular weight forms of DNA-PKcs. (A) Over-exposed Western blot reveals high molecular weight forms of DNA-PKcs, which are dependent upon the PARsylating function of tankyrase 1. A high molecular weight "smear" appears above the primary DNA-PKcs band in the untreated control, which is absent with XAV939 treatment; a lower molecular weight "smear" appears below the primary DNA-PKcs band with XAV939 treatment (degradation products). **(B)** Western blot analysis of same samples (not over-exposed) and quantification of DNA-PKcs protein levels following 2, 4 and 6 hour SDS-PAGE run times for DMSO treated controls and XAV939 treated samples to separate high molecular weight forms. The control contains a large amount of DNA-PKcs in a modified, high molecular weight form, as seen by the reduction in DNA-PKcs band intensities over the longer run times (2hr compared to 4 and 6 hr run times; $p < 0.001$). No significant reduction of DNA-PKcs band intensity over longer run times was observed in XAV939 treated samples ($p > 0.05$). Relative expression (RE μ) are an average of two western blot analyses, which were used to calculate SEMs. P-values were determined by a *t test to compare two means*. **(C)** Graphical depiction of Western blot in panel B with associated SEMs.

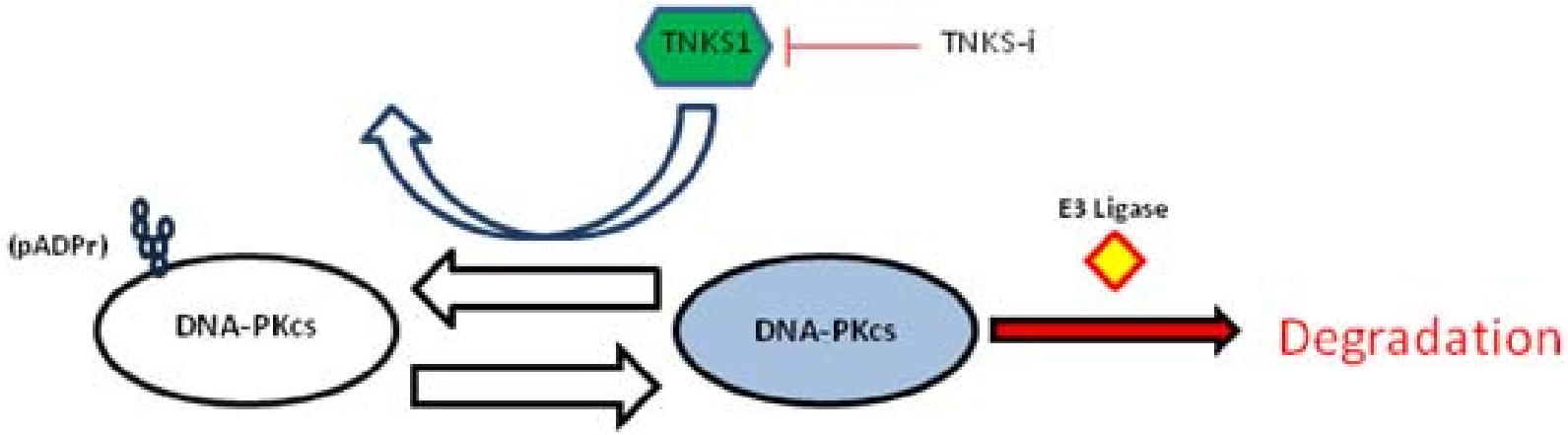


Figure 8. XAV939 inhibits tankyrase PARsylating activity. DNA-PKcs protein is not pADPr modified and forced toward proteasome-mediated degradation.

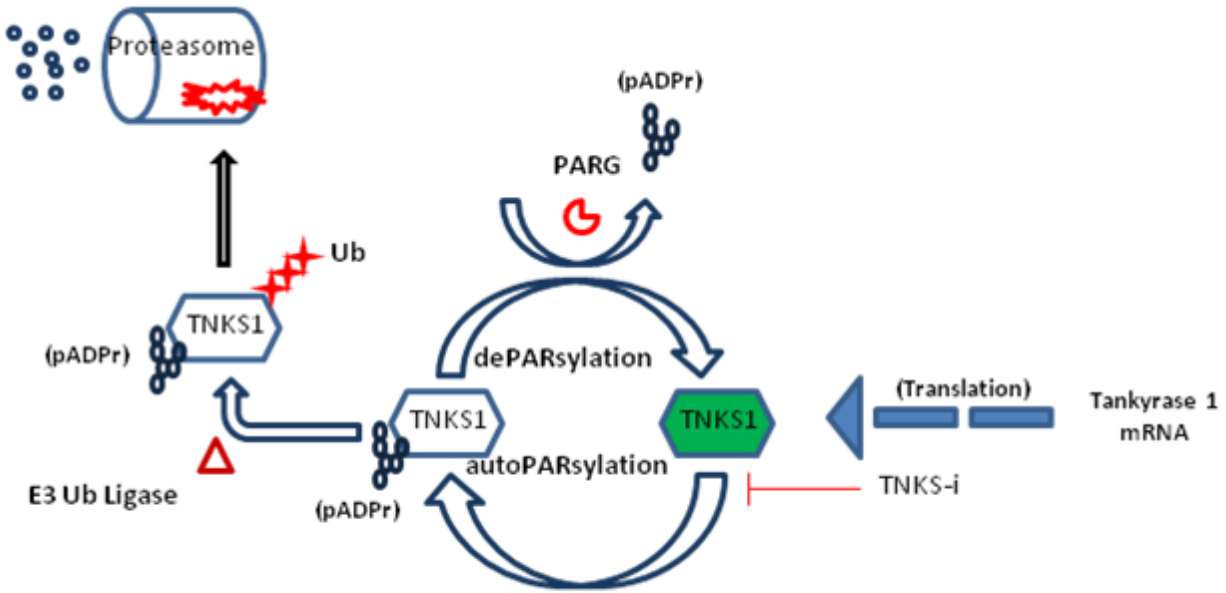


Figure 9. Inhibition of tankyrase 1 catalytic activity blocks automodification and the potential for ubiquitination. Tankyrase 1 protein levels are increased as the result of continued transcript translation and minimal protein degradation.

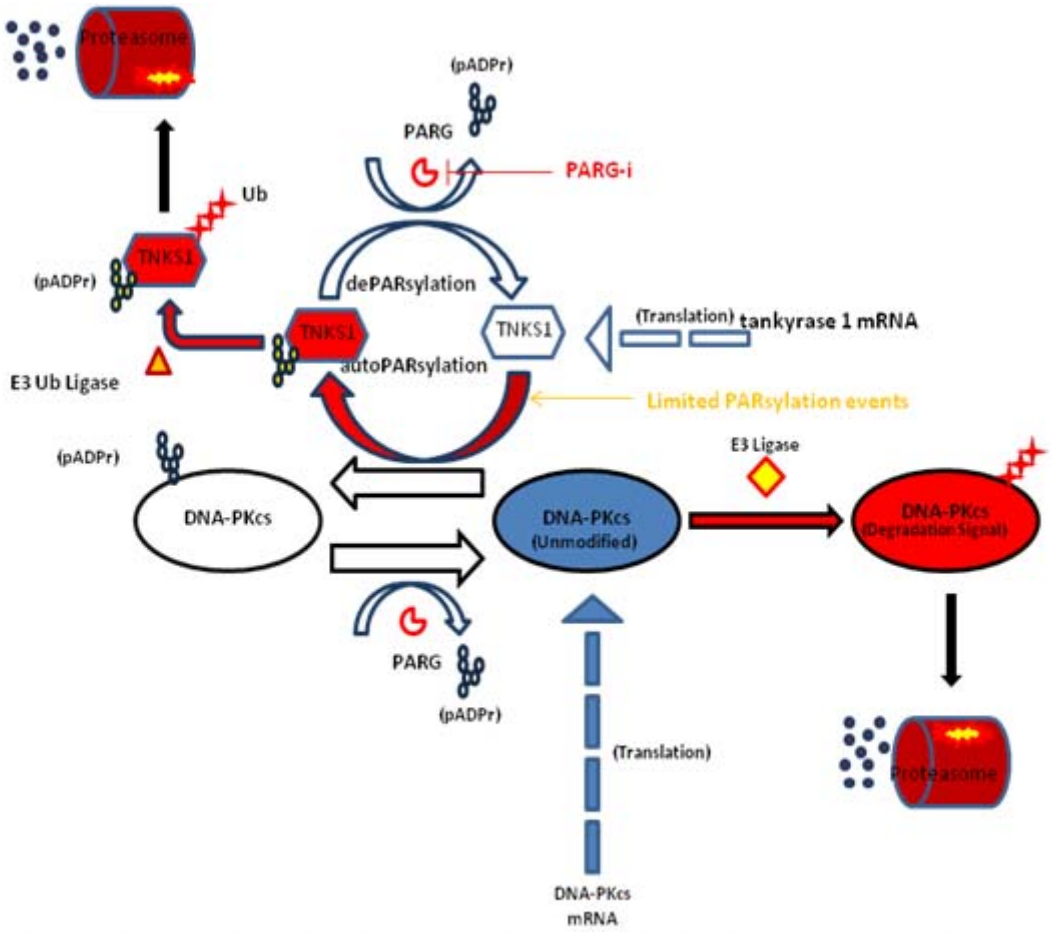


Figure 10. ADP-HPD PARG inhibition rapidly accumulates tankyrase 1 in an automodified form. The modified tankyrases are susceptible to ubiquitination and subsequent degradation. Consequentially, the tankyrase 1 protein is effectively and rapidly depleted.

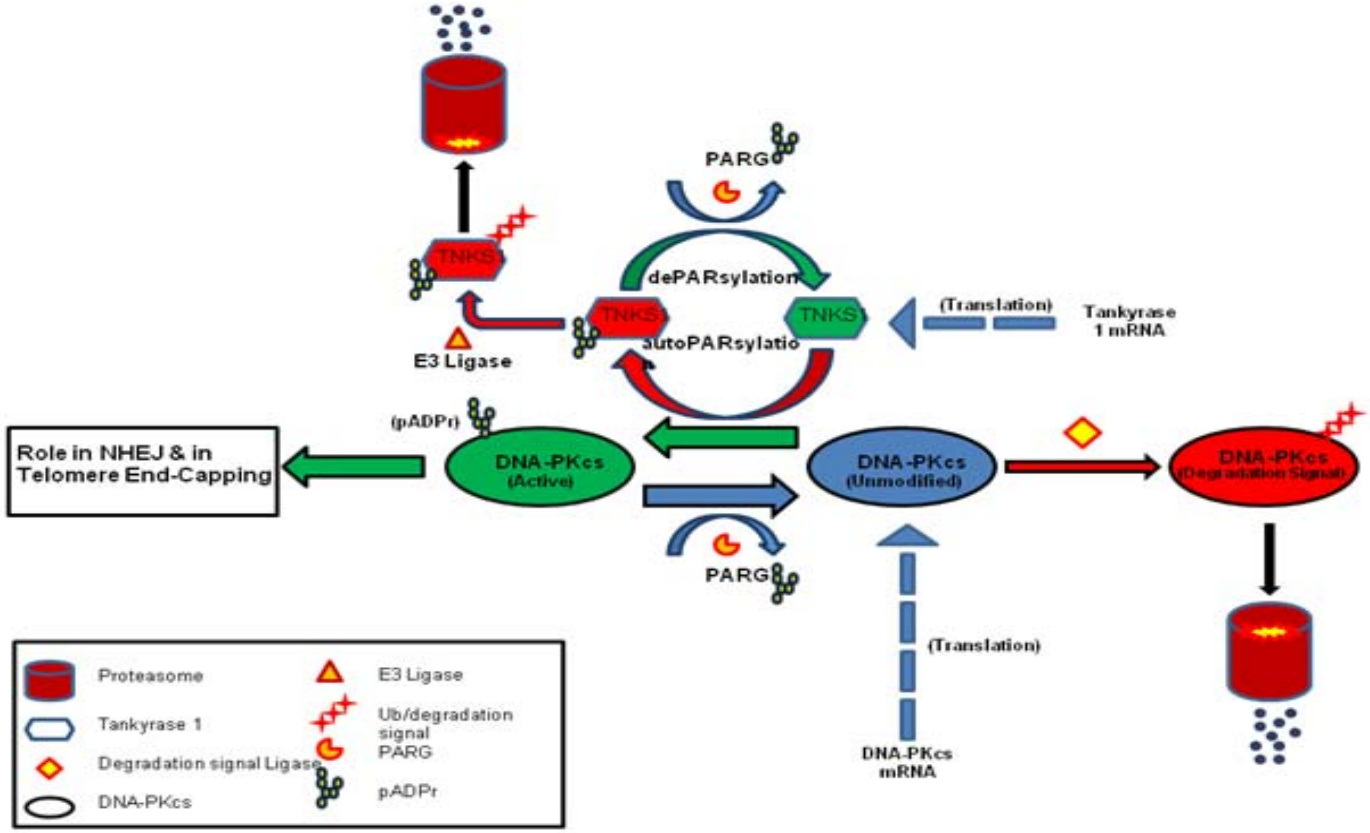


Figure 11. Model of DNA-PKcs existence in three dynamic pools; unmodified (blue), PARsylated (green), and marked for proteasome-mediated degradation (red). Unmodified pools of DNA-PKcs are PARsylated in a tankyrase 1 catalytic activity dependent manner (green arrow), a modification that can be reversed via PARG activity, resulting in unmodified DNA-PKcs (blue arrow). Once tankyrase 1 has PARsylated DNA-PKcs, tankyrase 1 auto-PARsylates (red) to dissociate from the multimerized-tankyrase complex. Tankyrase 1 relies on PARG activity to remove the auto-pADPr group and remain in an active state (green), otherwise, tankyrase 1 is ubiquitinated and targeted for degradation. The unmodified pool of DNA-PKcs is subject to being marked with a proteasome tag and subsequent degradation if not PARsylated by tankyrase 1. In conjunction with translation of new DNA-PKcs mRNA, there is a dynamic for DNA-PKcs PARsylation that shields a subpopulation of DNA-PKcs from degradation and perhaps represents the pool of kinase active DNA-PKcs. If this dynamic is disrupted by tankyrase 1 depletion or inhibition of its PARP catalytic activity, DNA-PKcs accumulates in the unmodified form and is forced to the right (degradation) resulting in depletion of DNA-PKcs protein.

REFERENCES

4.6.0 References

1. Dregalla RC, Zhou J, Idate RR, Battaglia CL, Liber HL, Bailey SM: **Regulatory roles of tankyrase 1 at telomeres and in DNA repair: suppression of T-SCE and stabilization of DNA-PKcs**. *Aging (Albany NY)* 2010, **2**(10):691-708.
2. Smith S, de Lange T: **Tankyrase promotes telomere elongation in human cells**. *Curr Biol* 2000, **10**(20):1299-1302.
3. Smith S, Giriat I, Schmitt A, de Lange T: **Tankyrase, a poly(ADP-ribose) polymerase at human telomeres**. *Science* 1998, **282**(5393):1484-1487.
4. Chang P, Coughlin M, Mitchison TJ: **Interaction between Poly(ADP-ribose) and NuMA Contributes to Mitotic Spindle Pole Assembly**. *Mol Biol Cell* 2009, **20**(21):4575-4585.
5. Sbodio JI, Chi NW: **Identification of a tankyrase-binding motif shared by IRAP, TAB182, and human TRF1 but not mouse TRF1. NuMA contains this RXXPDG motif and is a novel tankyrase partner**. *J Biol Chem* 2002, **277**(35):31887-31892.
6. Ame JC, Spenlehauer C, de Murcia G: **The PARP superfamily**. *Bioessays* 2004, **26**(8):882-893.
7. Smith S: **The world according to PARP**. *Trends Biochem Sci* 2001, **26**(3):174-179.
8. Ruscetti T, Lehnert BE, Halbrook J, Le Trong H, Hoekstra MF, Chen DJ, Peterson SR: **Stimulation of the DNA-dependent protein kinase by poly(ADP-ribose) polymerase**. *Journal of Biological Chemistry* 1998, **273**(23):14461-14467.
9. De Rycker M, Price CM: **Tankyrase polymerization is controlled by its sterile alpha motif and poly(ADP-ribose) polymerase domains**. *Mol Cell Biol* 2004, **24**(22):9802-9812.
10. De Rycker M, Venkatesan RN, Wei C, Price CM: **Vertebrate tankyrase domain structure and sterile alpha motif (SAM)-mediated multimerization**. *Biochem J* 2003, **372**(Pt 1):87-96.
11. Yeh TY, Meyer TN, Schwesinger C, Tsun ZY, Lee RM, Chi NW: **Tankyrase recruitment to the lateral membrane in polarized epithelial cells: regulation by cell-cell contact and protein poly(ADP-ribosyl)ation**. *Biochem J* 2006, **399**(3):415-425.
12. Juarez-Salinas H, Sims JL, Jacobson MK: **Poly(ADP-ribose) levels in carcinogen-treated cells**. *Nature* 1979, **282**(5740):740-741.

13. Alvarez-Gonzalez R, Jacobson MK: **Characterization of polymers of adenosine diphosphate ribose generated in vitro and in vivo**. *Biochemistry* 1987, **26**(11):3218-3224.
14. Davidovic L, Vodenicharov M, Affar EB, Poirier GG: **Importance of poly(ADP-ribose) glycohydrolase in the control of poly(ADP-ribose) metabolism**. *Exp Cell Res* 2001, **268**(1):7-13.
15. Jacobson EL, Antol KM, Juarez-Salinas H, Jacobson MK: **Poly(ADP-ribose) metabolism in ultraviolet irradiated human fibroblasts**. *J Biol Chem* 1983, **258**(1):103-107.
16. Miwa M, Sugimura T: **Splitting of the ribose-ribose linkage of poly(adenosine diphosphate-robose) by a calf thymus extract**. *J Biol Chem* 1971, **246**(20):6362-6364.
17. Ueda K, Hayaishi O: **ADP-ribosylation**. *Annu Rev Biochem* 1985, **54**:73-100.
18. Wielckens K, Schmidt A, George E, Bredehorst R, Hilz H: **DNA fragmentation and NAD depletion. Their relation to the turnover of endogenous mono(ADP-ribosyl) and poly(ADP-ribosyl) proteins**. *J Biol Chem* 1982, **257**(21):12872-12877.
19. Hsiao SJ, Smith S: **Tankyrase function at telomeres, spindle poles, and beyond**. *Biochimie* 2008, **90**(1):83-92.
20. Chi NW, Lodish HF: **Tankyrase is a golgi-associated mitogen-activated protein kinase substrate that interacts with IRAP in GLUT4 vesicles**. *J Biol Chem* 2000, **275**(49):38437-38444.
21. Huang SM, Mishina YM, Liu S, Cheung A, Stegmeier F, Michaud GA, Charlat O, Wiellette E, Zhang Y, Wiessner S *et al*: **Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling**. *Nature* 2009, **461**(7264):614-620.
22. Sbodio JI, Lodish HF, Chi NW: **Tankyrase-2 oligomerizes with tankyrase-1 and binds to both TRF1 (telomere-repeat-binding factor 1) and IRAP (insulin-responsive aminopeptidase)**. *Biochem J* 2002, **361**(Pt 3):451-459.
23. Chang P, Coughlin M, Mitchison TJ: **Tankyrase-1 polymerization of poly(ADP-ribose) is required for spindle structure and function**. *Nat Cell Biol* 2005, **7**(11):1133-1139.
24. Gaglio T, Saredi A, Bingham JB, Hasbani MJ, Gill SR, Schroer TA, Compton DA: **Opposing motor activities are required for the organization of the mammalian mitotic spindle pole**. *J Cell Biol* 1996, **135**(2):399-414.
25. Merdes A, Ramyar K, Vechio JD, Cleveland DW: **A complex of NuMA and cytoplasmic dynein is essential for mitotic spindle assembly**. *Cell* 1996, **87**(3):447-458.
26. Lade AG, Monga SP: **Beta-catenin signaling in hepatic development and progenitors: Which way does the WNT blow?** *Dev Dyn* 2011, **240**(3):486-500.
27. Dajani R, Fraser E, Roe SM, Yeo M, Good VM, Thompson V, Dale TC, Pearl LH: **Structural basis for recruitment of glycogen synthase kinase 3beta to the axin-APC scaffold complex**. *Embo J* 2003, **22**(3):494-501.
28. Ding Y, Dale T: **Wnt signal transduction: kinase cogs in a nano-machine?** *Trends Biochem Sci* 2002, **27**(7):327-329.

29. Liu C, Li Y, Semenov M, Han C, Baeg GH, Tan Y, Zhang Z, Lin X, He X: **Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism**. *Cell* 2002, **108**(6):837-847.
30. Xing Y, Clements WK, Kimelman D, Xu W: **Crystal structure of a beta-catenin/axin complex suggests a mechanism for the beta-catenin destruction complex**. *Genes Dev* 2003, **17**(22):2753-2764.
31. Gagne JP, Isabelle M, Lo KS, Bourassa S, Hendzel MJ, Dawson VL, Dawson TM, Poirier GG: **Proteome-wide identification of poly(ADP-ribose) binding proteins and poly(ADP-ribose)-associated protein complexes**. *Nucleic Acids Res* 2008, **36**(22):6959-6976.
32. Pleschke JM, Kleczkowska HE, Strohm M, Althaus FR: **Poly(ADP-ribose) binds to specific domains in DNA damage checkpoint proteins**. *J Biol Chem* 2000, **275**(52):40974-40980.
33. Milam KM, Thomas GH, Cleaver JE: **Disturbances in DNA precursor metabolism associated with exposure to an inhibitor of poly(ADP-ribose) synthetase**. *Exp Cell Res* 1986, **165**(1):260-268.
34. Purnell MR, Whish WJ: **Novel inhibitors of poly(ADP-ribose) synthetase**. *Biochem J* 1980, **185**(3):775-777.
35. Rock KL, Gramm C, Rothstein L, Clark K, Stein R, Dick L, Hwang D, Goldberg AL: **Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules**. *Cell* 1994, **78**(5):761-771.
36. Okita N, Ashizawa D, Ohta R, Abe H, Tanuma S: **Discovery of novel poly(ADP-ribose) glycohydrolase inhibitors by a quantitative assay system using dot-blot with anti-poly(ADP-ribose)**. *Biochem Biophys Res Commun* 2010, **392**(4):485-489.
37. Nooren IM, Thornton JM: **Structural characterisation and functional significance of transient protein-protein interactions**. *J Mol Biol* 2003, **325**(5):991-1018.
38. Dragovich PS, Zhou R, Skalitzky DJ, Fuhrman SA, Patick AK, Ford CE, Meador JW, 3rd, Worland ST: **Solid-phase synthesis of irreversible human rhinovirus 3C protease inhibitors. Part 1: Optimization of tripeptides incorporating N-terminal amides**. *Bioorg Med Chem* 1999, **7**(4):589-598.
39. Alvarez-Gonzalez R, Althaus FR: **Poly(ADP-ribose) catabolism in mammalian cells exposed to DNA-damaging agents**. *Mutat Res* 1989, **218**(2):67-74.
40. Ruscetti T, Lehnert BE, Halbrook J, LeTrong H, Hoekstra MF, Chen DJ, Peterson SR: **Stimulation of the DNA-dependent protein kinase by poly(ADP-ribose) polymerase**. *Journal of Biological Chemistry* 1998, **273**(23):14461-14467.
41. Nooren IM, Thornton JM: **Diversity of protein-protein interactions**. *Embo J* 2003, **22**(14):3486-3492.
42. Kaminker PG, Kim SH, Taylor RD, Zebarjadian Y, Funk WD, Morin GB, Yaswen P, Campisi J: **TANK2, a new TRF1-associated poly(ADP-ribose) polymerase, causes rapid induction of cell death upon overexpression**. *J Biol Chem* 2001, **276**(38):35891-35899.

43. Galande S, Kohwi-Shigematsu T: **Poly(ADP-ribose) polymerase and Ku autoantigen form a complex and synergistically bind to matrix attachment sequences**. *J Biol Chem* 1999, **274**(29):20521-20528.
44. Peng Y, Woods RG, Beamish H, Ye R, Lees-Miller SP, Lavin MF, Bedford JS: **Deficiency in the catalytic subunit of DNA-dependent protein kinase causes down-regulation of ATM**. *Cancer Res* 2005, **65**(5):1670-1677.
45. Cook BD, Dynek JN, Chang W, Shostak G, Smith S: **Role for the related poly(ADP-Ribose) polymerases tankyrase 1 and 2 at human telomeres**. *Mol Cell Biol* 2002, **22**(1):332-342.
46. Rattan SI: **Synthesis, modification and turnover of proteins during aging**. *Adv Exp Med Biol* 2010, **694**:1-13.
47. Burger AM, Kona F, Amemiya Y, Gao Y, Bacopulos S, Seth AK: **Role of the BCA2 ubiquitin E3 ligase in hormone responsive breast cancer**. *Open Cancer J* 2010, **3**(1):116-123.
48. Slama JT, Aboul-Ela N, Goli DM, Cheesman BV, Simmons AM, Jacobson MK: **Specific inhibition of poly(ADP-ribose) glycohydrolase by adenosine diphosphate (hydroxymethyl)pyrrolidinediol**. *J Med Chem* 1995, **38**(2):389-393.
49. Slama JT, Aboul-Ela N, Jacobson MK: **Mechanism of inhibition of poly(ADP-ribose) glycohydrolase by adenosine diphosphate (hydroxymethyl)pyrrolidinediol**. *J Med Chem* 1995, **38**(21):4332-4336.
50. Althaus FR, Richter C: **ADP-ribosylation of proteins. Enzymology and biological significance**. *Mol Biol Biochem Biophys* 1987, **37**:1-237.
51. Levy JA, Virolainen M, Defendi V: **Human lymphoblastoid lines from lymph node and spleen**. *Cancer* 1968, **22**(3):517-524.
52. Veuger SJ, Curtin NJ, Richardson CJ, Smith GC, Durkacz BW: **Radiosensitization and DNA repair inhibition by the combined use of novel inhibitors of DNA-dependent protein kinase and poly(ADP-ribose) polymerase-1**. *Cancer Res* 2003, **63**(18):6008-6015.
53. Veuger SJ, Curtin NJ, Smith GC, Durkacz BW: **Effects of novel inhibitors of poly(ADP-ribose) polymerase-1 and the DNA-dependent protein kinase on enzyme activities and DNA repair**. *Oncogene* 2004, **23**(44):7322-7329.
54. Canudas S, Houghtaling BR, Kim JY, Dynek JN, Chang WG, Smith S: **Protein requirements for sister telomere association in human cells**. *Embo J* 2007, **26**(23):4867-4878.

CHAPTER 5

Future Directions – Implications of poly(ADP-ribosyl)ation in Non-Homologous End-Joining pathways

The following figures are original productions contributed by the author and are unpublished.

5.1.0 pADPr is a diverse posttranslational protein modification with multiple functional implications

We have found that the telomeric PARP family member PARP-5a, better known as tankyrase 1, operates as a key regulator of DNA-PKcs protein stability via poly(ADP-ribosyl)ation (pADPr/PAR) modification [1]. In this modified form, DNA-PKcs maintains a proteasome-resistant conformation that is dynamically regulated by the contributions of tankyrase 1, poly(ADP-ribose) glycohydrolase (PARG) and the proteasome [1]. When tankyrase 1 is depleted or catalytically inhibited, intracellular DNA-PKcs is rapidly de-PARsylated (via PARG activity) and so becomes a substrate for proteasome-mediated degradation, resulting in the rapid and significant reduction of DNA-PKcs protein.

Interestingly, there are examples of tankyrase-dependent pADPr protein modifications that result in destabilization and degradation of the modified protein [2-5]. In the case of DNA-PKcs however, pADPr addition is a positive regulator of the protein, joining a select few other proteins which respond to PARsylation in a similar fashion [6,

7]. So, although not the first protein found to be stabilized via pADPr addition, the story regarding DNA-PKcs and pADPr -modification is just emerging and so, is incomplete.

In a manner similar to phosphorylation, the addition of a pADPr chain to a protein is thought to initiate a conformational change in the acceptor protein via the net two negative charges of each APD-ribose monomer in the chain [8, 9]. Following pADPr addition, the fate of the pADPr-modified proteins have been shown to include: additional/secondary modification, recruitment of additional proteins, scaffolding between proteins and/or activation of the PARsylated protein [6-15].

Ubiquitination is one such possible secondary protein modification following pADPr-addition, as seen in the classic Telomere Repeat-binding Factor 1 (TRF1)-pADPr model [13]. Here, TRF1 is pADPr-modified by tankyrase 1 to release TRF1 from the telomere, allowing access to telomerase as well as progression of the replication fork through the telomere [13]. Once released, PARsyalted-TRF1 becomes a substrate for E3 ubiquitin ligase and subsequent proteasome degradation if not dePARsyalted via PARG activity [13].

A classic example of protein recruitment to a pADPr-modified protein is that of PARP-1 in the base-excision repair and single-strand break repair (BER/SSB) pathways, where, hypo-autoPARsylation is used to electrostatically recruit Ligase III and XRCC1 to the DNA-damage site (reviewed in [8]). Immediately following, hyper-autoPARsylation stimulates PARP-1 release from the DNA via heavy negative electrostatic repulsion between the pADPr groups and the negatively charged DNA (reviewed in [9]). Thus, PARP-1 utilizes pADPr as a mechanism for DNA-repair protein recruitment to the

damage site, releasing itself from the DNA once recruitment is completed, allowing for the appropriate repair proteins to access the damage site.

A more recently described novel feature of pADPr addition is utilization of the electrostatic nature of the group to facilitate non-covalent scaffolding between adjacent proteins to maintain stable protein-protein interactions. The nuclear mitotic apparatus protein (NuMA) has been identified as a protein that requires tankyrase 1-dependent PARsylation for appropriate complex organization [6]. Each NuMA monomer recruited to the greater NuMA complex is PARsylated and interacts with the adjacent NuMA and/or associated tankyrase 1 monomers non-covalently through the pAPDr chain [6]. When tankyrase 1 function is disrupted, the NuMA complex does not organize properly, leading to failure of spindle-pole organization and dynamics. Earlier studies identified a variety of proteins that contain pADPr-interaction motifs, which are now recognized as potential players in protein complex scaffolding [16, 17]. Interestingly, amongst the proteins found to contain pADPr-interacting motifs are the non-homologous end-joining (NHEJ) proteins Ku70 and DNA-PKcs [17].

Protein modification by pADPr-addition has also been shown to activate some acceptor proteins. The transcription factor, nuclear factor of activated T-cells (NFAT), accepts pADPr via PARP-1 activity and is positively regulated as a result, increasing NFAT-dependent transcription [7]. Further, *in vitro* studies have demonstrated that DNA-PKcs is capable of pADPr-modification, and the corresponding functional consequence of this modification is increased DNA-PKcs kinase activity [12].

It is clear that pADPr modification of proteins can have multiple purposes. The addition of pADPr can result in subsequent protein modification by an additional

enzymes (i.e. E3 ubiquitin ligase) [3, 4, 13], recruitment of additional proteins electrostatically (i.e., PARP-1) [15, 18-20], non-covalent pADPr-dependent protein interactions (i.e. NuMA) [6, 11] or protein activation (i.e. NFAT & DNA-PKcs) [7, 12]. Thus, the role of DNA-PKcs PARsylation very likely extends beyond protein stabilization. Currently, we understand that modification of DNA-PKcs via tankyrase 1-dependent PARsylation has a dynamic, stabilizing role by protecting DNA-PKcs from proteasome-mediated degradation. However, the functional implication of this modification has yet to be established.

5.2.0 pADPr-modification in DNA-PK integrity

5.2.1 DNA-PK components interact with pADPr and are PARP substrates

Recruitment of the Ku70/Ku80 heterodimer to the site of a double-stranded break (DSB) is imperative for initiation of DNA-PK-mediated NHEJ [21]. The localization of the Ku70/80 heterodimer to a DSB results in the subsequent recruitment of DNA-PKcs, forming the DNA-PK holoenzyme (reviewed in [22]). However, the means of DNA-PKcs recruitment to the Ku heterodimer, holoenzyme stabilization and mechanism of its activation is not currently understood.

Studies have provided evidence of pADPr involvement in DNA-PK protein components [12, 16, 17]. Most recently, PARP-3 has been shown to be a key factor in recruitment and stabilization of XRCC4 & Ligase IV at the DSB following DNA-PK dissociation, possibly via pADPr-dependent processes [23]. Components of the DNA-PK holoenzyme have also been shown to accept pADPr *in vivo* [12, 16]. Additionally, Ku70 and DNA-PKcs possess non-covalent pADPr-interacting motifs, suggesting a potential mechanism for DNA-PK scaffolding & stabilization [17]. Based on these findings and

our characterization of tankyrase 1-mediated DNA-PKcs pADPr modification, we hypothesize that pADPr modification of the DNA-PK proteins contributes to DNA-PK function in NHEJ-mediated DSB-repair from three perspectives: *holoenzyme activation*, *protein recruitment and stabilization*.

5.2.2 DNA-PKcs kinase function in the DNA-PK holoenzyme requires tankyrase 1-dependent pADPr-modification

The first insight into a potential role for pADPr modified DNA-PKcs came from a previous study *in vitro* that demonstrated PARsylated DNA-PKcs possessed increased kinase activity compared to unmodified forms [12]. A protein complex immunoprecipitation (Co-IP) pull-down of PARP-1 identified DNA-PKcs as an intracellular binding partner, leading to an investigation as to the possible purpose of such a complex [12]. Analysis of PARP-1 catalytic activity revealed that DNA-PKcs is an acceptor of pADPr residues in a DNA-independent manner. Further, pADPr-modified forms of DNA-PKcs demonstrated an up-regulation in kinase function of approximately 7-fold over unmodified forms, regardless of the presence or absence of DNA in the reaction. This study provided preliminary evidence in support of DNA-PKcs being a pADPr-modified member of the proteome with functional implications.

Proteome-wide interrogation of pADPr-modified proteins has verified DNA-PKcs as a pADPr-modified protein [16]. We find that pADPr-modification is fundamental to maintaining DNA-PKcs protein stability, whereas *in vitro* studies submitted that there is a functional role for this modification in the regulation of DNA-PKcs kinase activity [12]. To date, evidence for the role of pADPr-modified DNA-PKcs from a functional biochemical perspective is lacking.

We postulate that tankyrase 1-dependent pADPr-modification of DNA-PKcs results in a proteasome resistant, kinase active form of the protein that is favored in DNA-PKcs-dependent biochemical processes, DNA-repair specifically. Considering that the role of DNA-PKcs is to serve as the active kinase in DNA-PK-mediated NHEJ we speculate that pADPr-modified DNA-PKcs exists in a dynamic proteasome resistant pool, providing a readily available 'reserve' of catalytically active DNA-PKcs 'primed' for immediate response to the Ku heterodimer (at the DSB) and the initiation of DNA-PK-mediated NHEJ.

5.2.3 PARP-1 activity in the recruitment of DNA-PKcs to the Ku heterodimer

The mechanism by which DNA-PKcs is recruited to the Ku heterodimer at the site of a DSB is not understood. We postulate that the recruitment of DNA-PKcs to the Ku heterodimer is a pADPr dependent event on two separate fronts. The first requisite is that DNA-PKcs exist in a pADPr-modified form. Provided this, we suspect an additional pADPr-modified component of DNA-PK is necessary for appropriate holoenzyme assembly and stabilization. We propose PARsylated Ku80 as a candidate to complete pADPr scaffolding within the DNA-PK holoenzyme, as it has also been shown to be a PARsylated member of the proteome [16].

The Ku heterodimer and PARP-1 each recognize the chemical signatures of exposed DNA-ends and so, it has been proposed they compete for DSB-ends and determine pathway choice: classical NHEJ or alternative PARP-1-mediated NHEJ [21]. Failure to assemble the DNA-PK holoenzyme is known to result in ligase IV-dependent NHEJ [24] and/or PARP-1-mediated 'alternative NHEJ' [25]. Consistent with such studies, we imagine that PARP-1 acts as an accessory protein to the DNA-PK

holoenzyme [26] by initiating recruitment of DNA-PKcs to the DNA-bound Ku heterodimer via pADPr-modification of Ku80.

Although association of the Ku heterodimer with the DSB reduces the affinity of PARP-1 for the exposed-DNA ends [21], it does not necessarily completely inhibit the ability for PARP-1 to briefly bind DNA. Once bound to the DSB, the Ku heterodimer translocates distal to the break [27], allowing restricted access of PARP-1 to the DSB. As a consequence, PARP-1 would form a short lived intermediate complex with the DSB and Ku80 and thus, the association of PARP-1 with the exposed ends of the break is not long enough to recruit PARP-1-associated DNA-repair proteins (as it presumably would in alternative NHEJ [25]). However, this model does associate Ku80 with a PARP family member in a site-specific manner, suggesting a mechanism by which the reported PARP-1-Ku80 complex forms [28] and Ku80 becomes PARsylated [17] (Figure 1).

We propose pADPr modification of Ku80, at the DSB specifically, may in fact serve as a mechanism for recruitment of DNA-PKcs and its subsequent activation, similar to PARP-1 automodification in the recruitment to SSB-repair proteins [25]. This model depicts the co-localization of PARP-1 and the Ku heterodimer at the DSB and the ensuing PARsylation of Ku80, providing a plausible basis for the 'DNA-dependent' nature of the DNA-dependent Protein Kinase; i.e., DNA-PK is activated upon recruitment and binding of DNA-PKcs to the pADPr-modified, DNA-bound Ku heterodimer.

5.2.4 pADPr acts as a scaffold between DNA-PK protein components

We propose that PARP-1, the 'competitor' with the Ku heterodimer for DSBs [21], co-localizes with the Ku heterodimer at the DSB and subsequently PARsylates Ku80. The pADPr-modification of Ku80 provides a practical means of recruitment for

DNA-PKcs. The pADPr modification has been shown to be essential in non-covalent scaffolding between proteins [6], which requires a pADPr interacting motif [17]. Interestingly, both Ku70 and DNA-PKcs have been shown to possess these motifs and are capable of forming complexes with PARsylated proteins [17].

We speculate that pADPr provides the core scaffolding structure within the DNA-PK holoenzyme and is necessary for stability and function of the enzyme in NHEJ-mediated DNA-repair. Consistent with our model, the pADPr chain extending from Ku80 (PARP-1-dependent) would serve as a platform for a strong non-covalent interaction with the pADPr-interacting motif(s) of DNA-PKcs. Ku80 in a PARsylated form favors recruitment of pADPr-modified DNA-PKcs (with enhanced kinase activity [12]) to complete pADPr-dependent DNA-PK scaffolding; the pADPr chain extending from DNA-PKcs interacts with the Ku70 pADPr-interacting motif [16, 17] (Figure 1).

5.2.5 pADPr modification in the activation, recruitment and stabilization of DNA-PK components

Here, we propose to investigate pADPr-modification as a key feature of functional DNA-PK. The model we intend to test involves pADPr-modification of Ku80 at DSBs specifically, perhaps by catalytic activity of PARP-1, co-localized to the DSB. The PARsylated heterodimer electrostatically recruits DNA-PKcs to the DSB site in a similar fashion as hypo-autoPARsylated PARP-1 recruits SSB-repair proteins (reviewed [8]). Next, the newly recruited DNA-PKcs protein interacts with the pADPr chain extending from Ku80 via its pADPr interaction motif. However, the affinity of DNA-PKcs for the Ku heterodimer is likely dependent on the PARsylated state of DNA-PKcs. DNA-PKcs recruited in PARsylated state will interact electrostatically (through the

pADPr chain) with the pADPr-interacting motif of Ku70. In this model, pADPr modification is responsible for DNA-PKcs recruitment to the Ku heterodimer, as well as the stabilization of the DNA-PK holoenzyme where it acts as a scaffold between the subunits. Further, DNA-PKcs may be recruited by Ku80-associated pADPr, but DNA-PK scaffolding would be incomplete without pADPr associated DNA-PKcs. Thus, the selection of DNA-PKcs in a PARsylated, kinase active state is necessary to establish a stable, catalytically active DNA-PK complex (Figure 1).

Corresponding to the model proposed above, deficiency in PARP-1 activity would result in failure to modify Ku80 via pADPr. The consequences of this deficiency would in many ways mirror that of failed DNA-PKcs pADPr modification or DNA-PKcs deficiency. Here, the inability to PARsylate Ku80 would deter DNA-PKcs recruitment and therefore fail to assemble the functional DNA-PK holoenzyme. In the event DNA-PKcs was recruited to the heterodimer by chance, the incomplete scaffolding of DNA-PK components would again result in a short lived DNA-PK complex. This model provides a potential explanation as to why the DNA-PK holoenzyme does not localize to DSBs preassembled; PARP-1-dependent PARsylation of Ku80 at the DSB provides reasoning as to the why the recruitment of DNA-PKcs to the heterodimer is 'DNA-dependent'.

Our overall goal is to move forward from our initial observation of tankyrase 1-dependent PARsylation of DNA-PKcs being required for proteasome resistance, to pADPr modification being critical for appropriate recruitment, scaffolding and activation of DNA-PK and 'classic-NHEJ'. We aim to investigate the involvement of pADPr-modified forms of DNA-PKcs in DNA-PK holoenzyme assembly and function. Further,

we aim to provide evidence of PARP-1 as a critical accessory protein to DNA-PK assembly.

Under conditions in which DNA-PKcs is not recruited in a pADPr-modified form, DNA-PK holoenzyme scaffolding is incomplete, unstable and catalytically inactive. Further, we postulate dysfunction of PARP-1 would result in deficient DNA-PKcs recruitment. However, in this case, successful binding of DNA-PKcs with the heterodimer despite lacking Ku80 pADPr modificaiton would result in a catalytically active but unstable holoenzyme. Our model presented here poses a mechanism for DNA-PK holoenzyme formation, stabilization and activation. Deficiencies in PARsylating events would result in the shift from DNA-PK-mediated end-joining to PARP-1 mediated DSB-repair.

5.3.0 PARP-1-mediated 'alternative' NHEJ or salvage pathway?

We propose a potential model for PARP-1 acting as an accessory protein to the DNA-PK holoenzyme assembly which can potentially explain the findings of previous studies suggesting PARP-1 and DNA-PK function in conjunction with one another for efficient end-joining repair [26], not as competitors as speculated by others [21]. This relationship between PARP-1 and DNA-PK may also involve PARP-2, as PARP-2 has been historically recognized as a PARP with 'redundant' function to PARP-1 [29-31].

In the absence of DNA-PKcs, Ku-mediated end-joining involving ligase IV is known to occur in a PARP-1 independent fashion [24, 32, 33]. Thus, although the mechanism of DSB-repair may differ, failure to recruit DNA-PKcs results in residual Ku-mediated, Ligase IV/XRCC4-dependent end-joining. Failure to resolve DSBs by DNA-PK and/or Ligase IV dependent end-joining results in the initiation of PARP-1-mediated

alternative-NHEJ, involving several PARP-1 interacting single-stranded break (SSB) repair proteins [25].

PARP-1 is known to interact with DNA for brief periods of time during its role in DNA-repair (SSB and DSB-repair). PARP-1 readily binds single-strand breaks in a hypo-PARsylated form for the purpose of recruiting the required SSB-repair components, XRCC1 and Ligase III [8, 9, 34]. DNA-bound PARP-1 is stimulated to further auto-PARsylate, resulting in hyper-PARsylated PARP-1 and release from the DNA through electrostatic repulsion [9, 35]. Considering DNA-bound PARP-1 stimulates the release of PARP-1, it is unlikely PARP-1 binding to the each side of the DSB is sustained for a long enough period of time to facilitate a true alternative NHEJ pathway.

Second, PARP-1 access to the DNA break may not be entirely inhibited by the DNA-bound Ku heterodimer. In fact, it has been postulated that PARP-1 functions concurrently with DNA-PK to achieve NHEJ [26]. The Ku heterodimer recognizes the chemistry of DSBs and translocates ~14bps upstream from the damage site [27]. Due to this, there is a degree of interference introduced by Ku that antagonizes PARP-1 association with the DNA. However, this does not necessarily mean PARP-1 is entirely blocked from the DNA-break. Rather, the affinity of PARP-1 for the exposed DNA ends may be reduced, owing to steric hinderance resulting from Ku-bound DNA. Further, the longer Ku70/80 is in association with DNA, the greater the chance for the heterodimer to dissociate from the DNA or translocate distal from the break and failure to form the DNA-PK holoenzyme.

Lastly, we know the alternative method of NHEJ is employed in the event of Ku, Ligase IV and in some cases DNA-PKcs deficiencies [36-39]. Compared to ‘classical’,

DNA-PK-mediated NHEJ, 'alternative' NHEJ is slow and error-prone, resulting in translocations between chromosomes [40-42]. The combination of these alternative NHEJ characteristics results in an elevated frequency of chromosomal translocations [40] and thus, increased carcinogenic potential (reviewed in [40, 43]). The question to be addressed is what drives the 'switch' from 'classic' to 'alternate' NHEJ pathways, and what contributes to the reduced fidelity of end-joining function in the latter pathway? Recent research proposes the machinery involved in 'alternative' NHEJ is comprised largely of SSB-repair proteins [25, 44, 45] and thus, we suspect PARP-1-mediated DSB repair may in fact be the result of SSB-repair events.

5.4.0 PARP-1-mediated DSB-repair via alternative NHEJ

5.4.1 Alternative NHEJ as DSB-salvage pathway mediated by SSB-repair events

In vitro analysis has shown PARP-1 affinity for exposed DNA-ends is higher than that of DNA-PK, indicating broken DNA (double- or single-stranded) will 'preferentially' bind PARP-1 over the DNA-PK holoenzyme [46]. However, inhibition of either PARP-1 or DNA-PKcs has been shown to decrease (not entirely diminish) DSB-repair capability to a remarkably similar extent, suggesting PARP-1 and DNA-PK work in conjunction to rapidly repair DSBs [26, 47]. We suggest PARP-1 does not compete with the Ku heterodimer for DSBs [21] (DNA-PK verses PARP-1-mediated NHEJ) but rather, persists as an accessory protein to the DNA-PK holoenzyme. Failure to assemble a functionally active DNA-PK holoenzyme results in PARP-1-mediated DSB-repair.

We postulate PARP-1 and the Ku heterodimer interact at DSBs concurrently, allowing for PARP-1/2 mediated PARsylation of Ku80. Failure to recruit DNA-PKcs and activate the DNA-PK holoenzyme will ultimately favor PARP-1 association with the

DSB, independent of the Ku heterodimer resulting in the 'switch' to alternative NHEJ. PARP-1 binding to DNA inhibits association of the Ku heterodimer with the DSB and activates PARP-1 catalytic activity, subsequent automodification (hypoPARsylates) and the recruitment of SSB-repair machinery [44, 45]. In both alternative NHEJ and SSB-repair, PARP-1 automodification recruits XRCC1 [34], Ligase III and DNA polymerase β (POL-β) [8], perhaps on each side of the DNA duplex to facilitate SSB-repair. Thus, PARP-1-mediated DSB-repair requires time to recruit the necessary repair proteins and close proximity of the broken ends (Figure 2). Our model provides an explanation for the error-prone nature of PARP-1-mediated DSB end-joining, which often results in translocation events between chromosomes [40, 48, 49].

We explain the characteristics of PARP-1-dependent alternative NHEJ as the result of slower DSB-repair due to the restrictions of SSB-repair processes in repairing DSBs. As time elapses following the induction of DSBs, PARP-1-mediated repair fails to end-join the originally paired DNA-strands, as they become increasingly far apart. Thus, the ends of the break can 'drift' toward an entirely different DSB, where PARP-1-mediated SSB-repair events can end-join the proximal DNA-strands, resulting in a translocation event. This explanation of the alternative-NHEJ process as a salvage pathway, using two independent SSB-repair events provides a potential explanation as to the reduced efficiency and error-prone nature of PARP-1-mediated DSB repair.

5.5.0 DNA-PKcs pADPr-modified residue(s)

Beyond the scope of PARP-1-mediated DSB-repair, it will be critical to establish the precise location/s of tankyrase 1-dependent DNA-PKcs pADPr modification. Identification of these pADPr modified residues in DNA-PKcs will be essential to

understanding DNA-PKcs deficiencies (protein level and/or function), telomere uncapping and impaired NHEJ capability. As with our study investigating the impact of single-nucleotide polymorphisms (SNPs) within the murine *Prkdc* allele and telomere-uncapping, a SNP in a critical domain/motif is capable of abolishing protein function [50]. Variant forms of DNA-PKcs resulting from SNPs and possibly influencing pADPr modification sites of DNA-PKcs directly or tankyrase 1 interacting motifs, would be crucial to developing biomarkers for IR sensitivity, estimating risk of carcinogenesis and even understanding accelerated aging. The inability to PARsylate DNA-PKcs as the consequence of a SNP impacting tankyrase 1 interaction and/or pADPr accepting residues would result in critically low levels of DNA-PKcs, as it becomes a substrate for E3 ligase ubiquitination.

Relevant to our model proposing pADPr-dependent DNA-PK activation, recruitment and scaffolding, variant forms of DNA-PKcs incapable of being PARsylated, would fail to form a stable, activated DNA-PK holoenzyme capable of performing DNA-PK-mediated NHEJ function. Hence, cellular IR sensitivity would be increased and background mutagenesis would further enhance carcinogenic risk. Additionally, variant forms of tankyrase 1 resulting from SNPs in the *TNKS* allele would result in similar, if not identical phenotypes.

5.6.0 Significance and Relevance to Cancer

Identification of the mechanism by which the DNA-PK holoenzyme is assembled, stabilized and activated is of critical importance with respect to carcinogenesis. Carcinogenesis is characterized by the accumulation of mutations, genomic instability and inappropriate cell-cycle regulation. Our proposed investigation would provide

valuable mechanistic insight into pADPr-dependent DNA-PK function and effective NHEJ. According to our model, DNA-damage induced in a background where pADPr-acceptor residues in DNA-PKcs and/or Ku80 are mutated would result in a significantly increased rate of mutagenesis due to inefficient 'classical' NHEJ. The inability to assemble a catalytically active DNA-PK holoenzyme, as seen in Ku deficient cells, would result in PARP-1-mediated end-joining throughout the cell cycle [40]. This method of end-joining is slow, inefficient and error prone, resulting in increased translocation [40-42]. Inherently, translocations between chromosomes has been shown to correlate with increased carcinogenic potential; often playing a role in oncogene activation. The Philadelphia chromosome, a 9:22 translocation responsible for CML, provides a classic example (reviewed in [43]). Our model would be the first to explain the shift from DNA-PK-mediated NHEJ to PARP-1-mediated end-joining and the corresponding increase in translocation events and subsequent carcinogenic phenotypes as the result of mis-regulated pADPr-dependent recruitment, scaffolding and activation of the DNA-PK holoenzyme.

LIST OF FIGURES

5.7.0 Figures

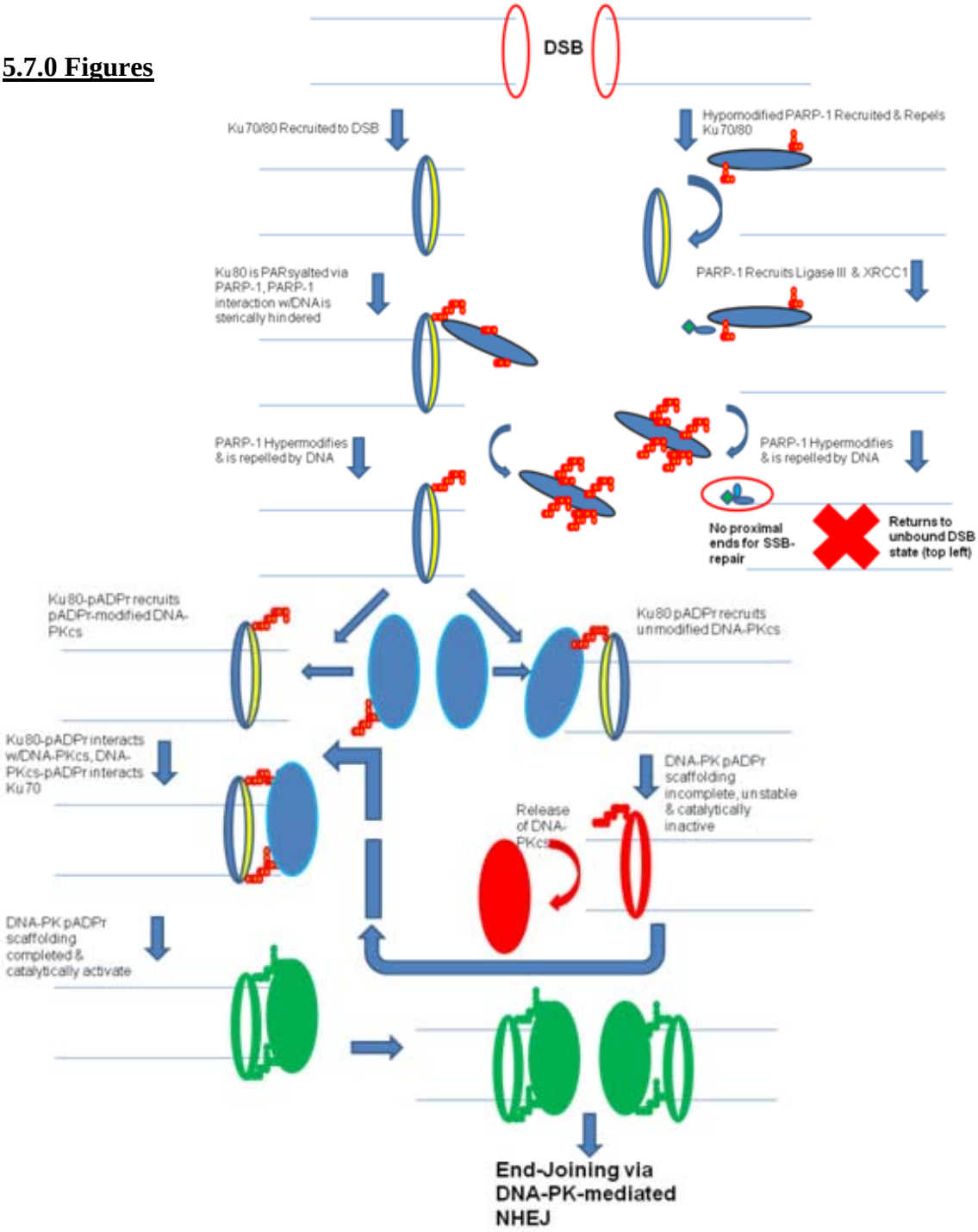


Figure 1. DNA-PK-mediated DSB-repair via pADPr-modification. PARP-1 acts as a DNA-PK holoenzyme accessory protein, PARsylating Ku80 for DNA-PKcs recruitment, interacting via the pADPr interaction motif of DNA-PKcs. pADPr modified forms of DNA-PKcs are favored to complete pADPr-based DNA-PK holoenzyme scaffolding and enhanced kinase activity.

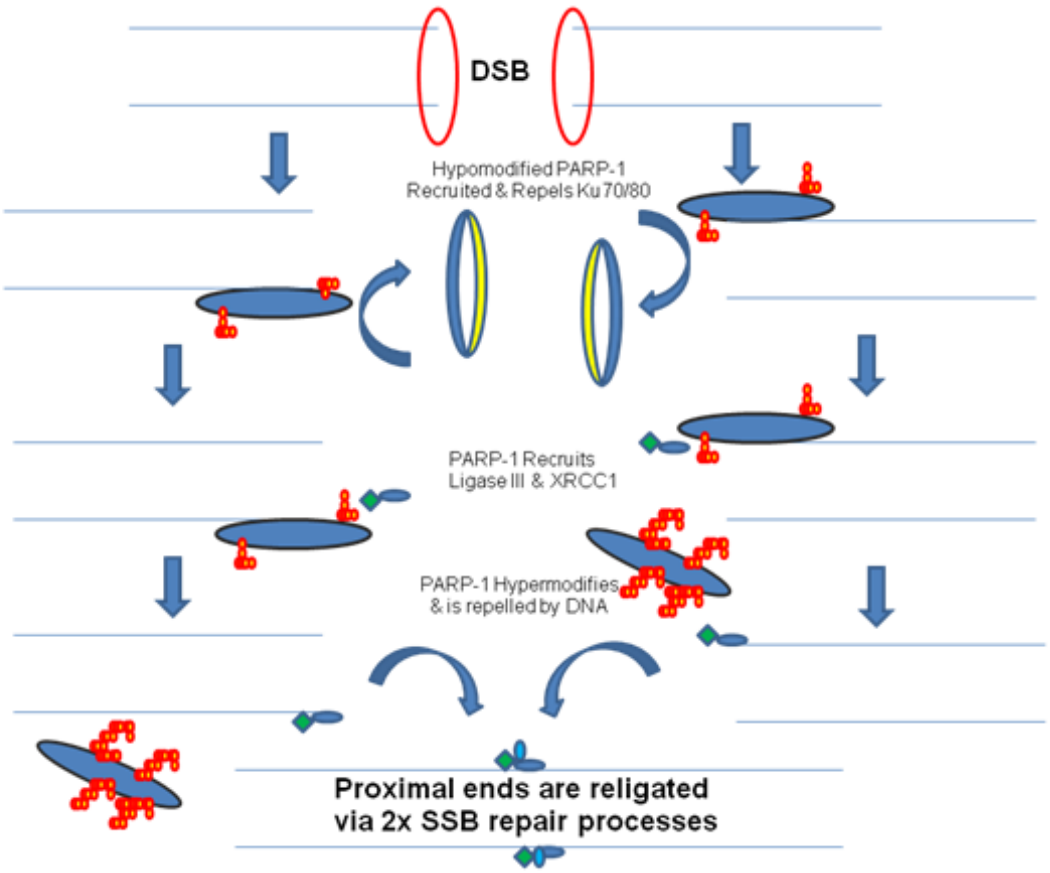


Figure 2. PARP-1-mediated 'Alternative NHEJ' by single-stranded break repair pathways. PARP-1 is recruited to the DSB in the absence of the Ku heterodimer. Recognition of exposed-DNA chemistry initiates automodification of PARP-1, followed by the recruitment of single-stranded break repair machinery (Ligase III and XRCC1). If additional exposed DNA-ends (DSBs) are proximal, end-joining will be performed by two independent single-strand break repair processes.

REFERENCES

5.8.0 References

1. Dregalla RC, Zhou J, Idate RR, Battaglia CL, Liber HL, Bailey SM: **Regulatory roles of tankyrase 1 at telomeres and in DNA repair: suppression of T-SCE and stabilization of DNA-PKcs**. *Aging (Albany NY)* 2010, **2**(10):691-708.
2. De Rycker M, Price CM: **Tankyrase polymerization is controlled by its sterile alpha motif and poly(ADP-ribose) polymerase domains**. *Mol Cell Biol* 2004, **24**(22):9802-9812.
3. Smith S, de Lange T: **Tankyrase promotes telomere elongation in human cells**. *Curr Biol* 2000, **10**(20):1299-1302.
4. Smith S, Giriat I, Schmitt A, de Lange T: **Tankyrase, a poly(ADP-ribose) polymerase at human telomeres**. *Science* 1998, **282**(5393):1484-1487.
5. Yeh TY, Meyer TN, Schwesinger C, Tsun ZY, Lee RM, Chi NW: **Tankyrase recruitment to the lateral membrane in polarized epithelial cells: regulation by cell-cell contact and protein poly(ADP-ribosyl)ation**. *Biochem J* 2006, **399**(3):415-425.
6. Chang P, Coughlin M, Mitchison TJ: **Interaction between Poly(ADP-ribose) and NuMA Contributes to Mitotic Spindle Pole Assembly**. *Mol Biol Cell* 2009, **20**(21):4575-4585.
7. Olabisi OA, Soto-Nieves N, Nieves E, Yang TT, Yang X, Yu RY, Suk HY, Macian F, Chow CW: **Regulation of transcription factor NFAT by ADP-ribosylation**. *Mol Cell Biol* 2008, **28**(9):2860-2871.
8. Ame JC, Spenlehauer C, de Murcia G: **The PARP superfamily**. *Bioessays* 2004, **26**(8):882-893.
9. Smith S: **The world according to PARP**. *Trends Biochem Sci* 2001, **26**(3):174-179.
10. Chang P, Coughlin M, Mitchison TJ: **Tankyrase-1 polymerization of poly(ADP-ribose) is required for spindle structure and function**. *Nat Cell Biol* 2005, **7**(11):1133-1139.
11. Hsiao SJ, Smith S: **Tankyrase function at telomeres, spindle poles, and beyond**. *Biochimie* 2008, **90**(1):83-92.
12. Ruscetti T, Lehnert BE, Halbrook J, Le Trong H, Hoekstra MF, Chen DJ, Peterson SR: **Stimulation of the DNA-dependent protein kinase by poly(ADP-ribose) polymerase**. *Journal of Biological Chemistry* 1998, **273**(23):14461-14467.
13. Chang W, Dynek JN, Smith S: **TRF1 is degraded by ubiquitin-mediated proteolysis after release from telomeres**. *Genes Dev* 2003, **17**(11):1328-1333.

14. Bouchard VJ, Rouleau M, Poirier GG: **PARP-1, a determinant of cell survival in response to DNA damage**. *Exp Hematol* 2003, **31**(6):446-454.
15. Dantzer F, de La Rubia G, Menissier-De Murcia J, Hostomsky Z, de Murcia G, Schreiber V: **Base excision repair is impaired in mammalian cells lacking Poly(ADP-ribose) polymerase-1**. *Biochemistry* 2000, **39**(25):7559-7569.
16. Gagne JP, Isabelle M, Lo KS, Bourassa S, Hendzel MJ, Dawson VL, Dawson TM, Poirier GG: **Proteome-wide identification of poly(ADP-ribose) binding proteins and poly(ADP-ribose)-associated protein complexes**. *Nucleic Acids Res* 2008, **36**(22):6959-6976.
17. Pleschke JM, Kleczkowska HE, Strohm M, Althaus FR: **Poly(ADP-ribose) binds to specific domains in DNA damage checkpoint proteins**. *J Biol Chem* 2000, **275**(52):40974-40980.
18. Lavrik OI, Prasad R, Sobol RW, Horton JK, Ackerman EJ, Wilson SH: **Photoaffinity labeling of mouse fibroblast enzymes by a base excision repair intermediate. Evidence for the role of poly(ADP-ribose) polymerase-1 in DNA repair**. *J Biol Chem* 2001, **276**(27):25541-25548.
19. Leppard JB, Dong Z, Mackey ZB, Tomkinson AE: **Physical and functional interaction between DNA ligase IIIalpha and poly(ADP-Ribose) polymerase 1 in DNA single-strand break repair**. *Mol Cell Biol* 2003, **23**(16):5919-5927.
20. Prasad R, Lavrik OI, Kim SJ, Kedar P, Yang XP, Vande Berg BJ, Wilson SH: **DNA polymerase beta -mediated long patch base excision repair. Poly(ADP-ribose)polymerase-1 stimulates strand displacement DNA synthesis**. *J Biol Chem* 2001, **276**(35):32411-32414.
21. Wang M, Wu W, Rosidi B, Zhang L, Wang H, Iliakis G: **PARP-1 and Ku compete for repair of DNA double strand breaks by distinct NHEJ pathways**. *Nucleic Acids Res* 2006, **34**(21):6170-6182.
22. Mahaney BL, Meek K, Lees-Miller SP: **Repair of ionizing radiation-induced DNA double-strand breaks by non-homologous end-joining**. *Biochem J* 2009, **417**(3):639-650.
23. Rulten SL, Fisher AE, Robert I, Zuma MC, Rouleau M, Ju L, Poirier G, Reina-San-Martin B, Caldecott KW: **PARP-3 and APLF function together to accelerate nonhomologous end-joining**. *Mol Cell* 2011, **41**(1):33-45.
24. Smogorzewska A, Karlseder J, Holtgreve-Grez H, Jauch A, de Lange T: **DNA ligase IV-dependent NHEJ of deprotected mammalian telomeres in G1 and G2**. *Curr Biol* 2002, **12**(19):1635-1644.
25. Mansour WY, Rhein T, Dahm-Daphi J: **The alternative end-joining pathway for repair of DNA double-strand breaks requires PARP1 but is not dependent upon microhomologies**. *Nucleic Acids Res* 2010, **38**(18):6065-6077.
26. Mitchell J, Smith GC, Curtin NJ: **Poly(ADP-Ribose) polymerase-1 and DNA-dependent protein kinase have equivalent roles in double strand break repair following ionizing radiation**. *Int J Radiat Oncol Biol Phys* 2009, **75**(5):1520-1527.
27. Jones JM, Gellert M, Yang W: **A Ku bridge over broken DNA**. *Structure* 2001, **9**(10):881-884.

28. Galande S, Kohwi-Shigematsu T: **Poly(ADP-ribose) polymerase and Ku autoantigen form a complex and synergistically bind to matrix attachment sequences**. *J Biol Chem* 1999, **274**(29):20521-20528.
29. Ame JC, Rolli V, Schreiber V, Niedergang C, Apiou F, Decker P, Muller S, Hoger T, Menissier-de Murcia J, de Murcia G: **PARP-2, A novel mammalian DNA damage-dependent poly(ADP-ribose) polymerase**. *J Biol Chem* 1999, **274**(25):17860-17868.
30. de Murcia JMN, Ricoul M, Tartier L, Niedergang C, Huber A, Dantzer F, Schreiber V, Ame JC, Dierich A, LeMeur M *et al*: **Functional interaction between PARP-1 and PARP-2 in chromosome stability and embryonic development in mouse**. *Embo J* 2003, **22**(9):2255-2263.
31. Shieh WM, Ame JC, Wilson MV, Wang ZQ, Koh DW, Jacobson MK, Jacobson EL: **Poly(ADP-ribose) polymerase null mouse cells synthesize ADP-ribose polymers**. *J Biol Chem* 1998, **273**(46):30069-30072.
32. Lieber MR: **The mechanism of human nonhomologous DNA end joining**. *J Biol Chem* 2008, **283**(1):1-5.
33. Lieber MR, Yu K, Raghavan SC: **Roles of nonhomologous DNA end joining, V(D)J recombination, and class switch recombination in chromosomal translocations**. *DNA Repair (Amst)* 2006, **5**(9-10):1234-1245.
34. Okano S, Lan L, Caldecott KW, Mori T, Yasui A: **Spatial and temporal cellular responses to single-strand breaks in human cells**. *Mol Cell Biol* 2003, **23**(11):3974-3981.
35. Zahradka P, Ebisuzaki K: **A shuttle mechanism for DNA-protein interactions. The regulation of poly(ADP-ribose) polymerase**. *Eur J Biochem* 1982, **127**(3):579-585.
36. Gao Y, Chaudhuri J, Zhu C, Davidson L, Weaver DT, Alt FW: **A targeted DNA-PKcs-null mutation reveals DNA-PK-independent functions for KU in V(D)J recombination**. *Immunity* 1998, **9**(3):367-376.
37. Gao Y, Sun Y, Frank KM, Dikkes P, Fujiwara Y, Seidl KJ, Sekiguchi JM, Rathbun GA, Swat W, Wang J *et al*: **A critical role for DNA end-joining proteins in both lymphogenesis and neurogenesis**. *Cell* 1998, **95**(7):891-902.
38. Gu Y, Sekiguchi J, Gao Y, Dikkes P, Frank K, Ferguson D, Hasty P, Chun J, Alt FW: **Defective embryonic neurogenesis in Ku-deficient but not DNA-dependent protein kinase catalytic subunit-deficient mice**. *Proc Natl Acad Sci U S A* 2000, **97**(6):2668-2673.
39. Taccioli GE, Amatucci AG, Beamish HJ, Gell D, Xiang XH, Torres Arzayus MI, Priestley A, Jackson SP, Marshak Rothstein A, Jeggo PA *et al*: **Targeted disruption of the catalytic subunit of the DNA-PK gene in mice confers severe combined immunodeficiency and radiosensitivity**. *Immunity* 1998, **9**(3):355-366.
40. Simsek D, Jasin M: **Alternative end-joining is suppressed by the canonical NHEJ component Xrcc4-ligase IV during chromosomal translocation formation**. *Nat Struct Mol Biol* 2010, **17**(4):410-416.
41. Yan CT, Boboila C, Souza EK, Franco S, Hickernell TR, Murphy M, Gumaste S, Geyer M, Zarrin AA, Manis JP *et al*: **IgH class switching and translocations use a robust non-classical end-joining pathway**. *Nature* 2007, **449**(7161):478-482.

42. Zhu C, Mills KD, Ferguson DO, Lee C, Manis J, Fleming J, Gao Y, Morton CC, Alt FW: **Unrepaired DNA breaks in p53-deficient cells lead to oncogenic gene amplification subsequent to translocations**. *Cell* 2002, **109**(7):811-821.
43. Quintas-Cardama A, Cortes J: **Molecular biology of bcr-abl1-positive chronic myeloid leukemia**. *Blood* 2009, **113**(8):1619-1630.
44. D'Amours D, Desnoyers S, D'Silva I, Poirier GG: **Poly(ADP-ribosyl)ation reactions in the regulation of nuclear functions**. *Biochem J* 1999, **342 (Pt 2)**:249-268.
45. Lindahl T, Satoh MS, Poirier GG, Klungland A: **Post-translational modification of poly(ADP-ribose) polymerase induced by DNA strand breaks**. *Trends Biochem Sci* 1995, **20**(10):405-411.
46. D'Silva I, Pelletier JD, Lagueux J, D'Amours D, Chaudhry MA, Weinfeld M, Lees-Miller SP, Poirier GG: **Relative affinities of poly(ADP-ribose) polymerase and DNA-dependent protein kinase for DNA strand interruptions**. *Biochim Biophys Acta* 1999, **1430**(1):119-126.
47. Veuger SJ, Curtin NJ, Smith GC, Durkacz BW: **Effects of novel inhibitors of poly(ADP-ribose) polymerase-1 and the DNA-dependent protein kinase on enzyme activities and DNA repair**. *Oncogene* 2004, **23**(44):7322-7329.
48. Bennardo N, Cheng A, Huang N, Stark JM: **Alternative-NHEJ is a mechanistically distinct pathway of mammalian chromosome break repair**. *PLoS Genet* 2008, **4**(6):e1000110.
49. Greaves MF, Wiemels J: **Origins of chromosome translocations in childhood leukaemia**. *Nat Rev Cancer* 2003, **3**(9):639-649.
50. Fabre KM, Ramaiah L, Dregalla RC, Desaintes C, Weil MM, Bailey SM, Ullrich RL: **Murine Prkdc Polymorphisms Impact DNA-PKcs Function**. *Radiat Res* 2011.

CHAPTER 6

Final Discussion

6.1.0 Overview of Findings

6.1.1 DNA-PKcs-dependent telomere end-capping requires the leucine zipper domain

Our investigation of the two single-nucleotide polymorphisms (SNPs) residing in the *Prkdc* allele of the BALB/c mouse revealed that the leucine zipper domain is essential for effective telomere end-caping. Consistent with our prior studies, we find the DNA-PKcs-deficient BALB/c mouse experiences telomere uncapping and fusion events involving DSBs following IR exposure [1]. The BALB/c mouse harbors two *Prkdc* SNPs, one within the phosphatidylinositol-3-related-kinase (PIKK) (M3844V) domain and the other within the protein interacting leucine zipper motif (R2140C) [2]. Here, we investigated the DNA-PKcs SNPs responsible for BALB/c telomere dysfunction using the LEWES mouse, which contains only the *Prkdc* SNP within the PIKK domain (M3844V) [3]. The frequency of telomere-based fusions, both spontaneous and IR induced, in LEWES mirrored those observed in the control C57BL/6 mouse [3]. Based on these findings, we concluded that DNA-PKcs end-capping function relies on the leucine zipper motif rather than the catalytic kinase activity, perhaps suggesting an important new role for conformational changes in activating the kinase function.

The transcriptional (mRNA) expression of the BALB/c *Prkdc* allele does not significantly differ from C57BL/6 *Prkdc* allele expression [4]. However, the level of

detectable DNA-PKcs protein is significantly less in the BALB/c cell line compared to that of C57BL/6. Thus, the SNPs within the leucine zipper and PIKK domain of the *Prkdc* allele in the BALB/c mouse negatively impacts DNA-PKcs protein stability [2, 4]. The SCID mouse shows a more dramatic DNA-PKcs deficiency and instability phenotypes [5] and unlike BALB/c, SCID contains a truncated form of the DNA-PKcs protein that results in lowered protein levels and a kinase null protein [6]. Interestingly, the BALB/c DNA-PKcs protein is not truncated and contains all functional domains but protein levels are reduced as the consequence of a posttranscriptional defect [2, 4, 7]. We sought to separate function of the BALB/c *Prkdc* SNPs present in the leucine zipper motif (R2140C) and PIKK domain (M3844V).

Considering our evidence demonstrating DNA-PKcs protein stability dependence on tankyrase 1 poly(ADP-ribosyl)ation (PARsylation/pADPr) [8], we speculated that specific SNPs within the *Prkdc* allele might interfere with the ability to PARsylate DNA-PKcs. Although, we recognize that the tankyrase 1-DNA-PKcs relationship has not yet been investigated in the mouse model and that tankyrases are known to play differing roles in the mouse compared to humans [9], if the relationship between tankyrase 1 and DNA-PKcs identified in human cell lines holds true in the mouse, then it is possible that the leucine zipper motif is critical for the association of DNA-PKcs with tankrase 1. An inability to initiate docking between the two proteins as a consequence of SNPs in the leucine zipper domain could result in the inability to pADPr-modify DNA-PKcs and subsequent proteasome-mediated degradation, explaining the dramatically reduced level of detectable DNA-PKcs protein in the BALB/c mouse [2]. Further, telomeric end-capping and DNA-PK-mediated NHEJ would be impaired as the consequence of the

inability for DNA-PKcs to accept pADPr. This model would explain the observed sensitivity of BALB/c cells to IR, increased mutagenesis and telomere-uncapping [1, 2, 4, 7, 10, 11]. Extrapolation of this model to the human, argues particular *PRKDC* polymorphisms in domains that are essential for tankyrase-1 binding and/or pADPr addition of the DNA-PKcs protein would result in robust genome and telomere instability phenotypes.

6.1.2 Tankyrase 1 depletion results in telomeric-recombination and genomic instability phenotypes

We identified tankyrase 1 as a key player in maintaining genomic integrity by regulating stability of the DNA-repair protein DNA-PKcs. Such a role for tankyrase 1 is novel in that the tankyrase subfamily of PARPs lack a DNA-binding domain, unlike the DNA-repair-associated PARPs 1 and 2 [12-14]. Initially identified and understood to be a regulator of telomere length, tankyrase 1 PARsylates the telomere-repeat binding factor 1 (TRF1), releasing it from the telomere and providing access to telomerase [15, 16]. Consistent with our initial hypothesis and supporting reports describing the necessity for tankyrase 1 in telomere stability [17], tankyrase 1 siRNA-mediated protein depletion resulted in elevated frequencies of cytogenetically visible telomere sister chromatid exchanges (T-SCEs) [8]. However, we did not observe increased frequencies of genomic-sister chromatid exchanges (G-SCE) above background under similar tankyrase 1 knockdown conditions [8], suggesting the role of tankyrase 1 in regulating these recombination events is specific to the telomere. Provided the well characterized role of tankyrase 1 in dissociating TRF1 from telomeric double-stranded DNA [15, 16], tankyrase 1 knockdown would result in the failure to dissociate TRF1, stalling the

replication fork progression through the telomere. To circumnavigate the obstruction posed by TRF1, sister chromatid exchanges within the telomeric sequence would allow for short advancements, a 'by-pass' by the DNA-polymerase.

n addition to the telomere-specific function of tankyrase 1 in terms of regulating sister chromatid exchanges, tankyrase 1 knockdown also resulted in an array of unanticipated genomic instability phenotypes. First, the depletion of tankyrase 1 protein levels resulted in increased IR-sensitivity in multiple cell-types (over the mock transfected controls). This finding suggested that the impact of tankyrase 1 depletion reaches beyond increased telomere-recombination (T-SCE) and may involve a role in DNA-repair. In support of this, our analysis of mutation frequency in human lymphoblasts (WTK1) under tankyrase 1 knockdown conditions revealed elevated frequencies of thymidine kinase (TK) mutations. Tankyrase 1 depletion also resulted in increased terminal deletions and telomere-based chromosome fusions following exposure to both low- and high- linear energy transfer (LET) radiation-types (over the siRNA mock transfection) [8]. Therefore, the depletion of tankyrase 1 protein resulted in a significant increase over the background of several genomic instability phenotypes.

Western blot analysis of classical non-homologous end-joining (C-NHEJ) proteins Ku80 and DNA-PKcs following tankyrase 1 knockdown revealed a significant reduction in DNA-PKcs protein levels; however, levels of Ku80 were unaffected by tankyrase 1 protein depletion. To investigate the underlying mechanism by which the DNA-PKcs protein was depleted, we determined the relative level of mRNA transcripts for tankyrase 1, tankyrase 2 and DNA-PKcs via qRT-PCR analysis. As expected, tankyrase 1 mRNA levels were diminished whereas tankyrase 2 and DNA-PKcs

transcript levels were unaffected. These findings demonstrated the specificity of the knockdown (tankyrase 1-specific), and that DNA-PKcs protein stability relies on tankyrase 1 at the protein level. Further, the converse knockdown of DNA-PKcs showed that tankyrase 1 protein levels were unaffected. Based on these findings, we concluded that DNA-PKcs protein stability is dependent on the catalytic activity of tankyrase 1. We found that the depletion of tankyrase 1 leads to increased telomere recombination events and further, the depletion or catalytic inhibition of tankyrase 1 results in genomic and telomere instability phenotypes that emerge as a consequence of DNA-PKcs depletion.

6.1.3 DNA-PKcs protein stability is dynamically regulated by tankyrase 1-dependent pADPr-modification and PARG activity

Instability phenotypes emerging as the consequence of tankyrase 1 siRNA-mediate depletion stems from the combination of telomere uncapping and DNA double-stranded break (DSB)-repair deficiencies. Most importantly, we identified the role of tankyrase 1 in DNA-repair associated genomic instability as one that is indirect in nature; i.e., DNA-PKcs protein stability is dependent on the tankyrase 1 protein. Preliminary evidence also suggested that the relationship between tankryase 1 and DNA-PKcs was not stoichiometric in nature which would require the stable interaciton of tankyrase 1 with DNA-PKcs, as Co-IP failed to demonstrate a stable complex between the two proteins. In support or a transient interaction, we found that high concentrations of the broad-range PARP inhibitor 3-aminobenzamide (3-AB) (20 mM, more than that necessary to inhibit PARP-1 and PARP-2 [18, 19]) was most effective in the depletion of DNA-PKcs protein levels; suggesting the activity of a PARP family member other than PARP-1 and/or PARP-2 is responsible for DNA-PKcs protein stability.

To investigate further, we turned to small molecule inhibitors of tankyrase 1 and poly(ADP-ribose) glycohydrolase (PARG) enzymatic activity, XAV939 and ADP-HPD respectively. We found that inhibition of tankyrase PARP catalytic activity resulted in the rapid and prominent reduction of DNA-PKcs protein levels. This observation supported the concept that DNA-PKcs protein stability is dependent on tankyase 1 and more importantly, builds on the findings of the 3-AB studies suggesting tankyrase 1 catalytic activity as a key factor in DNA-PKcs protein stability. We concluded that the stability of DNA-PKcs is dependent on tankyrase 1 PARsylating activity specifically, rather than the simple presence of the tankyrase 1 protein alone. Furthermore, the PARsylating activity of tankyrase 1 in DNA-PKcs protein stability does not appear to be redundant across PARP-family members; i.e., 3-AB and XAV939 studies indicated the activity of other PARPs do not contribute to the stability of DNA-PKcs.

Additional studies, including western blot analysis (over-exposed) with fluorescent immunolabeling of DNA-PKcs, revealed high molecular weight forms of DNA-PKcs that failed to migrate with the dominant 470kD band. Further electrophoretic separation of the 470kD DNA-PKcs protein band revealed that DNA-PKcs was highly heterogeneous with respect to molecular weight. Lysates from cells treated with tankyrase and/or PARG inhibitors (XAV939 & ADP-HPD, respectively) demonstrated that the heterogeneity of DNA-PKcs molecular weight is dependent on tankyrase 1 enzymatic modification (PARsylation). Therefore, we concluded that DNA-PKcs was PARsylated via tankyrase 1, resulting in a proteasome-resistant form of DNA-PKcs, in what might arguably be a catalytically active form of DNA-PKcs [20].

In addition to identification of a mechanistic role for tankyrase 1 in DNA-PKcs stability, we clearly demonstrated the fundamental importance in maintaining the dynamic nature of the pADPr modification and thus, the appropriate regulation of the pADPr acceptor protein, DNA-PKcs. In our model, the outcome of disrupting the critical dynamics of pADPr addition and removal proved to be detrimental from two separate approaches: pADPr-dependent DNA-PKcs stability and appropriate tankyrase 1 autoregulation.

Beyond demonstrating the dependency of DNA-PKcs protein stability on tankyrase 1 PARsylation, XAV939 tankyrase inhibitor studies revealed the importance of the transient, dynamic nature of the pADPr-modification. Inhibition of tankyrase 1 enzymatic activity resulted in the depletion of DNA-PKcs over a short time course (<12 hours) compared to the siRNA studies, in which *maximum* depletion of DNA-PKcs required approximately 24 hours. Inhibition of the tankyrase PARP domain rapidly destabilized the DNA-PKcs protein despite the increase in intracellular tankyrase 1 protein levels [8]. Provided PARG activity is rapid enough to dePARsylate all pADPr modified proteins in <60 seconds following PARP inhibition proteaome-wide [21, 22], we suspected that pADPr modification of DNA-PKcs was short-lived with a rapid turn over between 'PARsylated' and 'dePARsylaed' states.

Inhibition of tankyrase 1 PARsylating activity forces the rapid PARG-mediated dePARsylation of DNA-PKcs, favoring proteasome-mediated degradation. We do not suspect every monomer of DNA-PKcs to be PARsylated intracellularly but rather acts dynamically with a rapid on/off rate, supporting the concept that posttranslational

modifications (e.g., phosphorylation) with regulatory implications are generally transient and in nature [23, 24].

Over the course of our investigation, we repeatedly illustrated that the regulation of DNA-PKcs protein stability is dependent on pADPr-addition by tankyrase 1 & removal by PARG [8]. Targeting of DNA-PKcs for proteasome-mediated degradation appears to depend on several factors, including the state of DNA-PKcs PARsylation in respect to time and space. In order to be targeted for the proteasome, PARG activity against DNA-PKcs PARsylation and E3-Ligase acitivty must be close in time and space such that the respective DNA-PKcs protein unit exists in a *completely* dePARsylated state at the instantaneous moment of the encounter with an E3-Ligase. PARsylated forms of DNA-PKcs that encounter an E3-Ligase therefore will have no consequence, illustrating the importance for the dynamics of pADPr addition and removal. Changing the rate of DNA-PKcs PARsylation (siRNA knockdown and/or catalytic inhibition) resulted in an increased frequency of DNA-PKcs in a dePARsyalted form, available to modification by E3-Ligase and subsequent protein degradation by the proteasome.

In studies investigating inhibition of PARG activity (ADP-HPD), we disrupted the ability for a PARsylated target to revert back to its original, unmodified state. Due to the fact that pADPr additions are short-lived under normal conditions [25], inhibition of PARG abrogated the ability to use pADPr-protein modification in a transient, regulated fashion. We found that PARG inhibition resulted in rapid DNA-PKcs depletion, mirroring the effect of tankyrase 1 siRNA-mediated knockdown and XAV939 inhibition, exposing the consequence of disrupting the appropriate regulation of pADPr automodification in regards to tankyrase 1. Tankyrase 1 binds and PARsylates target

proteins in multimerized hetero- and homo-complexes of tankyrases 1 & 2 interacting through their sterile alpha motifs (SAM) [26, 27]. The subsequent event is autoPARsylation, which disrupts tankyrase-tankyrase SAM interactions and dissociates the automodified tankyrase from the tankyrase-complex [26]. Under normal cellular conditions, a considerable proportion of automodified tankyrase would be dePARsylated by the activity of PARG and maintain an active status. Monomers of pADPr-modified tankyrase 1 that are not dePARsylated become substrates for ubiquitination and degradation [28]. Therefore, the addition of the PARG inhibitor (ADP-HPD) to cell culture results in the rapid accumulation of tankyrases in a PARsylated state, followed by ubiquitination and degradation. As a consequence, tankyrase 1 protein levels are abolished over a short-time course, coupled with the concurrent loss of DNA-PKcs protein levels. Though the mechanism of tankyrase 1 depletion differed, PARG inhibition resulted in loss of DNA-PKcs as the result of reduced tankyrase 1 levels, similar to DNA-PKcs depletion by siRNA knockdown of tankyrase 1.

6.2.0 Implications of tankyrase 1-dependent pADPr modification of DNA-PKcs in carcinogenesis and aging

Revealing tankyrase 1 as an important regulator of DNA-PKcs protein stability is informative regarding the array of possible instability phenotypes that would emerge as a consequence of polymorphic forms of either protein. We have not yet determined the relevant protein interaction sites and specific residue(s) of DNA-PKcs that are PARsylated, which would provide insight into the domains of the *PRKDC* and *TNKS* alleles in which SNPs would result in defective protein variants, potentially impacting the intracellular roles of DNA-PKcs in end-capping and/or NHEJ.

Our analysis of DNA-PKcs protein domains that contribute to mammalian telomere end-capping suggested that the leucine zipper motif of DNA-PKcs, rather than the PIKK domain, is essential for this particular function. It remains unclear what role each DNA-PKcs domain plays in NHEJ and downstream DNA-repair-dependent genomic stability. For future investigations of DNA-PKcs pADPr-modification in the appropriate recruitment, scaffolding and activation in the DNA-PK holoenzyme, it will be important to consider the several functional domains of the *PRKDC* allele that could be influenced by SNPs. We speculate the relevant domains may involve a combination of those necessary to facilitate protein interactions (leucine zipper motif), kinase function (PIKK domain) and pADPr-acceptor/interacting residues. Additionally, SNPs in the *TNKS* allele that impact protein interactions (ankyrin-like repeat domains) may also affect the efficiency and consistency of tankyrase 1-mediated DNA-PKcs pADPr-modification. Interestingly, several SNPs in the human *PRKDC* allele have been associated with human cancers [29], supporting the possibility of disrupted tankyrase 1 interaction and/or pADPr modification. SNPs in the *TNKS* allele that correlate to various cancers have been identified, and involve telomere erosion. [30, 31].

Identification of the mechanism by which DNA-PKcs is regulated [8] has important implications in respect to carcinogenesis and aging, both of which can be characterized by the accumulation of mutations paired with increasing genomic instability, and inappropriate cell-cycle regulation over time as the result of DNA-mis-repair or chronic damage [32-34]. Mutations occurring in the *PRKDC* and/or *TNKS* alleles that disrupt the transient interaction between tankyrase 1 and DNA-PKcs and/or PARsylation of specific DNA-PKcs residues would be expected to result in reduced

levels of DNA-PKcs, similar to the phenomenon seen in the SCID and BALB/c mouse [2, 6]. As a consequence, both telomere and genomic instability phenotypes would emerge, including increased mutation frequency and deficient DNA-PK-mediated NHEJ repair [8], which may further advance carcinogenic potential. If a translocation event were to occur within a tumor suppressor gene resulting in the inactivation of the downstream proteins function, or the activation of a proto-oncogene, the risk of carcinogenic potential will become significantly elevated [32], corresponding with previous reports regarding the DNA-PKcs deficient BALB/c mouse [4, 7, 10].

The loss of DNA-PKcs would result in telomere uncapping [1, 5, 35, 36] and DSB-repair mediated by PARP-1 [37]. DSB-repair by the PARP-1-dependent alternative NHEJ pathway is characterized by slower end-joining processivity and an elevated frequency of translocation events [38], at least some of which could result from telomere-DSB fusion events.

Evidence from Maria Blasco and colleagues suggested that deficiencies of DNA-PKcs, Ku80/86 or PARP-1 all contribute to accelerated aging phenotypes in mice with a telomerase negative background [39]. This study provides additional evidence supporting the findings of ours and others that suggest a DNA-PK NHEJ model that incorporates PARP-1 as an accessory protein to the holoenzyme [40]. In the study by Blasco and colleges, Ku80/86 and DNA-PKcs knockout mice were characterized by 'early-aging' phenotypes compared to mice containing only telomerase deficiencies, suggesting that the loss of DNA-PK-mediated NHEJ accelerates aging phenotypes. Although PARP-1 knockout mice displayed early-aging phenotypes compared to controls, they did not pose accelerated aging phenotypes to the same extent observed in

Ku80/86 and DNA-PKcs deficient mice. Regarding the classical NHEJ model, the loss of PARP-1 alone should not impact the effectiveness of DNA-PK-mediated NHEJ. We propose the loss of PARP-1 contributes to the slight aging phenotypes observed by Blasco et al. as a consequence of failed PARP-1 dependent pADPr-modification of Ku80/86. Pertaining to our proposed PARP-1-DNA-PK NHEJ model (Chapter 5), we speculated that failure of Ku80 pADPr modification (by PARP-1) would result in the inability to efficiently recruit *and* maintain the catalytically active pADPr-modified DNA-PKcs [20].

Regulation of DNA-PKcs protein stability by tankyrase 1 catalytic PARP activity provides us with a novel approach to investigating cancer and aging phenotypes pertaining to deficient telomere end-capping and DNA-PK-mediated end-joining repair. We next seek to determine the functional attributes of pADPr-modification of DNA-PKcs in classical NHEJ. We suspect the consequence of impaired DNA-PKcs pADPr-modification (beyond protein destabilization and degradation) is likely to be one of increased mutagenesis and persistent chromosome instability, thereby facilitating carcinogenesis on the one hand, and limited cellular proliferation on the other, contributing to accelerated aging.

There are many diseases characterized by premature aging phenotypes in childhood and juvenile cancer cases. Most of these aging phenotypes have been attributed to deficiencies in DNA-repair and replication proteins such as ATM, BLM and WRN [41-45]. Fitting into this scheme is the inability to perform NHEJ in a DNA-PK-dependent manner. Inherited germ-line mutations in DNA-PKcs pADPr-acceptor residues, tankyrase 1 binding domains or tankyrase 1 catalytic function would result in an

individual with an elevated risk of the early onset of cancers and aging phenotypes in affected tissues. Further, validation of our proposed PARP-1 associated DNA-PK model would suggest interference with non-covalent pADPr-interacting sites of DNA-PKcs and Ku70 as well as Ku80/86 pADPr accepting residue(s), would contribute to deficient end-joining, perpetuating aging and carcinogenic phenotypes. Cumulatively, our discovery of the mechanism underlying tankyrase-1-dependent DNA-PKcs regulation via PARsyaltion, coupled with the important implications of defects in either protein, provides valuable insight into an additional mechanism by which both accelerated aging and cancer phenotypes may emerge.

REFERENCES

6.3.0 References

1. Williams ES, Klingler R, Ponnaiya B, Hardt T, Schrock E, Lees-Miller SP, Meek K, Ullrich RL, Bailey SM: **Telomere dysfunction and DNA-PKcs deficiency: characterization and consequence**. *Cancer Res* 2009, **69**(5):2100-2107.
2. Yu Y, Okayasu R, Weil MM, Silver A, McCarthy M, Zabriskie R, Long S, Cox R, Ullrich RL: **Elevated breast cancer risk in irradiated BALB/c mice associates with unique functional polymorphism of the Prkdc (DNA-dependent protein kinase catalytic subunit) gene**. *Cancer Res* 2001, **61**(5):1820-1824.
3. Fabre KM, Ramaiah L, Dregalla RC, Desaintes C, Weil MM, Bailey SM, Ullrich RL: **Murine Prkdc Polymorphisms Impact DNA-PKcs Function**. *Radiat Res* 2011.
4. Okayasu R, Suetomi K, Yu Y, Silver A, Bedford JS, Cox R, Ullrich RL: **A deficiency in DNA repair and DNA-PKcs expression in the radiosensitive BALB/c mouse**. *Cancer Res* 2000, **60**(16):4342-4345.
5. Bailey SM, Meyne J, Chen DJ, Kurimasa A, Li GC, Lehnert BE, Goodwin EH: **DNA double-strand break repair proteins are required to cap the ends of mammalian chromosomes**. *Proc Natl Acad Sci U S A* 1999, **96**(26):14899-14904.
6. Danska JS, Holland DP, Mariathasan S, Williams KM, Guidos CJ: **Biochemical and genetic defects in the DNA-dependent protein kinase in murine scid lymphocytes**. *Mol Cell Biol* 1996, **16**(10):5507-5517.
7. Ponnaiya B, Cornforth MN, Ullrich RL: **Radiation-induced chromosomal instability in BALB/c and C57BL/6 mice: the difference is as clear as black and white**. *Radiat Res* 1997, **147**(2):121-125.
8. Dregalla RC, Zhou J, Idate RR, Battaglia CL, Liber HL, Bailey SM: **Regulatory roles of tankyrase 1 at telomeres and in DNA repair: suppression of T-SCE and stabilization of DNA-PKcs**. *Aging (Albany NY)* 2010, **2**(10):691-708.
9. Chiang YJ, Hsiao SJ, Yver D, Cushman SW, Tessarollo L, Smith S, Hodes RJ: **Tankyrase 1 and tankyrase 2 are essential but redundant for mouse embryonic development**. *PLoS One* 2008, **3**(7):e2639.
10. Ullrich RL: **Tumor induction in BALB/c female mice after fission neutron or gamma irradiation**. *Radiat Res* 1983, **93**(3):506-515.
11. Ullrich RL, Bowles ND, Satterfield LC, Davis CM: **Strain-dependent susceptibility to radiation-induced mammary cancer is a result of differences in epithelial cell sensitivity to transformation**. *Radiat Res* 1996, **146**(3):353-355.

12. Ame JC, Rolli V, Schreiber V, Niedergang C, Apiou F, Decker P, Muller S, Hoger T, Menissier-de Murcia J, de Murcia G: **PARP-2, A novel mammalian DNA damage-dependent poly(ADP-ribose) polymerase**. *J Biol Chem* 1999, **274**(25):17860-17868.
13. Ame JC, Spenlehauer C, de Murcia G: **The PARP superfamily**. *Bioessays* 2004, **26**(8):882-893.
14. Smith S: **The world according to PARP**. *Trends Biochem Sci* 2001, **26**(3):174-179.
15. Smith S, de Lange T: **Tankyrase promotes telomere elongation in human cells**. *Curr Biol* 2000, **10**(20):1299-1302.
16. Smith S, Giriat I, Schmitt A, de Lange T: **Tankyrase, a poly(ADP-ribose) polymerase at human telomeres**. *Science* 1998, **282**(5393):1484-1487.
17. Hsiao SJ, Smith S: **Sister telomeres rendered dysfunctional by persistent cohesion are fused by NHEJ**. *J Cell Biol* 2009, **184**(4):515-526.
18. Jacobson EL, Antol KM, Juarez-Salinas H, Jacobson MK: **Poly(ADP-ribose) metabolism in ultraviolet irradiated human fibroblasts**. *J Biol Chem* 1983, **258**(1):103-107.
19. Purnell MR, Whish WJ: **Novel inhibitors of poly(ADP-ribose) synthetase**. *Biochem J* 1980, **185**(3):775-777.
20. Ruscetti T, Lehnert BE, Halbrook J, Le Trong H, Hoekstra MF, Chen DJ, Peterson SR: **Stimulation of the DNA-dependent protein kinase by poly(ADP-ribose) polymerase**. *Journal of Biological Chemistry* 1998, **273**(23):14461-14467.
21. Alvarez-Gonzalez R, Jacobson MK: **Characterization of polymers of adenosine diphosphate ribose generated in vitro and in vivo**. *Biochemistry* 1987, **26**(11):3218-3224.
22. Davidovic L, Vodenicharov M, Affar EB, Poirier GG: **Importance of poly(ADP-ribose) glycohydrolase in the control of poly(ADP-ribose) metabolism**. *Exp Cell Res* 2001, **268**(1):7-13.
23. Nooren IM, Thornton JM: **Structural characterisation and functional significance of transient protein-protein interactions**. *J Mol Biol* 2003, **325**(5):991-1018.
24. Nooren IM, Thornton JM: **Diversity of protein-protein interactions**. *Embo J* 2003, **22**(14):3486-3492.
25. Juarez-Salinas H, Sims JL, Jacobson MK: **Poly(ADP-ribose) levels in carcinogen-treated cells**. *Nature* 1979, **282**(5740):740-741.
26. De Rycker M, Price CM: **Tankyrase polymerization is controlled by its sterile alpha motif and poly(ADP-ribose) polymerase domains**. *Mol Cell Biol* 2004, **24**(22):9802-9812.
27. De Rycker M, Venkatesan RN, Wei C, Price CM: **Vertebrate tankyrase domain structure and sterile alpha motif (SAM)-mediated multimerization**. *Biochem J* 2003, **372**(Pt 1):87-96.
28. Yeh TY, Meyer TN, Schwesinger C, Tsun ZY, Lee RM, Chi NW: **Tankyrase recruitment to the lateral membrane in polarized epithelial cells: regulation by cell-cell contact and protein poly(ADP-ribosyl)ation**. *Biochem J* 2006, **399**(3):415-425.

29. Bhatti P, Struewing JP, Alexander BH, Hauptmann M, Bowen L, Mateus-Pereira LH, Pineda MA, Simon SL, Weinstock RM, Rosenstein M *et al*: **Polymorphisms in DNA repair genes, ionizing radiation exposure and risk of breast cancer in U.S. Radiologic technologists**. *Int J Cancer* 2008, **122**(1):177-182.
30. Mirabello L, Yu K, Kraft P, De Vivo I, Hunter DJ, Prescott J, Wong JY, Chatterjee N, Hayes RB, Savage SA: **The association of telomere length and genetic variation in telomere biology genes**. *Hum Mutat* 2010, **31**(9):1050-1058.
31. Varadi V, Brendle A, Brandt A, Johansson R, Enquist K, Henriksson R, Svenson U, Tavelin B, Roos G, Hemminki K *et al*: **Polymorphisms in telomere-associated genes, breast cancer susceptibility and prognosis**. *Eur J Cancer* 2009, **45**(17):3008-3016.
32. Lee EY, Muller WJ: **Oncogenes and tumor suppressor genes**. *Cold Spring Harb Perspect Biol* 2010, **2**(10):a003236.
33. Poehlmann A, Roessner A: **Importance of DNA damage checkpoints in the pathogenesis of human cancers**. *Pathol Res Pract* 2010, **206**(9):591-601.
34. Rieder CL: **Mitosis in vertebrates: the G2/M and M/A transitions and their associated checkpoints**. *Chromosome Res* 2010.
35. Bailey SM, Brenneman MA, Halbrook J, Nickoloff JA, Ullrich RL, Goodwin EH: **The kinase activity of DNA-PK is required to protect mammalian telomeres**. *DNA Repair (Amst)* 2004, **3**(3):225-233.
36. Bailey SM, Murnane JP: **Telomeres, chromosome instability and cancer**. *Nucleic Acids Res* 2006, **34**(8):2408-2417.
37. Bennardo N, Cheng A, Huang N, Stark JM: **Alternative-NHEJ is a mechanistically distinct pathway of mammalian chromosome break repair**. *PLoS Genet* 2008, **4**(6):e1000110.
38. Mansour WY, Rhein T, Dahm-Daphi J: **The alternative end-joining pathway for repair of DNA double-strand breaks requires PARP1 but is not dependent upon microhomologies**. *Nucleic Acids Res* 2010, **38**(18):6065-6077.
39. Espejel S, Franco S, Sgura A, Gae D, Bailey SM, Taccioli GE, Blasco MA: **Functional interaction between DNA-PKcs and telomerase in telomere length maintenance**. *Embo J* 2002, **21**(22):6275-6287.
40. Mitchell J, Smith GC, Curtin NJ: **Poly(ADP-Ribose) polymerase-1 and DNA-dependent protein kinase have equivalent roles in double strand break repair following ionizing radiation**. *Int J Radiat Oncol Biol Phys* 2009, **75**(5):1520-1527.
41. Hickson ID: **RecQ helicases: caretakers of the genome**. *Nat Rev Cancer* 2003, **3**(3):169-178.
42. Opresko PL, Mason PA, Podell ER, Lei M, Hickson ID, Cech TR, Bohr VA: **POT1 stimulates RecQ helicases WRN and BLM to unwind telomeric DNA substrates**. *J Biol Chem* 2005, **280**(37):32069-32080.
43. Panda S, Isbatan A, Adami GR: **Modification of the ATM/ATR directed DNA damage response state with aging and long after hepatocyte senescence induction in vivo**. *Mech Ageing Dev* 2008, **129**(6):332-340.
44. Rossi ML, Ghosh AK, Bohr VA: **Roles of Werner syndrome protein in protection of genome integrity**. *DNA Repair (Amst)* 2010, **9**(3):331-344.

45. Vaziri H, West MD, Allsopp RC, Davison TS, Wu YS, Arrowsmith CH, Poirier GG, Benchimol S: **ATM-dependent telomere loss in aging human diploid fibroblasts and DNA damage lead to the post-translational activation of p53 protein involving poly(ADP-ribose) polymerase**. *Embo J* 1997, **16**(19):6018-6033.