THE ROLE OF DNA REPLICATION FORK REMODELING PROTEINS IN LYMPHOMAGENESIS AND HEMATOPOIETIC CELL REPLICATION STRESS

By:
Matthew Vincent Puccetti

Dissertation
Submitted to the Faculty of the
Graduate School of Vanderbilt University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY
in

Pathology
August 10, 2018
Nashville, Tennessee, USA

Approved:
Larry Swift, Ph.D. (Chair)
Christine M. Eischen, Ph.D. (Thesis advisor)
David Cortez, Ph.D.
William Tansey, Ph.D.
Deborah Lannigan, Ph.D.

ACKNOWLEDGEMENTS

Ph.D. training is a monumental undertaking that would be impossible to complete without the guidance, assistance and support of others. There are several people in particular I wish to thank who have directly contributed to multiple facets of my graduate education and to whom I owe a significant debt of gratitude.

First and foremost, I would like to sincerely thank my thesis advisor, Dr. Christine Eischen. The training of graduate students involves an enormous effort on the part of the mentor and Dr. Eischen's commitment to the success and growth of her students is extraordinary. Without her unyielding support, my own educational advancements and this work itself would not have been possible. She has made innumerable contributions to my scientific training, from expanding my intellectual aptitude and my ability to think critically, to honing my technical skills and offering career guidance. I will forever be grateful for the significant investment she has made in my education.

I would also like to thank the members of my thesis advisory committee: Dr. Larry Swift, Dr. David Cortez, Dr. William Tansey and Dr. Deborah Lannigan. I am deeply appreciative for their input, guidance and critical analysis of my data over the past five years. Having such an engaged and helpful committee has been a significant boon to my scientific training.

My training would also have been incomplete without the support and friendship of my colleagues in the Eischen lab over the years. I would particularly like to acknowledge Dr. Clare Adams, Dr. Brian Grieb and Dr. Mick Edmonds; you each have made significant contributions to this project, to my technical training and/or have offered invaluable advice and support for which I will always be grateful. I would also like to thank Pia Arrate for her significant technical contributions and assistance with many aspects of my thesis work.

I would also like to acknowledge the Vanderbilt University Medical Scientist Training Program, particularly the former and current program directors Dr. Terence Dermody and Dr. Christopher Williams. The programmatic supported offered by the Vanderbilt MSTP has been invaluable to my education and I will forever be appreciative of the opportunity I've been afforded to train in such an exceptional program.

And finally, I thank my parents, my wife Deirdre and all my friends and family for your support and encouragement over the years.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Figure **Page**

LIST OF TABLES

Table **Page**

LIST OF ABBREVIATIONS

‘3-OH	3’-hydroxyl group
4-OHT/4HT	4-hydroxytamoxifen
5-FU	5-fluorouracil
7-AAD	7-aminoactinomycin D
9-1-1	RAD9-RAD1-HUS1 complex
γH2AX	phosphorylated histone H2A, variant X
ANOVA	Analysis of variance
APIM	AlkB homology 2 PCNA interacting motif
ARF	Alternative reading frame of the INK4A/ARF locus; $p14^{ARF}$
ATP	Adenosine tri-phosphate
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related protein
ATRIP	ATR-interacting protein
B220	Protein tyrosine phosphatase, receptor type, C
BAX	BLC-2 associated X
BCL-2	B-cell lymphoma 2
BCL-X_L	B-cell lymphoma extra-large/B-cell lymphoma 2-like protein 1
BCL-W	B-cell lymphoma 2-like protein 2
BER	Base excision repair
BIM	BCL-2-like 11
BIR	Break-induced replication
BLM	Bloom syndrome RecQ-like helicase

BRCA1	Breast cancer 1/breast cancer type 1 susceptibility protein
BRCA2	Breast cancer 2/breast cancer type 2 susceptibility protein
BrdU	5-bromo-2’-deoxyuridine
C57Bl/6	C57 black 6 mouse
CC3/7	Cleaved-caspase 3/7
CD	Cluster of differentiation
CD3	CD3 antigen, epsilon chain
CD4	CD4 antigen
CD8	CD8 antigen, alpha chain
CD11b	Integrin alpha M
CD19	CD19 antigen
CD34	CD34 antigen
CD43	Sialophorin
CD48	CD48 antigen
CD45	Protein tyrosine phosphatase, receptor type, C
CD127/Il7R	Interleukin 7 receptor
CD135/Flt3	FMS-like tyrosine kinase 3
CD150	Signaling lymphocytic activation molecule family member 1
CDC6	Cell division cycle 6
CDC7	Cell division cycle 7
CDC25	Cell division cycle 25
CDC45	Cell division cycle 45
CDK	Cyclin-dependent kinase

CDK2	Cyclin-dependent kinase 2
CDK4	Cyclin-dependent kinase 4
CDK6	Cyclin-dependent kinase 6
CDT1	Chromatin licensing and DNA replication factor 1
CFS	Common fragile site
CHK1	Checkpoint kinase 1
CldU	5-chloro-2'-deoxyuridine
c-kit	KIT proto-oncogene receptor tyrosine kinase
Cs^{137}	Cesium-137
D-loop	Displacement loop
DAPI	4',6-diamadino-2-phenylindole, dihydrochloride
DDR	DNA damage response
DDT	DNA damage tolerance
DMEM	Dulbecco's Modified Eagle medium
DNA	Deoxyribose nucleic acid
DNA-PK	DNA-dependent protein kinase
DP	Double positive
DSB	Double-stranded DNA break
E-box	Enhancer box (sequence CACGTG)
ETAA1	Ewing tumor-associated antigen 1
EtOH	Ethanol
E2F	Transcription factor E2F
FANCJ	Fanconi anemia complementation group J

FANCM	Fanconi anemia complementation group M
G1	Gap 1 phase
G2	Gap 2 phase
GFP	Green fluorescent protein
Gr1	Lymphocyte antigen 6 complex, locus G
Gy	Gray
H2A	Histone H2A
H2AX	Histone H2A, variant X
HepA	ATP-dependent helicase HepA
HL	Hodgkin lymphoma
HLTF	Helicase-like transcription factor
HR	Homologous recombination
HSPC	Hematopoietic stem and progenitor cell
HU	Hydroxyurea
ICL	Inter-strand crosslink
IdU	5-iodo-2’-deoxyuridine
IgD	Immunoglobulin heavy constant D
IgM	Immunoglobulin heavy constant M
IL-7	Interleukin-7
IL-7Rα	Interleukin-7 receptor alpha
IR	Irradiation
Lin	Lineage
LSK	Lineage-negative, Sca1-positive, cKit-positive

LT-HSC	Long-term hematopoietic stem cell
M	Mitosis
MAX	MYC-associated factor X
Mb	Myc-box
MCM	Mini chromosome maintenance
MDM2	Murine double minute 2
MEF	Mouse embryonic fibroblast
miRNA	micro-RNA
MIZ-1	Zinc finger and BTB domain-containing protein 17
MPP	Multi-potent progenitor
MRE11	Double-strand break repair protein MRE11A
mRNA	Messenger RNA
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
Myc	Myelocytomatosis oncogene
MyER	Myc estrogen receptor fusion protein
NER	Nucleotide excision repair
NHL	non-Hodgkin lymphoma
NZF	NPL4 zinc-finger
ORC	Origin recognition complex
p53	Tumor protein 53
$p15^{INK4b}$	Cyclin-dependent kinase inhibitor 4B
$p21^{Cip1}$	Cyclin-dependent kinase inhibitor 1A
$p27^{Kip1}$	Cyclin-dependent kinase inhibitor 1B

PCNA	Proliferating cell nuclear antigen
PI	Propidium iodide
PIKK	phosophatidylinositol-3-kinase-related kinase
PIP	PCNA interacting protein
polα	DNA polymerase alpha
polδ	DNA polymerase delta
polε	DNA polymerase epsilon
pre-RC	pre-replication complex
RAD17	RAD17 checkpoint clamp loader component
RAD51	RAD51 recombinase
RB	Retinoblastoma protein
RECQ1	RecQ-like helicase 1
RNA	Ribonucleic acid
RNAPI	RNA polymerase I
RNAPII	RNA polymerase II
RNAPIII	RNA polymerase III
ROS	Reactive oxygen species
RPA	Replication protein A
RPA14/RPA3	Replication protein A3 (14 kDa subunit)
RPA32/RPA2	Replication protein A2 (32 kDa subunit)
RPA70/RPA1	Replication protein A1 (70 kDa subunit)
RPMI	Roswell Park Memorial Institute medium
S	Synthesis phase

Sca1 (Ly6A/E)	Lymphocyte antigen 6 complex, locus A
SCE	Sister-chromatid exchange
Ser/Thr	Serine/Threonine
shRNA	Short-hairpin RNA
SIOD	Schimke immuno-osseus dysplasia
SMARCAL1	SWI/SNF-related matrix associated, actin dependent regulator of chromatin, sub-family a-like 1
SNF	Sucrose non-fermenting
SP	Single positive
ssDNA	Single-stranded DNA
SWI/SNF	SWItch/sucrose non-fermenting
Ter119	Lymphocyte antigen 76
TNR	Tri-nucleotide repeat
TOPBP1	DNA topoisomerase binding protein II
TRRAP	Transformation/transcription domain associated protein
TLS	Trans-lesion synthesis
WRN	Werner syndrome RecQ-like helicase
WT	Wild-type
UV	Ultra-violet
ZRANB3	Zinc-finger, RAN-binding domain containing 3

CHAPTER I

INTRODUCTION

Part I: Cancer

Cancer statistics

Malignant neoplasms are the second leading cause of death in the United States (Center for Disease Control and Prevention 2016). In 2016, it is estimated that there were 1,685,210 new instances of cancer and 595,930 deaths (20.8% of all US deaths), which resulted in approximately $125 billion in health care costs (National Cancer Institute 2017). It is estimated that 39.6% of people will be diagnosed with cancer at some point in their lifetimes and by 2030, the annual number of new cancer diagnoses globally will rise from 14 million to 30 million with cancer deaths rising from 8 million to 13 million (National Cancer Institute 2017). Thus, cancer remains a major public health crisis and significant cause of human morbidity and mortality.

Cancer biology

Cancer is a collection of highly diverse diseases which share the biological commonality of unrestrained cell growth (Weinberg 2014). Normally, the processes of cell division, differentiation and cell death are tightly controlled events that ensure normal development and sustain healthy tissue structure and function. Cancer develops from normal cells which acquire the capacity to proliferate in an unrestrained manner while also developing

bypasses to internal tumor suppressive safeguards. In addition to forming tumors *in situ*, cancers possess the ability to grow and invade into surrounding, healthy tissue and most can metastasize to distant sites in other organs and tissues and disrupt their function, which is the leading cause of cancer-associated death (Weinberg 2014).

Malignant transformation is a multi-step processes that occurs over time and involves changes to the cellular genome both through DNA mutations and dysregulation of the epigenetic control of gene expression (Weinberg 2014). These processes generate the genetic diversity needed for the accumulation of cellular alterations that drive the transformation to malignancy. In 2000, Douglas Hanahan and Robert Weinberg provided a general overview of the features of cancer cells, which they termed "the Hallmarks of Cancer" (Hanahan and Weinberg 2000). These features are briefly described below.

Above all, cancer cells require sustained proliferative signaling independent of the normal homeostatic control of cell and tissue growth (Hanahan and Weinberg 2000). Cells obtain the ability to do this through a variety of mechanisms including oncogene mutation/activation, the over-production of growth factors, dysregulation of normal signaling pathways and disruption of growth-related negative feedback loops (Hanahan and Weinberg 2011). Additionally, cancer cells must evade growth suppressive signaling pathways, inactivate programmed cell death and enable replicative immortality. Normally, these processes are innately programmed within cells to control cell division and limit cellular life-span, which serves as a potent tumor-suppressive barrier (Hanahan and Weinberg 2000). Finally, cancer cells must induce angiogenesis to meet the high metabolic needs of rapidly growing cells while also triggering invasion and metastasis (Hanahan and Weinberg 2000).

A recent revision of this seminal publication has identified additional characteristics of many tumors that are integral to cancer biology (Hanahan and Weinberg 2011). In addition to the classical hallmarks described above, alterations to cellular energetics and metabolism are a distinct feature of many tumor types that are indispensable for unrestrained growth and proliferation (Hanahan and Weinberg 2011). Moreover, it is becoming clearer that the ability of cancer cells to avoid recognition and destruction by the immune system is essential for tumorigenesis, and modulation of the immune system is a promising and exciting therapeutic strategy currently under intensive study (Hanahan and Weinberg 2011, Couzin-Frankel 2013).

While these hallmarks provide a succinct description of the cellular states that underlie malignancy, there are also additional characteristics of cancer which function to facilitate the acquisition of these properties in neoplastic cells, termed "enabling hallmarks". Genome instability and mutation serves as the evolutionary driving force of cancer, endowing the tumor with the genetic diversity required for tumor progression and underlying the development of resistance to many current therapies (Hanahan and Weinberg 2011). Moreover, tumor-associated inflammation, which has been observed as inflammatory cell infiltrate in tumors for decades, is now understood to be a paradoxical driver of tumor development and progression and a direct contributor to neoplastic transformation (Hanahan and Weinberg 2011).

Thus, while cancer itself is an incredibly diverse set of diseases with a multitude of both environmental and genetic causes, these common themes underlie our understanding of cancer biology. Moreover, this general framework provides a solid foundation for

dissecting the molecular pathways and mechanisms that drive tumor formation and establish a conceptual base for therapeutic intervention and the treatment of cancer.

Lymphoma

Lymphoma is a general term encompassing a large class of diverse lymphoid neoplasms and is the cancer of interest in regards to the data presented in this dissertation. Historically, lymphoid cancers were classified as a lymphoma or leukemia based on tissue distribution. Lymphoma typically presented as a distinct tissue mass whereas leukemia exhibited wide-spread bone marrow and peripheral blood involvement; however, this distinction has blurred (Robbins et al. 2015). Lymphomas account for approximately 5% of all new cancer diagnoses in the United States and are responsible for over 20,000 U.S. deaths annually (National Cancer Institute 2017, Leukemia and Lymphoma Society 2018).

Clinically, lymphomas are classified as either *Hodgkins lymphoma (HL)* or *non-Hodgkins lymphomas (NHL).* HL accounts for approximately 10% of new lymphoma diagnoses and is classified based on classic histological findings, specific growth patterns, and spread through affected lymph nodes (Robbins et al. 2015). HL is common in young adults and in most instances, is successfully treated with radiation and chemotherapy. The remaining 90% of lymphoma diagnoses are broadly classified as NHL, which contains dozens of subtypes depending on the cell of origin, tissue distribution and molecular genetics (Armitage et al. 2017). Overall survival varies depending on the specific NHL subtype, however 10 year survival rates for NHLs in general are only about 60% (American Cancer Society 2017).

85-90% of lymphoid malignancies originate from B cells, with the remaining 10-15% being derived from T and NK cells (Armitage et al. 2017). Lymphomas in general present as painless lymphadenopathy but may also involve other lymphoid tissues (spleen, thymus), other viscera and may be associated with systemic symptoms. The vast majority of lymphomas are clonal in nature and occur after receptor rearrangement, which provides a valuable tool for diagnosis and detection of residual disease (Robbins et al. 2015). Chromosomal translocations are common in NHLs and the dysregulation of specific oncogenes via translocation (including MYC, BCL2 and Cyclin D_1) are characteristic of specific NHL sub-types (Armitage et al. 2017). However, while our understanding of the molecular nature of these diseases has greatly improved, there are still significant outstanding questions regarding the formation, diagnosis and treatment of lymphoid malignancies.

Part II: DNA replication

DNA replication is intimately tied to cancer biology as it can serve as a potential source of DNA damage and oncogenic mutations. Thus, the faithful duplication of the genome is essential for both cellular and organismal viability. DNA replication is a highly complex and controlled process which serves to ensure the fidelity of DNA duplication and its proper inheritance by daughter cells. Mechanistically, failure to properly replicate the genome can lead to DNA damage, chromosomal abnormalities, aneuploidy, amplifications and deletions, genomic instability and disease, including cancer and aging.

Background

Prokaryotic circular chromosomes possess a single replication origin sequence where the DNA replication machinery is loaded onto DNA to initiate and complete the replication of the entire bacterial chromosome (Lewis et al. 2016). Due to significantly larger genomic size and the presence of multiple chromosomes, DNA replication in eukaryotes must initiate from thousands of individual replication origins positioned throughout the genome (Fragkos et al. 2015). As such, eukaryotic DNA replication is a tightly coordinated and highly controlled process, as the cell must balance the number of actively replicating forks, the availability of replication-associated proteins and metabolites required for DNA synthesis, and must coordinate DNA replication with other biological processes occurring at DNA, such as transcription (Zeman and Cimprich 2014).

Replication origins are licensed in late mitosis/early G1 via the recruitment of replication initiating proteins (Fragkos et al. 2015) (Figure 1). These licensed origins prepare the chromatin for replication and fire during S-phase, resulting in the formation of active, bi-directional replication forks (DePamphilis et al. 2006, Parker et al. 2017) (Figure 1). Origin firing is an essential regulatory component of DNA synthesis and all origins do not fire simultaneously; origins are divided into early-S- and late-S-phase-firing origins in order to maintain a balance between replication accuracy, speed and the availability of replication-associated proteins and nucleotides (Mechali 2010, Cayrou et al. 2011, Fragkos et al. 2015). Moreover, the regulation of origin firing must be such that each active origin fires once, and only once, during S-phase in order to prevent re-replication and the regional amplification of DNA sequences (Blow and Dutta 2005). In fact, most licensed origins do not fire at all during a single cell cycle, but rather are held dormant in reserve only to be

activated upon instances of DNA replication stress where permanently stalled or collapsed replication forks must be rescued by a dormant origin to ensure the complete replication of the genome (Ge et al. 2007, Kawabata et al. 2011, Shima and Pederson 2017). Therefore, while normal cells have evolved a highly complex and regulated system to ensure the totality and fidelity of DNA replication during each cell division, there remain ample opportunities for dysfunction during this process which can contribute to human disease, such as in aging and cancer. Before exploring the dysfunctions that can occur during replication, it is first important to review the basic molecular mechanisms of DNA replication and events where the integrity of this process can be threatened.

Molecular mechanisms of DNA replication and cell cycle progression

As previously noted, replication begins in late mitosis/early G1 with the licensing of replication origins (Fragkos et al. 2015, Parker et al. 2017). Licensing involves the recruitment of a complex of proteins consisting first of the ORC complex, followed by the regulatory factors CDC6 and CDT1 (Bell and Stillman 1992, Mizushima et al. 2000, Speck and Stillman 2007, Duzdevich et al. 2015). Together, these proteins then facilitate the loading of the heterohexameric MCM2-7 complex, a multi-protein complex that serves as the functional helicase upstream of the replication fork, onto DNA (Evrin et al. 2009, Remus et al. 2009, Duzdevich et al. 2015, Yuan et al. 2017). This entire complex (termed the pre-replication complex; pre-RC), once loaded onto DNA, awaits activation by kinases during the G1/S transition (Figure 1).

Cell cycle progression is regulated by the highly coordinated expression of the cyclin family of proteins, their cyclin-dependent kinase (CDK) partners and the RB/E2F

signaling pathway (Malumbres and Barbacid 2009). Although a large degree of functional redundancy and compensatory ability exists between different cyclins and CDK members in vertebrates (Satyanarayana and Kaldis 2009), the classical model of mammalian cell cycle regulation is described here. Upon the initiation of mitogenic signaling in G1, levels of cyclin D family member proteins increase (Musgrove et al. 2011). D family cyclins then bind to their obligate CDK partner(s), CDK4 and/or CDK6, thereby activating their kinase activity (Matsushime et al. 1992, Meyerson and Harlow 1994). RB and other RB family member proteins are then phosphorylated by CDK4/6 leading to their partial inactivation and the increased expression of E family cyclins (Kato et al. 1993, Sherr and Roberts 2004). E cyclins bind to CDK2, which together with CDK4/6, hyper-phosphorylate RB and completely inactivate it (Lundberg and Weinberg 1998, Harbour et al. 1999). Normally, E2F transcription factors are bound to RB and kept inactive in its hypo-phosphorylated state. Hyper-phosphorylation of RB liberates the E2F transcription factors, which then drive the transcriptional program that facilitates the G1/S transition (Harbour and Dean 2000, Attwooll et al. 2004).

CDKs (particularly CDK4, CDK6 and CDK2) promote S-phase entry from G1 by acting upon a wide-array of downstream targets, including through the sequestration of the cell-cycle inhibitory proteins $p27^{Kip1}$ and $p21^{Cip1}$ (Otto and Sicinski 2017). Moreover, CDKs, in conjunction with the CDC7 kinase, also act upon the pre-RC and facilitate the recruitment of CDC45 and the GINS complex to the MCM complex, resulting in helicase activation and the unwinding of DNA (Masai et al. 2006, Montagnoli et al. 2006, Im et al. 2009, Remus and Diffley 2009, Labib 2010, Costa et al. 2011) (Figure 1). Origin firing coincides with the formation of a replication bubble and the establishment of a bi-

directional replication fork (Figure 1). Primase then forms short RNA primers which are elongated by polα, thereby initiating DNA synthesis. Additional factors are recruited to the nascent replisome, including the DNA clamp/processivity factor PCNA, polε, and polδ; the latter of which replace polα on the leading and lagging strands, respectively (Sclafani and Holzen 2007) (Figure 1). Moreover, the ssDNA formed during DNA unwinding by the helicase complex is bound by the heterotrimeric, ssDNA binding protein RPA (consisting of the RPA70/RPA1, RPA32/RPA2 and RPA14/RPA3 subunits). RPA functions to prevent the re-annealing of ssDNA prior to its use as a template for DNA synthesis, to inhibit the formation of secondary ssDNA structures which impede DNA replication, and to stabilize the unwound DNA (Wold and Kelly 1988, Zou et al. 2006). At this point, replication continues until all DNA has been successfully duplicated and cells enter mitosis. However, active replisomes can encounter obstacles that prevent their ability to successfully replicate DNA, which leads to a multitude of cellular responses to remedy the sources of the replication block.

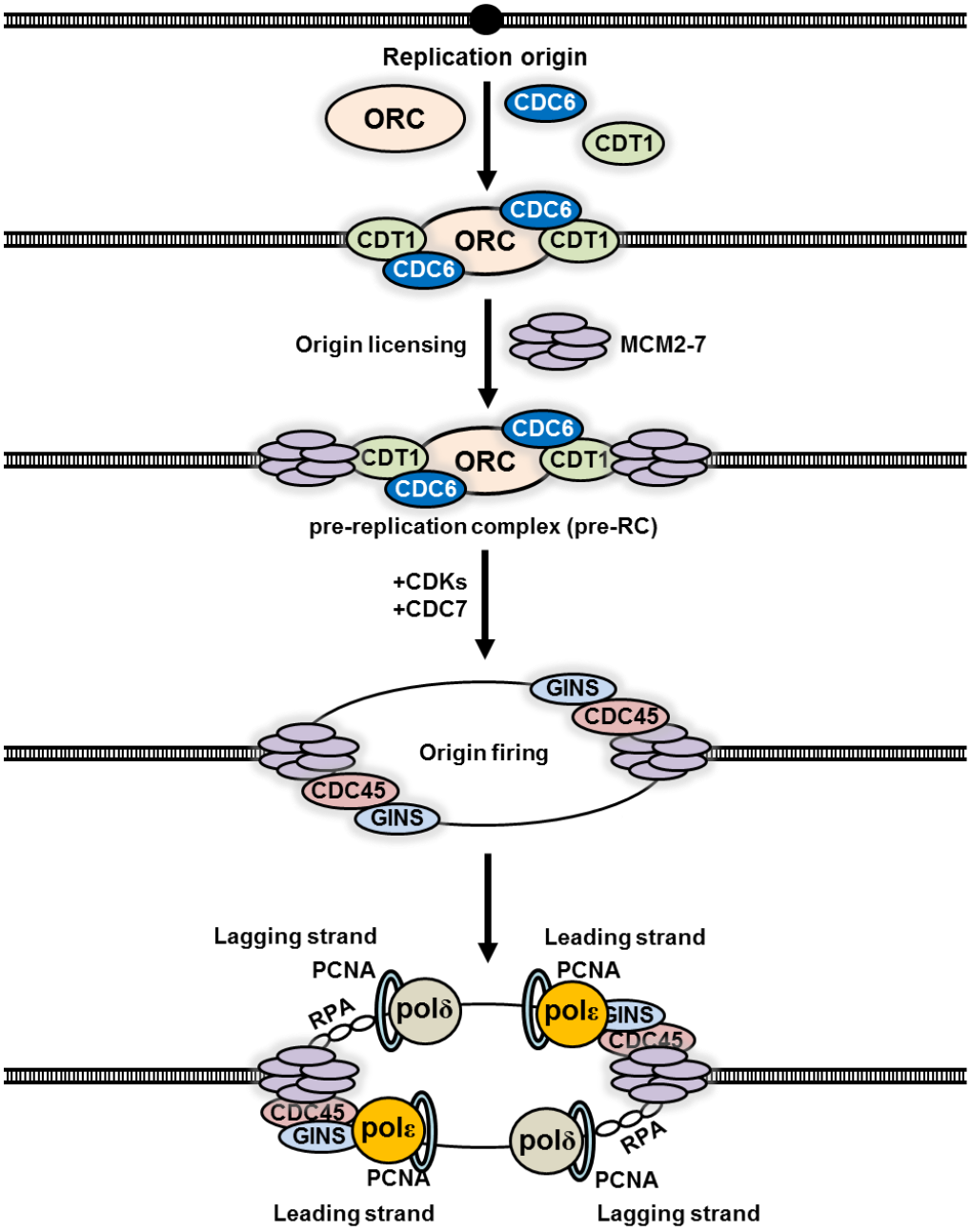

Figure 1. DNA replication in eukaryotes. Eukaryotic cells have thousands of replication origins located ubiquitously throughout the genome. DNA replication begins with origin licensing in late-M/early-G1. During licensing, the origin recognition complex (ORC) binds to origin sequences and together with CDC6 and CTD1, facilitates loading of the MCM2-7 complex onto DNA. This structure, termed the pre-replication complex (pre-RC), awaits phosphorylation by CDKs and CDC7, which promote loading of the GINS complex and CDC45 to the replication fork. This results in helicase activation, unwinding of the parental dsDNA and formation of an active replication bubble and bi-directional replication forks. Additional factors, including PCNA, polε and polδ are finally recruited to the nascent replisome and DNA synthesis commences.

DNA replication stress

As stated above, active replisomes encounter a wide variety of impediments which lead to replication fork slowing or stalling and the impairment of DNA synthesis – a process generally referred to as replication stress (Zeman and Cimprich 2014). The sources of replication stress are complex and varied and are both endogenous and exogenous in nature (Kotsantis et al. 2015). Specific sources of replication stress include damaged bases or mis-incorporated ribonucleotides, unresolved DNA damage, intrinsically difficult to replicate DNA sequences (including common fragile sites and sequences that form secondary DNA structures), altered nucleotide metabolism, collisions between the transcriptional machinery and replisomes, and the activities of oncogenes, such as MYC (Zeman and Cimprich 2014, Kotsantis et al. 2015) (Figure 2). These sources, and their contribution to cellular replication stress, will be briefly described below.

Many forms of replication stress are characterized by the functional uncoupling of the helicase complex and DNA polymerases. Under these conditions, the helicase complex continues to unwind the template DNA while the polymerases stall and are unable to synthesize new DNA (Figure 2). This results in the accumulation of excessive ssDNA adjacent to newly synthesized dsDNA. This ssDNA is rapidly bound by RPA, leading to the formation of RPA foci. In addition to forming a strong template for the recruitment of replication stress response factors (discussed below), this RPA-coated, persistent ssDNA threatens genomic integrity both through its inherently unstable nature (via its propensity for breakage during sustained fork stalling), but also through the processing of stalled forks by structure-specific endonucleases (Kotsantis et al. 2015). Replication stress can also

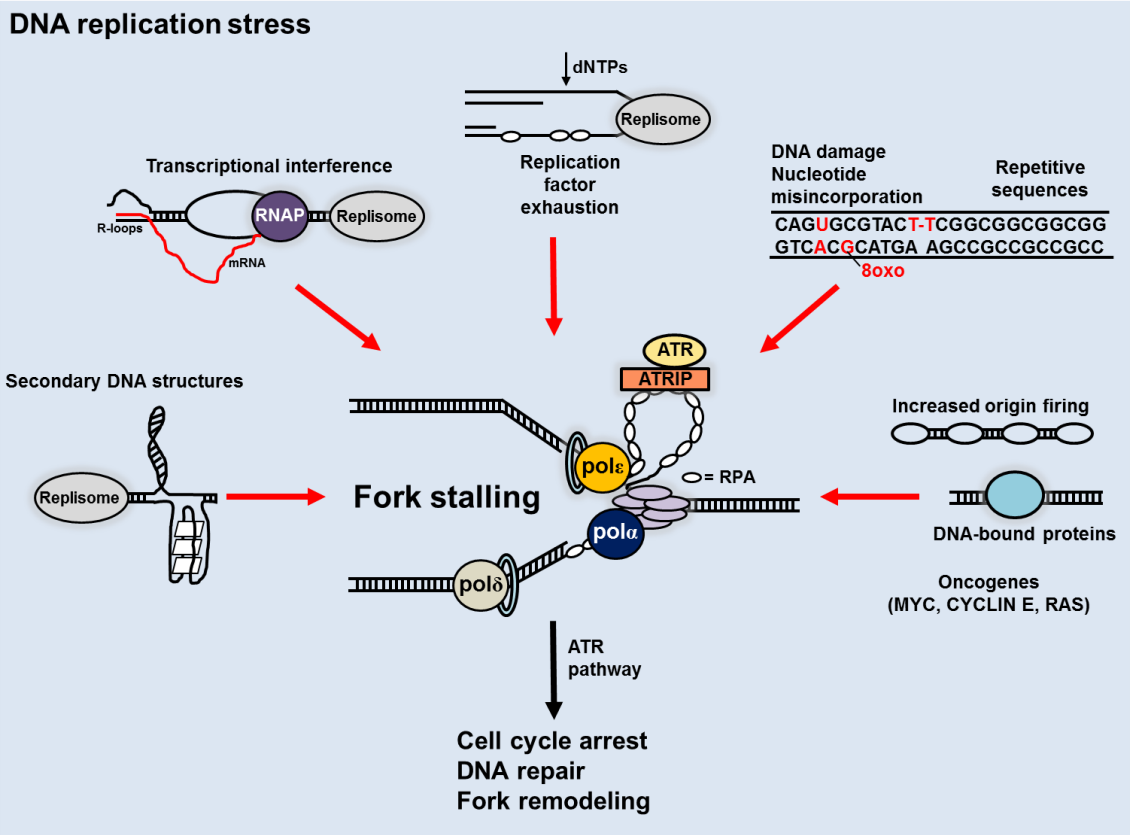

Figure 2. Common sources of DNA replication stress. Replication stress is a general term referring to a multitude of processes that inhibit replication fork progression and/or DNA synthesis and lead to fork stalling. In most instances, fork stalling is characterized by the uncoupling of the helicase and polymerase complexes, resulting in the excessive build-up of RPA-coated, ssDNA. This structure serves as a recruitment template for ATR via its interaction with ATRIP. The ATR kinase then coordinates the cellular response to replication stress. Fork stalling is the consequence of multiple obstacles impacting replication fork progression including: secondary DNA structures, transcriptional interference, DNA damage, replication factor/metabolite exhaustion, oncogene overexpression, alterations to cell cycle progression and interference with DNA-bound proteins.

occur without dissociation between the helicase and polymerase activities (Zeman and Cimprich 2014). For instance, interference between the replisome and DNA/protein complexes and inter-strand crosslinks can induce a robust replication stress response without the accumulation of ssDNA. However, the exact mechanism and pathways involved in the repair of these lesions remains incompletely understood.

One of the most common sources of replication stress is unrepaired DNA damage and base incorporation errors made during DNA replication (Barnes and Lindahl 2004). While considered "high fidelity" polymerases, polε and polδ occasionally mis-incorporate nucleotides into nascent DNA (error rates are approximately 10^{-4} to 10^{-5}) (Kunkel 2009). These insertions must be repaired by specialized endonucleases which can lead to fork stalling (Jiricny 2006). Moreover, DNA damage, such as ssDNA nicks caused by topoisomerases, ROS, ionizing radiation or excision repair pathways can lead to passive DSB formation and fork collapse via replication runoff (Kuzminov 2001, Cortes-Ledesma and Aguilera 2006, Hashimoto et al. 2011, Zeman and Cimprich 2014). Severe damage, like ICLs and UV-induced pyrimidine dimers can function as persistent barriers to replication fork progression, eliciting a strong replication stress response and requiring DDT pathways to circumvent the fork stalling lesion (Prakash et al. 2005, Lehmann et al. 2007, Bianchi et al. 2013, Huang et al. 2013, Quinet et al. 2014).

Specific regions of eukaryotic genomes are also inherently prone to replication stress. Common fragile sites (CFSs) are regions of the genome that are prone to breakage upon mild replication stress even with the cellular checkpoint/replication stress response intact (Gaillard et al. 2015). Multiple hypotheses have been put forth to explain their enhanced sensitivity to replication stress. It has been proposed that very long genes

contained in these regions have high collision rates between the transcriptional machinery and active replisomes (Helmrich et al. 2011). Moreover, additional studies have demonstrated that CFSs often reside in origin-poor regions, leading to a paucity of dormant origins available to rescue stalled forks, which eventually results in fork collapse and the under-replication of DNA (Letessier et al. 2011, Ozeri-Galai et al. 2011). DNA breakage within CFSs has been associated with multiple human diseases including several different types of cancer (Yunis and Soreng 1984, Tsantoulis et al. 2008, Burrow et al. 2009, Bignell et al. 2010).

Additionally, certain regions of DNA, such as tri-nucleotide repeats (TNRs) and G-quadruplexes, have been identified as intrinsic sources of replication stress-associated DNA damage (Wu et al. 2008, Sarkies et al. 2012, Follonier et al. 2013). TNRs can form secondary DNA structures that impede replisome progression and can also promote fork slippage, which contributes to the expansion or contraction of TNRs and genomic instability. G-quadruplex structures can serve as a physical impediment to replication fork progression (McMurray 2010, Kim and Mirkin 2013, Gaillard et al. 2015).

Interference between transcription and DNA replication is another common source of replication stress. Since these processes both occur at DNA, dysregulation or impaired coordination between either process can impair DNA synthesis and fork progression (Gaillard et al. 2013). Transcriptional interference with DNA replication can occur due to physical collisions between the competing complexes, leading to recombination and impaired DNA synthesis (Gottipati et al. 2008, Jones et al. 2013). Moreover, positive super-coiling associated with transcriptional progression along DNA, as well as the tethering of actively transcribed genes to the nuclear pore, can lead to torsional stress and

abnormal DNA structures resulting in fork stalling and replication stress (Wang 2002, Koster et al. 2010, Bermejo et al. 2011). Additionally, actively transcribed nascent RNA can form R-loops with complementary DNA sequences, which represents another form of topological stress that is unable to be navigated by active replisomes (Gan et al. 2011, Bhatia et al. 2014).

Cellular metabolism and the availability of replication-associated factors are also critical determinants of replication stress. Normal replication requires a multitude of replication factors including RPA, dNTPs, helicases, topoisomerases, histones and other proteins involved in replisome assembly, organization, and function of newly synthesized DNA. Depletion or exhaustion of any of these factors can lead to replication fork stalling and replication stress (Nelson et al. 2002, Ge et al. 2007, Ibarra et al. 2008, Bester et al. 2011, Steckel et al. 2012, Toledo et al. 2013, Zimmerman et al. 2013, Xie et al. 2014). This can occur due to altered cellular metabolism, where the synthesis of required metabolites is not coordinated with S-phase entry. Moreover, dysregulated cell cycle progression may lead to excessive origin firing resulting in exhaustion of critical replication-associated proteins and metabolites (Halazonetis et al. 2008, Bester et al. 2011, Jones et al. 2013, Srinivasan et al. 2013).

Finally, oncogenes are a noteworthy endogenous source of replication stress. Replication stress-induced by oncogenes is postulated to be a significant contributor to neoplastic transformation by promoting genomic instability, mutation and inactivation of tumor suppressive pathways (Bartkova et al. 2006, Di Micco et al. 2006, Dominguez-Sola et al. 2007). Oncogenes induce replicative stress through a variety of mechanisms, the details of which will be discussed in Chapter I, Part III.

Eukaryotic replication stress response

Upon replication stress, eukaryotic cells activate a complex kinase cascade that senses stalled forks and coordinates the cellular replication stress response in order to maintain replication fidelity, complete DNA synthesis and sustain cellular viability (Zeman and Cimprich 2014, Gaillard et al. 2015). The major kinase involved in this process is ATR (Saldivar et al. 2017). ATR is a member of the PIKK family of Ser/Thr kinases which also includes ATM and DNA-PK, the kinases primarily required for orchestrating the cellular response to DSBs (Blackford and Jackson 2017). Upon activation, ATR promotes an intra-S-phase cell cycle arrest and coordinates the repair and restart of stalled replisomes (Saldivar et al. 2017). Unlike ATM and DNA-PK, ATR is indispensable for proliferating cell survival (Brown and Baltimore 2000, de Klein et al. 2000). ATR deletion is embryonic lethal in mice while humans born with hypomorphic alleles of ATR present with Seckel Syndrome, a rare disease characterized by microcephaly, dwarfism, characteristic facial abnormalities and intellectual disability (Brown and Baltimore 2000, de Klein et al. 2000, O'Driscoll et al. 2003).

The major function of ATR is to coordinate multiple cellular processes in order to facilitate the accurate completion of DNA synthesis during stressful conditions (Saldivar et al. 2017). As previously noted, a canonical feature of many types of replication stress is the accumulation of RPA-coated ssDNA at the fork junction of stalled replisomes due to helicase/polymerase uncoupling. This specific structure serves as a recruitment platform for ATRIP (Zou and Elledge 2003), which in turn binds ATR and localizes it to stalled replication forks (Cortez et al. 2001, Ball et al. 2007). Following ATR localization, recruitment of the 9-1-1 clamp, in conjunction with the co-factors TOPBP1 and RAD17,

activates ATR kinase activity (Bermudez et al. 2003, Zou et al. 2003, Majka et al. 2006, Delacroix et al. 2007, Lee et al. 2007). Recent discoveries have also identified ETAA1 as an alternative activator of the ATR kinase that acts in parallel to the 9-1-1/RAD17/TOPBP1 complex, through its direct binding to RPA (Bass et al. 2016, Haahr et al. 2016).

Activation of ATR leads to the wide-spread phosphorylation of numerous downstream protein targets. The primary target of ATR is the kinase CHK1 which is phosphorylated by ATR on the Ser-317 and Ser-345 residues (Walworth and Bernards 1996, Liu et al. 2000, Lopez-Girona et al. 2001). Additional ATR phosphorylation targets include RPA and the histone H2A variant H2AX (γH2AX) (Brush et al. 1996, Wang et al. 2001, Ward and Chen 2001). However, H2AX is also phosphorylated by ATM and DNA-PK upon other sources of DNA damage, such as DSBs, and is a non-specific marker of ATR activation (Blackford and Jackson 2017).

Upon activation by ATR, CHK1 functions to coordinate the global cellular response to replication stress (Cimprich and Cortez 2008). Specifically, it phosphorylates its downstream targets to initiate cell cycle arrest, prevent the firing of late-S-phase origins and activate proteins involved in stabilizing and restarting stalled replication forks (Furnari et al. 1997, Peng et al. 1997, Sanchez et al. 1997, Feijoo et al. 2001, Heffernan et al. 2002, Smits et al. 2006, Maya-Mendoza et al. 2007). Biologically, these processes allow the cell time to repair DNA damage while inhibiting cell cycle progression and preventing the cell from entering mitosis with under-replicated or damaged DNA. Additionally, by suppressing origin firing and reducing DNA synthesis rates, the cell is able to preserve stores of critical replication components, such as RPA and nucleotides, and prevent their exhaustion – which can result in replication catastrophe and cell death.

In many instances, stalled forks can restart once the blocking lesion has been removed through the utilization of standard DNA repair mechanisms. For instance, NER and BER can repair nucleotide mismatch, misincorporation or modified bases (Marteijn et al. 2014, Wallace 2014). Moreover, damage on the lagging strand is less deleterious to replication than that on the leading strand, as the discontinuous nature of lagging strand synthesis and re-priming that continuously occurs with Okazaki fragment formation results in ssDNA gaps that bypass the lesion and allow for its repair at a later point (Kotsantis et al. 2015). For bulky or more persistent lesions, cells also utilize trans-lesion synthesis (TLS) pathways which either directly by-pass the stall-inducing lesion by leaving behind a ssDNA gap to be filled in by error-prone TLS polymerases (Prakash et al. 2005). This allows replication to continue while sacrificing fidelity, a trade-off that appears to be beneficial relative to the consequences of fork collapse and DSB formation (discussed below). Additionally, cells can utilize homologous recombination (HR), template switching or re-priming to restart stalled replication forks (Costes and Lambert 2012).

Other instances of fork stalling require specialized proteins to initiate proper fork restart. For instance, the RecQ family of helicases (BLM, WRN and RECQ1), certain Fanconi-anemia (FA) family member proteins (FANCM and FANCJ), as well as the specialized DNA translocases (SMARCAL1, ZRANB3 and HLTF) have been shown to promote fork restart on model DNA substrates *in vitro* through fork regression and branch migration (discussed below). However, the exact biochemical mechanism and the *in vivo* events that require each of these proteins are currently unclear and remain under intensive study.

If stalled replication forks are not stabilized or restarted in a timely manner, they are prone to collapse. Functionally, fork collapse refers to a replisome that has lost the ability to synthesize DNA. Mechanistically, this has been attributed to dissociation of the replication machinery or processing of the fork into a dsDNA break (Cortez 2015, Kotsantis et al. 2015). The efficient restart of stalled replisomes is essential for the maintenance of genomic integrity as the longer forks remain stalled the more likely they are to collapse (Saintigny et al. 2001, Petermann et al. 2010, Ragland et al. 2013).

As previously noted, collapse had historically been attributed to dissociation from or misplacement of the replication machinery from DNA, however recent data have challenged this view (Cobb et al. 2003, Lucca et al. 2004, Hashimoto et al. 2011, De Piccoli et al. 2012, Ragland et al. 2013, Yu et al. 2014). Moreover, stalled forks can be processed into DSBs by structure-specific endonucleases (Kotsantis et al. 2015). Fork cleavage has been proposed as a mechanism to restart replication using BIR, which involves D-loop formation with a homologous template followed by recruitment of the replication machinery (Zeman and Cimprich 2014, Sakofsky and Malkova 2017). However, in many instances, cleavage of stalled replication forks is associated with incapacity for fork restart (Kotsantis et al. 2015). This is likely due to inappropriate cleavage by structure specific endonucleases which aberrantly processes stalled replication forks into DSBs due to their structural similarity to DNA repair intermediates (Cotta-Ramusino et al. 2005, Hanada et al. 2007, Munoz et al. 2009, Kotsantis et al. 2015). Checkpoint activation during replication stress often phosphorylates many of these endonucleases to reduce their activity as the activity of these enzymes is normally limited to G2/M to prevent aberrant fork cleavage during S-phase (Matos et al. 2011, Sorensen and Syljuasen 2012). However, DSB

formation is a hallmark of prolonged replication stress even in checkpoint intact cells, highlighting the critical importance of replication fork restart and fork protection in maintaining genomic stability during replication stress (Zeman and Cimprich 2014).

Fork reversal

One important mechanism involved in limiting fork collapse that is of particular interest to the field is fork reversal (also known as fork regression). This process involves the re-annealing and reversal of both parental DNA and complimentary, newly synthesized DNA daughter strands into a "chicken-foot" structure, or a 4-way junction, at the replication fork (Neelsen and Lopes 2015). Fork reversal was originally identified only in checkpoint-deficient cells and was believed to be a pathological consequence of inactivation of the replication stress response (Sogo et al. 2002, Cotta-Ramusino et al. 2005, Bermejo et al. 2011). However, through the use of electron microscopy, several recent studies have observed fork reversal in a wide-range of normal vertebrate cells following exposure to multiple replication stress-inducing stimuli, thereby establishing fork reversal as a highly conserved, significant component of the cellular response to replication stress (Ray Chaudhuri et al. 2012, Neelsen et al. 2013, Ray Chaudhuri et al. 2015, Zellweger et al. 2015). Fork reversal is catalyzed by a wide-range of proteins *in vitro*, including helicases such as BLM and WRN as well as multiple, distant SNF2-family member, specialized DNA translocases (SMARCAL1, ZRANB3 and HLTF) (Machwe et al. 2006, Ralf et al. 2006, Machwe et al. 2007, Blastyak et al. 2010, Betous et al. 2012, Ciccia et al. 2012, Betous et al. 2013).

Mechanistically, fork reversal is believed to provide protection to stalled replisomes through multiple mechanisms. First, by re-annealing the parental DNA strands normally subjected to unwinding by helicases, fork reversal may prevent the accumulation of ssDNA at stalled replication forks, thereby precluding stalled forks to aberrant endonucleolytic cleavage and collapse (Ray Chaudhuri et al. 2012, Zellweger et al. 2015). Moreover, stabilization of stalled replication forks into reversed structures may replace the stall-inducing lesion into the context of dsDNA thereby allowing repair of the DNA damage by the normal error-free excision DNA repair pathways, while simultaneously preventing error-prone TLS pathways from bypassing lesions inducing fork stalling (Ray Chaudhuri et al. 2012, Neelsen and Lopes 2015, Zellweger et al. 2015). Additionally, retaining damaged replisomes in a reversed fork structure for extended periods of time may allow for replication rescue by dormant origins or reversed forks may serve as a template for DNA repair nucleases to initiate error-free, template-switching pathways needed to bypass persistently damaged DNA that is unable to be repaired in a timely fashion (Neelsen and Lopes 2015). However, while the existence of fork reversal has been established as a normal cellular response to replication fork stalling, the biological importance of the proteins that promote fork regression and their contribution to normal biological processes, such as hematopoiesis, aging and cancer remains unresolved. In particular, questions remain regarding the functional redundancy of proteins able to catalyze fork reversal, the requirements for their activity upon exposure to different sources of replication stress (both endogenous and exogenous) and their ability to influence biological processes such as tumorigenesis. Due to their close homology and similar *in vitro* functions, the SNF2 family

members SMARCAL1 and ZRANB3 are appealing targets to begin to address some of these outstanding questions.

SMARCAL1

SMARCAL1 (SWI/SNF-related matrix-associated, actin-dependent regular of chromatin, subfamily A-like 1; also known as HARP/HepA-related protein) is a 954 amino acid protein involved in maintaining replication fork integrity. Orthologs have been identified in higher eukaryotes ranging from *C. elegens* to mice; however no ortholog is readily identifiable in yeast (Coleman et al. 2000). Originally identified from bovine thymus isolate (Hockensmith et al. 1986), SMARCAL1 is a distant SWI/SNF family member which possesses sequence homology to the bacterial protein, HepA, a DNA-dependent ATPase (Coleman et al. 2000). Structurally, SMARCAL1 contains an N-terminal RPA binding domain, two central HARP domains and two C-terminus ATPase domains which are homologous to other SNF2 family members (Poole and Cortez 2017) (Figure 3). However, although possessing sequence similarity, unlike HepA, which is involved in transcriptional regulation, SMARCAL1 appears to have evolved specific functions at the replication fork.

Mutations in the *SMARCAL1* gene cause the rare, pleiotropic disorder Schimke immuno-osseous dysplasia (SIOD) in humans (Boerkoel et al. 2002). This disease manifests as progressive immuno-deficiency, spondyloepiphyseal dysplasia, facial dysmorphism, and severe, gradual nephropathy resulting in end-stage kidney disease (Spranger et al. 1991, Boerkoel et al. 2002, Clewing et al. 2007). Most patients do not survive past their early teens and succumb to severe, recurrent infections or complications

arising from renal transplantation (Spranger et al. 1991). Causative non-sense and missense mutations in SMARCAL1 have been identified in SIOD patients (Boerkoel et al. 2002, Clewing et al. 2007). The phenotypic severity of these mutations *in vitro* appears to correlate with the degree of retained SMARCAL1 protein function. Proteins from patients with missense mutations preserving partial ATPase activity display less severe phenotypes compared to mutants with abolished ATPase activity in *in vitro* fork reversal and ATPase assays (Betous et al. 2012). However, clinical severity has still been observed in patients with "mild" missense mutations and a complete understanding of the genotype/phenotype relationship in SIOD remains unresolved. While a few case reports have noted incidences of cancer in SIOD patients (Baradaran-Heravi et al. 2012, Carroll et al. 2013), the link between SMARCAL1 mutations and tumor development is currently unclear. Moreover, while experimental evidence has demonstrated that mutated SMARCAL1 proteins found in SIOD patients are unable to catalyze its enzymatic functions (Betous et al. 2012, Carroll et al. 2013), the precise biological mechanisms behind the observed cellular and clinical phenotypes of SIOD remains incompletely understood.

While the SMARCAL1 protein was originally identified in 2000, and its link to SIOD established in 2002, the first manuscript detailing a biochemical function of the protein was published in 2008. Using a plasmid-based assay, Yusufzai and Kadonaga demonstrated that SMARCAL1 is able to re-anneal complimentary sequences of single-stranded DNA (ssDNA) bound by the ssDNA binding protein RPA (Yusufzai and Kadonaga 2008). This function was ablated with inactivation of the ATPase domains (Betous et al. 2012). Further experimentation utilizing single-molecule molecular tweezers confirmed this fundamental function of SMARCAL1 (Betous et al. 2012, Burnham et al.

2017). Based upon this activity, SMARCAL1 was classified as a novel "annealing helicase".

SMARCAL1 is believed to primarily function in the replication stress response by stabilizing, repairing and remodeling stalled replication forks through its DNA-dependent ATPase/translocase activity (Poole and Cortez 2017). SMARCAL1 is recruited to stalled replication forks through its interaction with RPA32 via an RPA-binding domain present in its N-terminus (Bansbach et al. 2009, Ciccia et al. 2009, Postow et al. 2009, Yuan et al. 2009, Yusufzai et al. 2009) (Figure 3). This binding domain shares a high degree of sequence homology with other proteins known to interact with RPA and is essential for the recruitment of SMARCAL1 to sites of replication stress and for facilitating its fork repair/restart functions (Bansbach et al. 2009, Ciccia et al. 2009, Postow et al. 2009, Yuan et al. 2009, Bansbach et al. 2010, Matsuzaki et al. 2015, Bass et al. 2016, Haahr et al. 2016). SMARCAL1 shows the highest DNA binding affinity for forked junctions containing both ssDNA and dsDNA and does not bind DNA that is exclusively single-stranded or double-stranded in nature (Muthuswami et al. 2000, Yusufzai and Kadonaga 2008, Ghosal et al. 2011, Betous et al. 2012, Mason et al. 2014). DNA binding by SMARCAL1 requires both its ATPase and HARP domains, the latter of which function as substrate recognition domains to impart specificity for forked DNA structures (Betous et al. 2012, Mason et al. 2014). Mutations in the HARP2 domain result in a greater loss of ATP hydrolysis, DNA binding, and fork remodeling functions *in vitro* as compared to HARP1-mutant proteins. However, SIOD patients with HARP1 mutations still exhibit severe clinical phenotypes indicating the importance of both domains (Boerkoel et al. 2002, Betous et al. 2012).

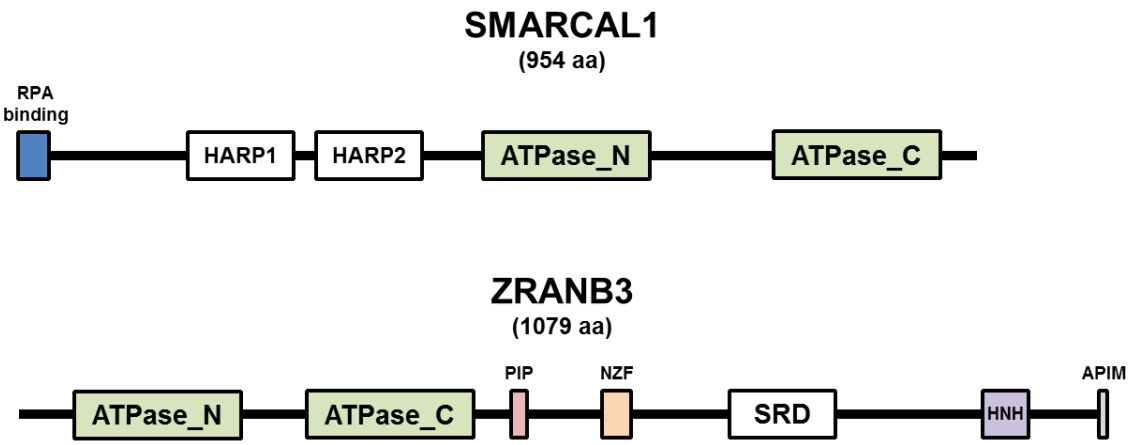

Figure 3. Functional domains of SMARCAL1 and ZRANB3. Schematic showing the identified functional domains of SMARCAL1 and ZRANB3. SMARCAL1 is localized to replication forks through an interaction of the N-terminal RPA binding domain with RPA. ZRANB3 localizes to replication through an interaction with poly-ubiquitinated PCNA which require its PIP, NZF and APIM domains.

SMARCAL1 is able to catalyze both fork regression of stalled replication forks as well as the reverse reaction, fork restoration (Betous et al. 2012, Betous et al. 2013, Bhat et al. 2015, Kolinjivadi et al. 2017). It is believed that this is the primary biochemical function of the protein as patient-derived SMARCAL1 mutants are impaired in their ability to catalyze these reactions *in vitro* (Betous et al. 2012, Carroll et al. 2013). Moreover, knockdown of SMARCAL1 alone in the absence of additional replication stress-inducing agents in cancer cell lines is sufficient to induce DSBs (Bansbach et al. 2009). However, whether or not this also occurs in untransformed cells is unclear.

Since inappropriate fork regression can result in DNA damage and impaired DNA replication, SMARCAL1 activity is tightly regulated by the cell. Overexpression experiments have demonstrated that SMARCAL1 hyperactivity also induces DSBs, as excessive recruitment of SMARCAL1 to normal replication forks likely results in aberrant fork remodeling and DSB formation (Bansbach et al. 2009). SMARCAL1 activity is regulated by phosphorylation and it is a known target of ATM, DNA-PK and ATR (Bansbach et al. 2009, Postow et al. 2009). Ser652 is phosphorylated by ATR and appears to be a major regulatory residue. Phosphorylation at this site leads to both reduced ATPase activity and reduced fork regression *in vitro* (Couch et al. 2013). Phosphorylation at Ser652 likely functions to maintain replication fork stability by properly balancing the amount of SMARCAL1-induced fork regression while also preventing aberrant or hyperactive fork remodeling (Couch et al. 2013, Poole and Cortez 2017). There are many additional phosphorylation sites on SMARCAL1, including the Ser889 residue which results in SMARCAL1 hyperactivity (Carroll et al. 2014). The biological significance of many of

the other phosphorylation sites, and the kinases responsible for these modifications, has not been elucidated at this point.

SMARCAL1 activity is also regulated through its substrate preferences, which are dictated in large part by the patterns of RPA binding at replication forks. At normally replicating forks, RPA is bound to the ssDNA gaps interspersed between Okazaki fragments on the lagging strand. This normal replication fork structure inhibits SMARCAL1 activity, thereby preventing abnormal remodeling of normal replication forks (Betous et al. 2013, Bhat et al. 2015). However, SMARCAL1 shows a strong preference for regressing forks with ssDNA gaps on the leading strand – a hallmark of many types of replication stress (Betous et al. 2013, Bhat et al. 2015) (Figure 2). Moreover, SMARCAL1 demonstrates a preference for restoring reversed forks that would lead to a three-way junction with a normal lagging strand ssDNA gap while also showing reduced restoration activity at reversed substrates that would result in a replication fork with a leading strand gap (Betous et al. 2013, Bhat et al. 2015). Thus, SMARCAL1 preferentially reverses stalled forks with a leading strand ssDNA gap while restoring reversed forks into a normal three-way junction with the proper lagging-strand gap.

SMARCAL1 does not show sequence specificity in its binding to DNA and there is some evidence that it travels with normally elongating replisomes, suggesting it is a general factor involved in the resolution of replication stress (Betous et al. 2012, Dungrawala et al. 2015). However, recent studies have shown that it has a specific function at telomeres, as SMARCAL1-deficient cells show elevated levels of telomere damage and evidence of abnormal telomeric replication (Poole et al. 2015, Cox et al. 2016). This function of SMARCAL1 appears to be independent of RPA binding, as N-terminal mutants

lacking the RPA-binding domain were still able to resolve telomeric replication stress and prevent c-circle formation (Poole et al. 2015).

Moreover, fork reversal mediated by proteins including SMARCAL1 and ZRANB3 has been implicated in genome stability in BRCA1/2-mutant cell lines. Upon fork reversal in normal cells, BRCA1 and BRCA2 function in tandem with RAD51 and other factors to stabilize reversed forks and prevent nucleolytic fork degradation into abnormal replication intermediates by the MRE11 nuclease (Hashimoto et al. 2010, Schlacher et al. 2011, Schlacher et al. 2012). However, in BRCA1/2-deficient cells, fork reversal by SNF-2 family member fork remodelers leads to an unprotected substrate for MRE11 leading to fork degradation and genomic instability (Kolinjivadi et al. 2017, Lemacon et al. 2017, Taglialatela et al. 2017). Thus, fork reversal mediated by SMARCAL1 and related proteins like ZRANB3 is an important contributor to genomic instability in BRCA1/2-deficient cells.

However, while many of the biochemical functions of SMARCAL1 have been elucidated, many outstanding questions remain. It is still unknown what other sources of endogenous replication stress require SMARCAL1 for resolution as is the role of SMARCAL1 *in vivo* (particularly in regards to normal biological processes like hematopoiesis, tumorigenesis and the etiology of the phenotypes of SIOD).

ZRANB3

ZRANB3 (Zinc-finger, RAN-binding domain containing 3) is a 1079 amino acid protein and member of the same class of distant SNF2 DNA translocases as SMARCAL1 and HLTF (Poole and Cortez 2017). Based on sequence homology, it is believed to be the most

closely related protein to SMARCAL1. Like SMARCAL1, ZRANB3 is thought to function as a fork remodeling/repair protein. However, unlike SMARCAL1, ZRANB3 does not possess an RPA-binding domain and is not recruited to replication forks by RPA (Yusufzai and Kadonaga 2010). Rather, ZRANB3 localizes to replication forks through an interaction with PCNA (Ciccia et al. 2012, Weston et al. 2012, Yuan et al. 2012). Structurally, ZRANB3 contains a conical PIP box as well as an APIM motif, both of which are required for mediating interactions with PCNA (Ciccia et al. 2012, Weston et al. 2012, Yuan et al. 2012, Sebesta et al. 2017) (Figure 3). These sequences show a high degree of homology with hundreds of other proteins that interact with PCNA (Mailand et al. 2013). Since PCNA is present at all active replisomes, ZRANB3 recruitment must be regulated in such a way that it does not localize to and aberrantly remodel normal replication forks. Upon replication stress, PCNA is ubiquitinated, which mediates the recruitment of multiple factors for fork repair and restart (Kanao and Masutani 2017). Interestingly, ZRANB3 also contains an NZF domain which specifically binds to polyubiquitin (Ciccia et al. 2012, Weston et al. 2012). Thus, while ZRANB3's association with PCNA requires its PIP box and APIM motif, its recruitment only occurs upon PCNA polyubiquitination at lysine 164 and engagement of the ZRANB3 NZF motif. Thus, the recruitment of ZRANB3 to replication forks is confined to damaged forks marked with polyubiquitinated PCNA during replication stress (Ciccia et al. 2012, Weston et al. 2012, Yuan et al. 2012, Sebesta et al. 2017).

ZRANB3 shares many biochemical similarities to SMARCAL1 in *in vitro* assays. As with SMARCAL1 loss, ZRANB3 depletion results in sensitivity to a wide-variety of DNA damaging agents and increased fork stalling when cells are treated with HU (Ciccia

et al. 2012, Weston et al. 2012, Yuan et al. 2012). Likewise, ZRANB3 possesses annealing helicase activity as it also is able to re-anneal complimentary, RPA bound ssDNA on plasmids (Yusufzai and Kadonaga 2010). Additionally, ZRANB3 is able to catalyze fork regression and restoration of replication forks *in vitro* (Ciccia et al. 2012, Betous et al. 2013, Vujanovic et al. 2017). Thus, ZRANB3, like SMARCAL1 is likely an important fork protection factor during replication stress.

ZRANB3, however, possess several unique functional differences from SMARCAL1. ZRANB3 does not possess HARP domains which confer forked DNA substrate recognition capabilities to SMARCAL1 (Betous et al. 2012, Mason et al. 2014). Rather, a recent study has demonstrated that ZRANB3 possesses a unique substrate recognition domain spanning amino acids 721-869 (Badu-Nkansah et al. 2016) (Figure 3). Like the HARP domains in SMARCAL1, this sequence imparts specificity for ZRANB3 binding to forked DNA structures (Badu-Nkansah et al. 2016).

ZRANB3 also possesses a C-terminus endonuclease domain (Weston et al. 2012, Badu-Nkansah et al. 2016, Sebesta et al. 2017). Biochemical analysis of its endonuclease function has shown a substrate preference of a ssDNA:dsDNA junction with at least twenty nucleotides of ssDNA. ZRANB3 then nicks the DNA two nucleotides into the dsDNA region (Weston et al. 2012). While the biological importance of this unique activity is unclear at this point, it has been proposed to function as a potential protection mechanism against DSB formation. (Weston et al. 2012). Under this proposal, ZRANB3 nicks the leading strand template upstream of a DNA lesion (Weston et al. 2012). It then catalyzes fork reversal to stabilize the fork, placing the lesion and nick back within the context of dsDNA and preventing endonucleolytic cleavage of the stalled fork. The exposed ‘3-OH

group is extended by a polymerase, the damaged DNA excised by a flap nuclease and the nick is then re-ligated to fully repair the lesion.

In addition to this distinctive endonuclease function, ZRANB3 is also able to catalyze the dissolution of D-loops (Ciccia et al. 2012). While SMARCAL1 is also able to dissolve D-loops after their formation, interestingly, ZRANB3 is able to block the formation of D-loops and dissolve pre-formed D-loops (Ciccia et al. 2012). Thus, it is likely that ZRANB3 functions to both resolve D-loops after HR (a function shared with SMARCAL1) while also regulating levels of strand-invasion and preventing aberrant recombination during replication (Poole and Cortez 2017). In support of this hypothesis, ZRANB3-deficient cells have been shown to have increased levels of sister chromatid exchanges (SCEs), signifying increased levels of uncontrolled recombination (Ciccia et al. 2012).

Less is known about the regulation of ZRANB3 than SMARCAL1. In addition to regulating its recruitment to stalled replisomes, PCNA also stimulates ZRANB3 nuclease activity (Sebesta et al. 2017). Moreover, RPA binding patterns at replication forks have also been shown to influence ZRANB3-mediated fork remodeling. Unlike SMARCAL1, which is driven to regress forks when RPA is bound to a ssDNA gap on the leading strand, ZRANB3 fork reversal is greatly inhibited by RPA on the leading strand (Betous et al. 2013). RPA bound to ssDNA gaps on the lagging strand has no effect on the ability of ZRANB3 to regress forks *in vitro*. Moreover, ZRANB3 is also inhibited from restoring stalled forks with a ssDNA gap on the lagging strand (Betous et al. 2013). While the full mechanism of how RPA directs the regression abilities of both proteins is still being elucidated, these data potentially suggest some degree of specificity for fork reversal by

either protein depending upon the location of RPA binding at stalled replication forks. However, more studies are needed to fully elucidate the biochemical functions of these proteins at replisomes.

As with SMARCAL1, there are still many outstanding questions about the biological functions of ZRANB3. Mutations in ZRANB3 have not been implicated in any human disease. Moreover, while some sequencing data exist suggesting ZRANB3 may be mutated in a subset of endometrial cancers (Lawrence et al. 2014), its impact on tumor development and role in tumorigenesis remains unknown at this point. Additionally, the functions of ZRANB3 in other normal biological process (such as hematopoiesis, stem cell biology, etc.) and sources of endogenous replication stress that require its functions for resolution are still unknown.

Replication stress in hematopoietic stem cells

Hematopoiesis is a hierarchical process where multi-potent long-term stem cells (LT-HSCs), through a process of asymmetric cell division, generate multiple progenitor cell populations that undergo significant expansion and differentiation to generate the entire range of mature hematopoietic cells (Bryder et al. 2006). Through the use of flow cytometry, multiple patterns of surface antigen expression have been elucidated to identify specific populations of hematopoietic stem and progenitor cells (HSPCs) (Challen et al. 2009) (Figure 4).

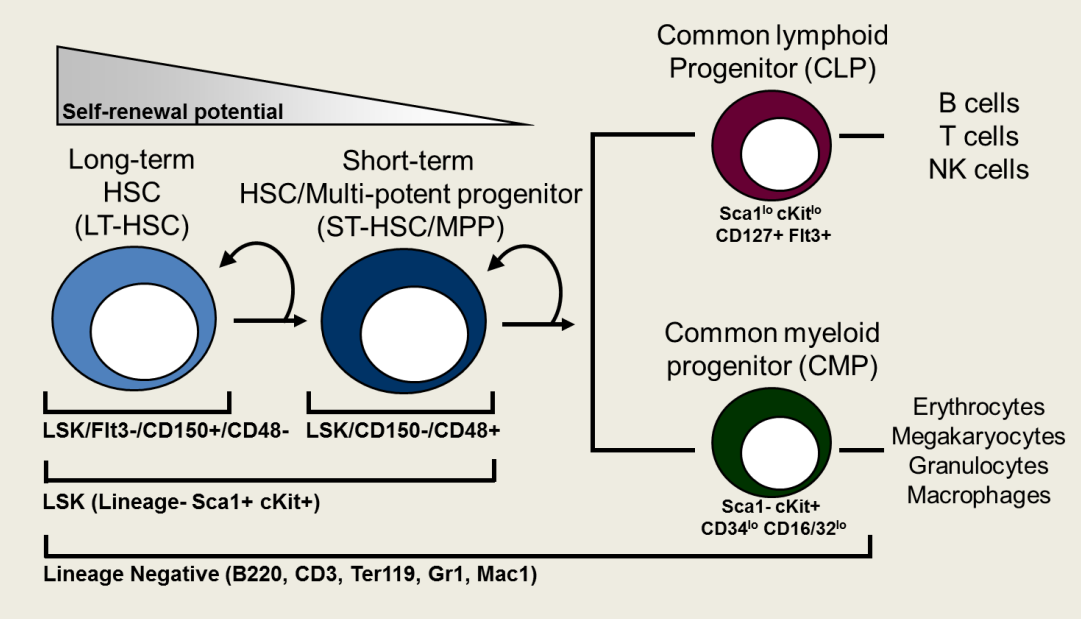

Figure 4. A simplified overview of hematopoiesis in mice. Long-term hematopoietic stem cells (LT-HSCs) have high self-renewal capacity and multi-lineage differentiation potential. Through a process of asymmetric cell division, they produce short-term hematopoietic stem cells (ST-HSCs) and multi-potent progenitors (MPPs) which give rise to the common progenitor cells of both the myeloid and lymphoid arms of the hematopoietic system. Common surface antigens found on each cell population are listed.

Briefly, LT-HSCs sit at the apex of the hematopoietic hierarchy and possess both self-renewal capacity and multi-lineage differentiation potential (Morita et al. 2010, Oguro et al. 2013, Wilson et al. 2015). LT-HSCs undergo asymmetric cell division where one daughter cell retains LT-HSC identity and complete self-renewal capacity (Orford and Scadden 2008, Wilson et al. 2015). LT-HSC populations must be maintained for the duration of life and LT-HSC loss and/or dysfunction is associated with aging (Dykstra et al. 2011, de Haan and Lazare 2018). The other daughter cell undergoes division and differentiation into short-term stem cells (ST-HSC) and multi-potent progenitors (MPPs). ST-HSCs and MPPs still possess multi-lineage differentiation potential but have reduced

self-renewal capacity and are capable of supporting hematopoiesis for only relatively short periods of time (Yang et al. 2005, Benveniste et al. 2010, Yamamoto et al. 2013). MPPs retain the ability to form cells from either the myeloid or lymphoid lineage. A critical cell fate decision is made during the differentiation from MPPs, as these cells form both common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs). CMPs are able to generate all cells within the myeloid lineage including cells within the megakaryocyte/erythroid lineage and granulocyte/monocyte lineage. CLPs differentiate into both B and T lymphocytes and NK cells. Additional lineage-restricted progenitors exist between these broad differentiation steps, the details of which are outside the scope of this review.

Replication stress has been identified as a significant contributor to HSPC dysfunction (Flach et al. 2014, Alvarez et al. 2015, Flach and Milyavsky 2018). In healthy, unstressed animals, the vast majority of LT-HSCs exist in a dormant state, rarely exiting quiescence to divide and form a ST-HSC (Arai and Suda 2007, Orford and Scadden 2008, Bernitz et al. 2016). Exit from dormancy alone in HSPCs is sufficient to induce replication stress, DNA damage and stem attrition (Walter et al. 2015). This phenotype has been associated with multiple stimuli including anemia following serial bleeding, administration of exogenous pro-growth cytokines, inflammatory responses, and as the consequence of certain oncogenic mutations (Cheshier et al. 2007, Essers et al. 2009, Baldridge et al. 2010, Takizawa et al. 2011, Itkin et al. 2012, An et al. 2015, Guryanova et al. 2016). Moreover, exit from dormancy and LT-HSC functional decline is associated with aging (Flach et al. 2014). Thus, replication stress is a critical physiologic threat to hematopoiesis and HSPC biology. Understanding the factors involved in the replication stress response in HSPCs is

critical for our understanding of hematology and has significant clinical implications for procedures such as bone marrow transplantation.

Part III: *MYC*

The *MYC* family of proteins consists of three potent, basic helix-loop-helix oncogenic transcription factors that contribute to the genesis and biology of the vast majority of human malignancies (Tansey 2014). *c-Myc*, the cellular *v-Myc* homolog, transcriptionally regulates approximately fifteen percent of genes in the human genome and influences a wide array of cellular processes including transcription, metabolism, cell proliferation, growth, DNA replication, and DNA damage (Dang et al. 2006, Zeller et al. 2006, Dang 2012). Research on *MYC* has greatly contributed to our understanding of the mechanisms of cellular transformation, oncogenes, differentiation, metabolism, and apoptosis. However, due to its far reaching biological functions and contribution to a plethora of cellular processes, there is still much to be learned regarding *MYC's* role in normal cellular biology and cancer.

History

Experiments performed in the twentieth century demonstrated that retroviruses can harbor genes capable of promoting transformation and tumorigenesis (Javier and Butel 2008). The first putative *Myc* gene was discovered in the 1970s through work with the avian retrovirus MC29 (Duesberg et al. 1977). Originally purified from a spontaneous

hematological malignancy in a chicken, subsequent propagation of this virus *in vivo* revealed that MC29-infected birds developed mycelocytomatosis, a type of myeloid malignancy, which gave the *Myc* gene its name (mycelocytomatosis). Subsequent molecular analysis of the MC29 virus led to the identification of the *v-Myc* oncogene in the late 1970s and identification of the cellular homolog, *c-Myc*, in 1982 (Mellon et al. 1978, Sheiness et al. 1978, Roussel et al. 1979, Sheiness and Bishop 1979, Vennstrom et al. 1982). MYC was almost immediately implicated in human cancer by its identification as the oncogenic driver of Burkitt lymphoma; this due to a reciprocal chromosomal translocation with an immunoglobulin gene locus and its subsequent overexpression in B cells (Dalla-Favera et al. 1982, Neel et al. 1982, Taub et al. 1982). Additional studies in the 1980s showed MYC was able to cooperate with RAS to induce transformation of fibroblasts, while genetic mouse models showed overexpression of Myc alone was sufficient to drive tumor formation (Land et al. 1983, Adams et al. 1985). Thus, these characterizations of *MYC* as a *bona fide* oncogene initiated the intensive study into understanding the biochemical and biological functions of this protein.

Structure

Myc-family proteins are highly conserved in metazoans and are critical for cellular viability and normal development. *Myc* knockout mice are embryonic lethal at day 9.5, likely due to importance of Myc for regulating cell cycle progression, growth, and metabolism (Davis et al. 1993). Mammals express 3 different MYC family member proteins, including c-Myc, N-Myc and L-Myc, which vary in their expression patterns, potency and dysregulation in malignancy (Legouy et al. 1987, Barrett et al. 1992, Nesbit

et al. 1998, Nesbit et al. 1999, Strieder and Lutz 2002). In general, c-MYC contributes to hematological malignances and solid organ tumors, N-MYC to cancers of neurological origin (such as neuroblastoma) and L-MYC to a subset of lung cancers (Tansey 2014).

MYC proteins contain an unstructured N-terminal transcription activation domain, a central domain, a nuclear localization sequence and a C-terminal DNA-binding domain (Tansey 2014). Alignment of the MYC family of proteins reveals multiple regions of high homology – five "Myc boxes" plus the conserved C-terminal DNA binding domain. The Myc boxes are termed MbI, MbII, MbIIIa, MBIIIb and MbIV. MbI and MbII are the most studied Myc boxes and lie within the transcriptional activation domain (Tansey 2014). MbI has been implicated in mediating aspects of the transcriptional functions of MYC as well as in the regulation of MYC protein stability (Stone et al. 1987, Lutterbach and Hann 1994, Welcker et al. 2004, Welcker et al. 2004, Herbst et al. 2005). MbII is the major domain involved in MYC-mediated transcription and is absolutely required for transformation and transcriptional activation/repression by MYC (Stone et al. 1987, Li et al. 1994, Hemann et al. 2005, Zhang et al. 2006). The c-terminal DNA-binding domain of MYC consists of a basic helix-loop-helix leucine zipper domain, which is characteristic of other DNA-binding transcription factors (Blackwell et al. 1990, Salghetti et al. 2001, Tansey 2014). However, unlike other proteins containing this structure, MYC does not form a homodimer to bind DNA, but rather forms a heterodimeric complex with its obligate binding partner MAX, a small basic helix-loop-helix leucine zipper protein which is absolutely required for MYC's oncogenic activity and its interaction with DNA (Blackwood and Eisenman 1991, Amati et al. 1993, Amati et al. 1993).

MYC and cancer

MYC dysregulation is found in a wide array of human cancers including lymphoma, breast cancer and neuroblastoma (Meyer and Penn 2008, Dang 2012). Unlike other oncogenes, such as *RAS*, *MYC* does not need to be mutated to unleash its oncogenic potential, and in fact, mutations are a relatively uncommon mechanism of *MYC* dysregulation in cancer (Tansey 2014). *In vivo* studies have shown that changes in expression levels of non-mutated MYC is sufficient to drive neoplastic transformation by itself and *MYC* overexpression is the most common form of *MYC* dysregulation in human malignancies.

MYC becomes dysregulated in cancer through a variety of mechanisms. As previously mentioned, the *MYC* gene was discovered through work on the avian MC29 retrovirus which encodes a v-gag-Myc chimeric protein (Lee and Reddy 1999). Additional studies in the 1980s confirmed the role of retroviral insertional mutagenesis in *MYC*-driven cancers through the discovery of viral sequences inserted into the *MYC* gene locus which drive its overexpression and the development of lymphoid malignancies (Hayward et al. 1981, Payne et al. 1982, Steffen 1984). In hematopoietic cancers, *MYC* overexpression is often the consequence of chromosomal translocations where the *MYC* gene is fused to the promotor sequence of a different gene (such as with the immunoglobulin genes in Burkitt lymphoma) (Dalla-Favera et al. 1982, Taub et al. 1982). These events occur in well over 90% of Burkitt lymphoma cases and are utilized for the clinical diagnosis of the disease (Dave et al. 2006). This translocation event has been modeled *in vivo* through the Eμ-*myc* mouse model, which overexpress *Myc* in B cells and develop clonal B-cell lymphomas (Adams et al. 1985). Translocations are rare in solid organ cancers that overexpress *MYC*, which instead typically show evidence of gene amplification. *MYC* amplification occurs in

breast, prostate and ovarian cancer as well as neuroblastoma and is associated with poor survival outcomes (Escot et al. 1986, Cher et al. 1996, Rao et al. 1998, Beroukhim et al. 2010, Stasik et al. 2010, Singhi et al. 2012). While these are the most common mechanisms of *MYC* dysregulation in cancer, they are not exclusive. There is evidence of *MYC* coding sequence mutations in some lymphomas (Johnston and Carroll 1992, Bhatia et al. 1993, Bhatia et al. 1994, Clark et al. 1994); however, these same cancers contain translocation events resulting in extremely high levels of *MYC* overexpression. As such, it is likely these are either passenger mutations or minor contributors to the pathology of the disease (Tansey 2014). Moreover, there are additional mechanisms that lead to increased levels of *MYC*, such as SNPs which stabilize the *MYC* mRNA as well as dysregulation of signaling pathways that control *MYC* transcription, however the significance of the contribution of these mechanisms to *MYC*-mediated transformation is currently debatable (Gregory and Hann 2000, Salghetti et al. 2001, Welcker et al. 2004, Welcker et al. 2004).

Thus, while *MYC* overexpression at the RNA or protein level is detectable in over 50% of human malignancies, it is likely that this fails to capture the true extent of *MYC* dysregulation in cancer. Due to the plethora of mechanisms that can lead to hyper-activated *MYC*, the frequency of its dysregulation in cancer and the frank survival advantages it confers upon cancer cells, it is likely that *MYC* is somehow involved in all human malignancies unless proven otherwise (Tansey 2014).

MYC biology

A unique aspect of *MYC* biology – a fact that likely underlies its potency as an oncogene – is the sheer diversity of cellular processes it impacts and /or regulates. A brief, but

certainly incomplete, overview of some of its most salient functions related to cancer and DNA replication, in particular, are outlined below (Figure 5).

Transcription

The vast majority of the biological functions of MYC are rooted in its role as a transcription factor. Together with its obligate binding partner MAX, MYC recognizes and binds a specific, high-affinity canonical sequence termed an "E-box" (Jones 2004). The E-box sequence is ubiquitously found throughout the genome and is present in the promotor regions of most genes transcriptionally regulated by MYC. However, MYC is also known to bind promotors lacking E-boxes as well as low-affinity, non-canonical E-box sequences (Zeller et al. 2006, Lin et al. 2012, Allevato et al. 2017). MYC transcriptional activity is also determined by additional factors such as the permissiveness of chromatin, specific epigenetic histone marks, *MYC* expression levels and interactions with other transcriptional regulatory complexes (Fernandez et al. 2003, Guccione et al. 2006, Zeller et al. 2006, Uribesalgo et al. 2011, Gerstein et al. 2012, Lin et al. 2012). Thus, while MYC is estimated to regulate approximately 15 percent of genes in the genome, the actual number could be far higher or lower depending on the status of a several different parameters in a particular cell at a given time. Thus, teasing out the precise nature of the MYC transcriptional network has proven to be extraordinarily difficult and is likely highly specific depending on the cellular context. However, in general, MYC-mediated transcription induces cell growth and proliferation.

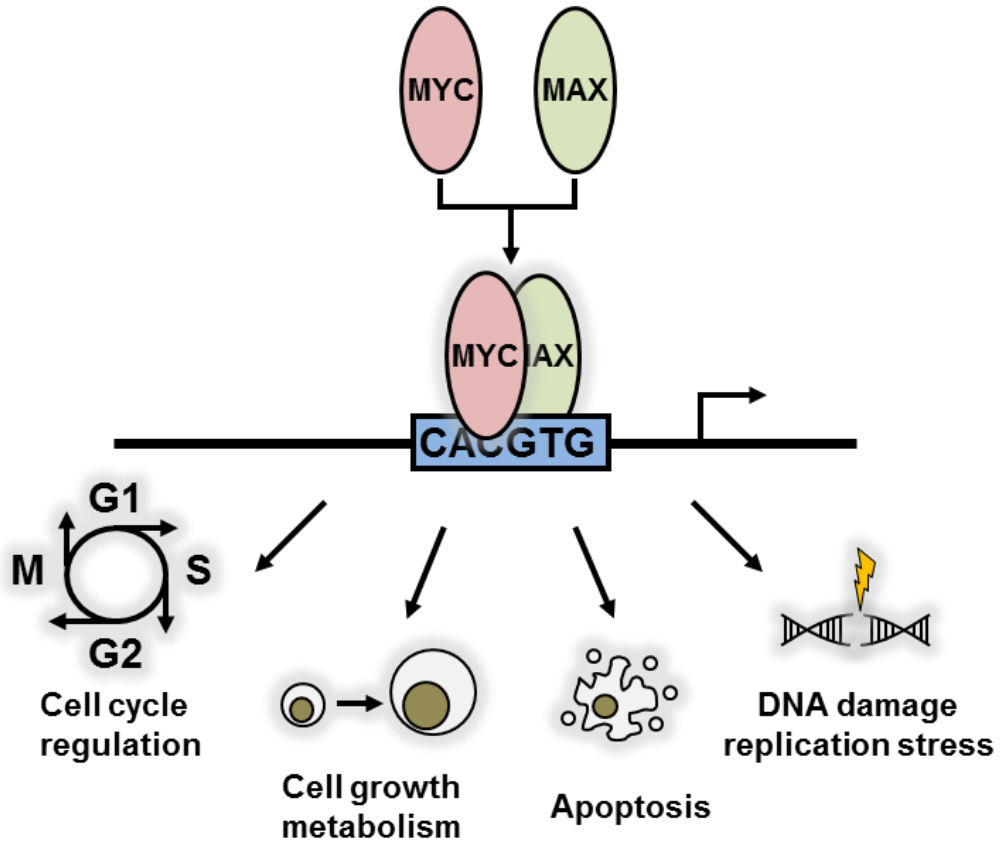

Figure 5. Biological functions of MYC. MYC is a basic helix-loop-helix transcription factor that forms a heterodimer with its obligate binding partner MAX and regulates approximately 15% of genes in the genome. The MYC transcriptional program impacts a wide array of cellular processes including cell cycle regulation, cell growth and metabolism, apoptosis, DNA replication and DNA damage.

Similar to other transcription factors, MYC functions as both a transcriptional activator and repressor. As an activator, MYC promotes transcription through multiple mechanisms. The MYC transcriptional cofactor TRRAP interacts with MbII and recruits histone acetyl transferase complexes which acetylate and relax chromatin, thereby permitting gene transcription (McMahon et al. 1998, McMahon et al. 2000, Frank et al. 2003). Moreover, MYC activity at promotors is involved in the phosphorylation of RNA polymerases, which relieves them from pausing and induces transcriptional elongation, thereby increasing gene expression (Eberhardy and Farnham 2001, Eberhardy and Farnham 2002).

MYC is able to stimulate transcription by RNA polymerases I, II and III. Upregulation of transcription by RNAPI and RNAPIII increases the synthesis of rRNA and tRNA, essential components for protein translation and cell growth, which serves as an integral component of the growth promoting nature of *MYC* (Gomez-Roman et al. 2003, Arabi et al. 2005, Grandori et al. 2005). Interestingly, MYC is a relatively weak activator of RNAPII target genes, as the increased expression of MYC target genes upon *MYC* overexpression is relatively modest (Levens 2002, Tansey 2014). Yet small changes in the expression of a wide variety of genes involved in tumorigenic processes underline the robust oncogenic nature of MYC.

Recent studies have proposed that rather than functioning as a transcription factor with a specific set of target genes, MYC may actually serve as a sequence-independent, general transcriptional amplifier. In this model, MYC lacks specificity as a transcription factor and instead serves to increase the expression of every gene being actively transcribed in the cell (Lin et al. 2012, Nie et al. 2012). While this model accounts for the wide-

reaching increase in transcriptional activity that is induced by MYC, it does not take in to account MYC's ability to transcriptionally repress genes (discussed below) nor does it explain the considerable evidence that MYC does in fact regulate the expression of specific genes. Moreover, questions have been raised about the normalization of the RNA sequencing data in these studies and the model is also unable to attribute the global amplification of mRNA production in *MYC*-overexpressing cells to MYC transcriptional activity directly, as this may simply be an indirect effect stemming from MYCs ability to induce cell growth (Dang 2014, Sabo et al. 2014).

In addition to its function as a transcriptional activator, MYC is also involved in the transcriptional repression of genes. In general, genes repressed by MYC tend to be anti-proliferative in nature (such as the CDK inhibitors p15, p21 and p27) and their repression by MYC co-operates with its pro-growth, oncogenic transcriptional activities to promote tumorigenesis (Staller et al. 2001, Seoane et al. 2002, Zhang et al. 2012). Mechanistically, MYC directly associates with histone deacetylases which remove acetyl groups from histones, leading to chromatin compaction and gene repression (Jiang et al. 2007, Kurland and Tansey 2008, Sun et al. 2014). Moreover, MYC can also repress genes through its interaction with the protein MIZ-1, which under contexts when it is not associated with MYC, functions as a transcriptional activator through its interactions with histone acetyl transferases near transcriptional start sites (Peukert et al. 1997). However, upon binding to MYC, these activating factors are replaced by repression cofactors, which promote heterochromatin formation and gene silencing (Herkert and Eilers 2010).

Apoptosis

Apoptosis is an integral safeguard against malignant transformation by oncogenes. In fact, during transformation, it is essential for cells to overcome and inactivate this tumor-suppressive barrier in order to fully progress to malignancy (Hanahan and Weinberg 2000). As with the overexpression of other oncogenes, hyper-activated MYC induces a robust apoptotic response in normal cells through a variety of different mechanisms (Askew et al. 1991, Evan et al. 1992, Shi et al. 1992). MYC directly regulates the expression of multiple BCL-2 family member proteins. MYC transcriptionally downregulates the anti-apoptotic proteins BCL-2 and BCL-X_L, while simultaneously upregulating the pro-apoptotic protein BIM (Eischen et al. 2001, Eischen et al. 2001, Maclean et al. 2003, Egle et al. 2004). As pro- and anti-apoptotic BCL-2 family members exist in a balance that either promotes or protects a cell from apoptosis, this alteration in the expression of multiple BCL-2 family members shifts the equilibrium towards apoptosis (Youle and Strasser 2008).

Recent studies from the Eischen laboratory have uncovered an additional novel mechanism of MYC-mediated apoptosis via its regulation of miRNAs and anti-apoptotic BCL-2 family members. In normal cells, MYC transcriptionally upregulates the expression of the let-7 and miR-15 miRNA families, of which BCL-2, BCL-X_L, and BCL-W are *bona fide* targets (Adams et al. 2016). The increase in levels of these miRNAs leads to the downregulation of BCL-2, BCL-X_L, and BCL-W, thereby driving apoptosis of the MYC-overexpressing cell (Adams et al. 2016).

Finally, an essential pathway for Myc-induced apoptosis involves the ARF-MDM2-p53 axis (Hermeking and Eick 1994, Wagner et al. 1994). ARF is a critical tumor suppressor that is an alternative reading frame of the Ink4a gene locus (Quelle et al. 1995,

Ozenne et al. 2010), which is commonly deleted in cancer (Kim and Sharpless 2006). ARF binds to MDM2, an E3 ubiquitin ligase which ubiquitinates p53 and targets it for proteosomal degradation, and inhibits its interaction with p53 (Nag et al. 2013). This leads to p53 stabilization and activation of the p53 transcriptional program, which induces cellular senescence and/or apoptosis (Vousden and Prives 2009). ARF expression is significantly enhanced upon activation of the MYC transcriptional program (Zindy et al. 1998). Thus, the activation of p53 is a direct consequence of MYC overexpression and serves a critical barrier to inhibit MYC-mediated tumorigenesis. *In vivo* mouse models of Myc-driven lymphomagenesis have highlighted the interconnected nature of these two pathways, as evidenced by the high rate of *Arf* and *p53* loss in Myc-driven B-cell lymphomas (Eischen et al. 1999).

Metabolism

One of the most potent effects of *MYC* overexpression is the global alteration to cellular metabolism that supports the enhanced cell growth mediated by *MYC*. *MYC*-overexpressing cells accumulate significantly more biomass than non-*MYC*-overexpressing cells, containing twice the amount of protein and mRNA (Rosenwald 1996, Iritani and Eisenman 1999, Nie et al. 2012). Moreover, since *MYC* drives cell growth and proliferation, it is not surprising that *MYC*-overexpressing cells show significant changes to metabolic flux and re-wirings of key metabolic pathways to support this growth. In general, two major changes to metabolism are most prevalent in cells overexpressing *MYC*. First, MYC upregulates genes involved in glucose uptake and glycolysis, which provides a growth advantage to nascent tumor cells, particularly in hypoxic environments (Shim et

al. 1997, Hu et al. 2011, Dang 2013). Second, *MYC* overexpression leads to widespread changes in glutamine metabolism via changes in expression of key metabolic enzymes (Wise et al. 2008, Gao et al. 2009). It is known that cells overexpressing MYC can become "addicted" to these two key metabolic shifts and drugging metabolic proteins involved in these processes is a promising avenue for treating *MYC*-driven malignancies (Sabnis et al. 2017). *MYC* is also involved in the metabolic regulation of key factors required for DNA synthesis, which will be discussed in more detail below.

DNA replication

While the aforementioned biological properties of MYC profoundly influence its behavior as an oncogene, it has acute effects on cell cycle progression and DNA replication (both directly and indirectly) and is a major source of oncogene-induced replication stress. As a cell cycle regulator, MYC accelerates S-phase entry and expedites transit through G1 and G2 (Karn et al. 1989, Shibuya et al. 1992, Mateyak et al. 1997, Amati et al. 1998). Overexpression of *MYC in vitro* asynchronously increases the percentage of cells in S-phase (Grandori et al. 2003, Robinson et al. 2009, Srinivasan et al. 2013). *MYC* is an immediate-early gene, and its mRNA is dramatically upregulated in quiescent cells upon the addition of mitogens (Kelly et al. 1983). Moreover, *MYC* overexpression alone is sufficient to induce re-entry of quiescent cells into S-phase (Eilers et al. 1989, Eilers et al. 1991). As a transcription factor, these activities are mainly mediated through the ability of MYC to transcriptionally control key cell cycle regulatory proteins. For example, MYC directly regulates D, A, and E family cyclin members as well as CDK4 and CDC25, which are required for entry into S-phase and cell cycle progression (Galaktionov et al. 1996,

Bouchard et al. 1999, Hermeking et al. 2000, Menssen and Hermeking 2002, Fernandez et al. 2003). Moreover, MYC also upregulates the expression of E2F family transcription factors, which together with MYC, drive the expression of additional proteins needed for cell cycle progression and DNA synthesis (Leone et al. 2001, Fernandez et al. 2003). In addition to upregulating the pro-proliferative genes listed above, Myc simultaneously represses critical cell cycle inhibitory proteins such as $p15^{INK4b}$ and p21 (Claassen and Hann 2000, Seoane et al. 2001, Staller et al. 2001, Seoane et al. 2002). Collectively, the transcriptional program of MYC functions to increase pro-proliferative genes involved in cell cycle progression, while simultaneously inhibiting checkpoint proteins to facilitate cell entry into the cell cycle.

In addition to altering cell cycle dynamics and S-phase progression, multiple groups have observed increased rates of origin firing in *MYC*-overexpressing cells (Dominguez-Sola et al. 2007, Srinivasan et al. 2013, Maya-Mendoza et al. 2015). This is likely to be, in part, due to the acceleration of S-phase caused by *MYC* overexpression and the need for additional origins to fire in order to facilitate DNA synthesis within the window of an abbreviated S-phase. However, a recent study has also identified MYC as interacting with components of the pre-RC, but not with other factors involved in DNA elongation such as RPA and PCNA (Dominguez-Sola et al. 2007). In these experiments, *MYC* overexpression resulted in increased origin firing and checkpoint activation, suggesting MYC may be involved in pre-RC assembly and/or origin firing (Dominguez-Sola et al. 2007). Through the use of DNA combing, studies have also demonstrated that *MYC* overexpression alters the spatiotemporal patterns of origin firing and increases the rate of early-S origin firing (Sankar et al. 2009, Srinivasan et al. 2013). This dysregulation leads to replication fork

asymmetry, replication stress and fork collapse. *MYC* overexpression also induces robust checkpoint activation and activation of the DNA damage response (DDR) (Felsher et al. 2000, Campaner and Amati 2012).

MYC, DNA damage and replication stress

MYC overexpression *in vitro* is associated with DDR activation and genomic instability (Campaner and Amati 2012). These observations have been replicated in *in vivo* studies revealing that DNA damage and genomic instability are significant consequences of *Myc* overexpression (Pusapati et al. 2006, Gorrini et al. 2007, Reimann et al. 2007). MYC-induced DNA damage likely arises as a consequence of several different aspects of MYC biology. First, MYC-induced DNA damage likely occurs in part due to elevated levels of ROS that are a byproduct of the enhanced metabolic rate of *MYC*-overexpressing cells (Vafa et al. 2002, Egler et al. 2005, Zhang et al. 2007). Moreover, *MYC* overexpression has been associated with amplifications and genome rearrangements (Mai et al. 1996, Felsher and Bishop 1999). By dysregulating cell cycle progression and untethering S-phase from mitosis, *MYC* overexpression can lead to endoreduplication and aneuploidy (Li and Dang 1999, Yin et al. 1999). Thus, genomic instability is a direct consequence of *MYC* dysregulation.

However, many of the effects of MYC on genomic stability are likely to arise due to its influences on DNA replication. As previously mentioned, MYC alters cell cycle progression, regulates origin firing and leads to replication fork asymmetry and collapse (see Chapter I, Part II – *DNA replication*). MYC-overexpressing cells activate a p53-induced G2 checkpoint, which is possibly due to the persistence of damaged DNA

generated during S-phase (Felsher et al. 2000). Mechanistically, MYC-induced replication stress likely causes DNA damage through a variety of mechanisms, many of which have been summarized in Chapter I, Part II. As a transcription factor, *MYC* overexpression leads to a global increase in transcription. This, coupled with the accelerated transit through S-phase, likely leads to significantly higher rates of collisions between transcriptional complexes and replisomes. Indeed, a recent study has described this exact event in cells overexpressing *MYC* or *Cyclin E* (Macheret and Halazonetis 2018). Moreover, the effect of MYC on origin firing may overwhelm the ability of the cell to cope with mis-timed and accelerated DNA replication (Rohban and Campaner 2015). This may lead to the exhaustion of critical replication factors (such as helicases, topoisomerases and nucleases) and/or other metabolites that can induce fork stalling. However, there is a lack of experimental evidence exploring this hypothesis in *MYC*-overexpressing cells, and in fact, there are some data that show MYC overexpression actually leads to enhanced anabolism of nucleotides, which may help mitigate the effects MYC has on origin firing and cell cycle progression (Bester et al. 2011).

However, the data show replication stress is a critical consequence of *MYC* overexpression. Indeed, *MYC*-overexpressing cells are highly sensitive to ATR/CHK1 inhibition, demonstrating their reliance on the replication stress response for survival (Cole et al. 2011, Hoglund et al. 2011, Murga et al. 2011, Ferrao et al. 2012, Schoppy et al. 2012). By identifying other factors that are involved in the cellular response to *MYC* overexpression, synthetic lethal approaches could potentially be designed to effectively kill *MYC*-overexpressing cells with high levels of replication stress while sparing normal, healthy tissues in the body.

CHAPTER II

DEFECTIVE REPLICATION STRESS RESPONSE INHIBITS LYMPHOMAGENESIS AND IMPAIRS LYMPHOCYTE RECONSTITUTION

This chapter from:

Puccetti, M.V.; Fischer, M.; Arrate, M.P.; Boyd, K.L.; Duszynski, R.J.; Betous, R.; Cortez, D.; and Eischen, C.M. *Oncogene*. 2016

Introduction

Genome instability is a hallmark of human cancer and contributes to tumor initiation and progression (Hanahan and Weinberg 2011). A poorly understood contributor to genome instability is DNA replication stress, which refers to processes that induce replication fork stalling and/or collapse and impede DNA synthesis (Zeman and Cimprich 2014). DNA damage is accrued through the processing of stalled forks into double-stranded DNA breaks, and incomplete DNA replication can result in deletions and chromosomal abnormalities (Ciccia and Elledge 2010, Zeman and Cimprich 2014). Replication stress has been identified in both pre-malignant and cancerous lesions and is associated with tumor development, progression, and evolution (Bartkova et al. 2006, Di Micco et al. 2006). Replication-associated DNA damage is thought to induce selective pressure to inactivate tumor suppressive programs in pre-malignant cells and provide a source of mutation within tumor cells (Macheret and Halazonetis 2015). However, in response to replication-associated DNA damage, cells activate a DNA damage response to facilitate

the completion of DNA replication and repair damaged DNA to minimize the threat to the genome (Ciccia and Elledge 2010, Nam and Cortez 2011). While replication stress has been linked to tumorigenesis, the contribution and function of specific replication stress response proteins in tumor development remain unknown.

Mammalian cells express several proteins that repair and restart stalled replication forks and promote genome stability during replication stress. One such protein, SMARCAL1, binds forked DNA structures (Yusufzai and Kadonaga 2008). It is recruited to stalled replication forks, through an interaction with the single-stranded (ss) DNA-binding protein replication protein A (RPA). There, SMARCAL1 promotes fork stabilization and repair by catalyzing the annealing of RPA-coated ssDNA to remodel stalled replication forks (Bansbach et al. 2009, Ciccia et al. 2009, Postow et al. 2009, Yuan et al. 2009, Yusufzai et al. 2009, Betous et al. 2012, Betous et al. 2013, Couch et al. 2013). Bi-allelic mutations in *SMARCAL1* cause the pleiotropic disorder Schimke Immuno-osseous Dysplasia (SIOD), which is characterized by immunodeficiency, spondyloepiphyseal dysplasia, facial dysmorphism, and progressive nephropathy (Boerkoel et al. 2002). While the biochemical function of SMARCAL1 at replication forks has been investigated, the *in vivo* functions of SMARCAL1, specifically regarding its role in tumorigenesis and the mechanism(s) driving the clinical phenotypes of SIOD, remain unresolved.

Here we report that Smarcal1 is a critical effector of the replication stress response in hematopoietic cells *in vivo.* In an irradiation (IR)/replication stress-induced model of T-cell lymphomagenesis, a deficiency in Smarcal1 resulted in elevated DNA damage and a significant delay in T-cell lymphoma development. Smarcal1 was required for

hematopoietic cell survival during forced proliferation from multiple stimuli and for repopulation of the thymus following IR. Thus, our data establishes Smarcal1 as a critical mediator of hematopoietic cell survival during acute replication stress via its genome-protecting functions. Moreover, these results also offer an explanation behind the immunodeficiency exhibited by SIOD patients.

Materials and Methods

Mice

C57Bl/6 *Smarcal1*$^{+/\Delta}$ mice were from Dr. Cornelius Boerkoel (University of British Columbia). Littermates (male and female) were used for all experiments. For IR-induced T-cell lymphomagenesis, littermates were irradiated (1.75 gray, ^{137}Cs) once weekly for four weeks at 28 days of age (+/-2 days). Mice were monitored for 500 days and sacrificed upon tumor development and/or signs of illness. Tissues were harvested for analysis. For 5-fluorouracil (5-FU) experiments, 6-8 week-old littermates were intraperitoneally injected with 5-FU (150 mg) once weekly for 5 weeks and sacrificed at humane endpoints. Competitive (1:1 ratio) bone marrow transplants were performed following standard procedures. Mouse studies were approved by the Vanderbilt University and Thomas Jefferson University Institutional Animal Care and Use Committees and adhered to all state and federal guidelines.

Cell culture and vectors.

Mouse embryonic fibroblasts (MEFs) were isolated and cultured as previously described (Zindy et al. 1998). U2OS cells were cultured in DMEM plus 7.5% FBS. To induce fork

stalling, U2OS cells were transfected with vectors encoding wild-type GFP-SMARCAL1 or GFP-SMARCAL1Δ and treated with 2 mM hydroxyurea (HU) for 4 hours as previously described (Bansbach et al. 2009).

Flow cytometry

HSPCs were harvested from femurs and identified with a biotinylated hematopoietic lineage kit (B220, CD3, Gr-1, CD11b and Ter119; eBioscience) and a panel of antibodies against specific HSPC surface markers. BrdU incorporation in thymi and bone marrow from 6-8 week old mice 1-3 days after a single 1.75 Gy dose of IR was performed according to manufacturer's instructions (BD Biosciences) four hours after intraperitoneal injection (1mg). Cells were incubated with FITC-Annexin-V and 7-AAD or Caspase 3/7 detection reagent according to the manufacturer (BD Biosciences or ThermoFisher, respectively). All samples were evaluated on a FACScalibur (BD Biosciences) and analyzed using FlowJo.

Western blotting

Western blotting of whole cell lysates from MEFs isolated from *Smarcal1*$^{+/+}$, *Smarcal1*$^{+/\Delta}$, and *Smarcal1*$^{\Delta/\Delta}$ embryos was performed as previously described (Eischen et al. 1999). Antibodies directed against the N- and C-terminus of Smarcal1 were used as previously described (Bansbach et al. 2009).

Fork reversal assay

Fork reversal assays were completed using gel-purified, radio-labeled DNA substrates

containing a leading strand gap and 0.5-2nM SMARCAL1Δ with or without 2nM wild-type SMARCAL1 in fork reversal reaction buffer as previously described (Betous et al. 2013).

Immunofluorescence

Immunofluorescence for γH2AX was performed using a standard protocol (details in Supplemental Material). Apoptosis was measured with the CellEvent Caspase 3/7 detection reagent (ThermoFisher) according to the manufacturer's instructions. Nuclei were counterstained with DAPI (Sigma-Aldrich) or ToPro3-iodide (Invitrogen) and images captured using microscopy (Zeiss LSM 510, Nikon A1R, or Nikon Eclipse 80i); a minimum of 110 cells per mouse were evaluated (blinded) for each experiment.

Neutral Comet Assays

Neutral comet assays with thymocytes and total bone marrow cells were performed as previously described (Alt et al. 2005, Bouska et al. 2008). A minimum of 50 cells per mouse were evaluated (blinded) per experiment.

Statistics

Log-rank tests (Figure 9), t-tests (Figure 22), one-way ANOVA followed by a Bonferroni correction (Figures 15, 17, 19, 21), and two-way ANOVA (Figure 22) were used to determine statistical significance.

Results

Smarcal1 knockout mice express a non-functional truncated protein

To investigate the *in vivo* functions of Smarcal1 during tumor development and acute replication stress, we evaluated *Smarcal1* knockout mice (*Smarcal1*$^{\Delta/\Delta}$) (Baradaran-Heravi et al. 2012). The targeting construct used to generate the *Smarcal1*$^{\Delta/\Delta}$ mice suggests an N-terminal truncated protein lacking amino acids 1-293 could be expressed; this region encodes the RPA-binding domain, the first HARP domain, and a portion of the second HARP domain (Figure 6A). Western blotting using an N-terminal specific Smarcal1 antibody showed half the levels of the 110 kDa full-length protein in *Smarcal1*$^{+/\Delta}$ MEFs and its expected loss in *Smarcal1*$^{\Delta/\Delta}$ MEFs (Figure 6B). A C-terminal specific antibody detected a ~70 kDa Smarcal1 protein in these same cells (Figure 6B), verifying the expression of truncated Smarcal1 (Figure 6B).

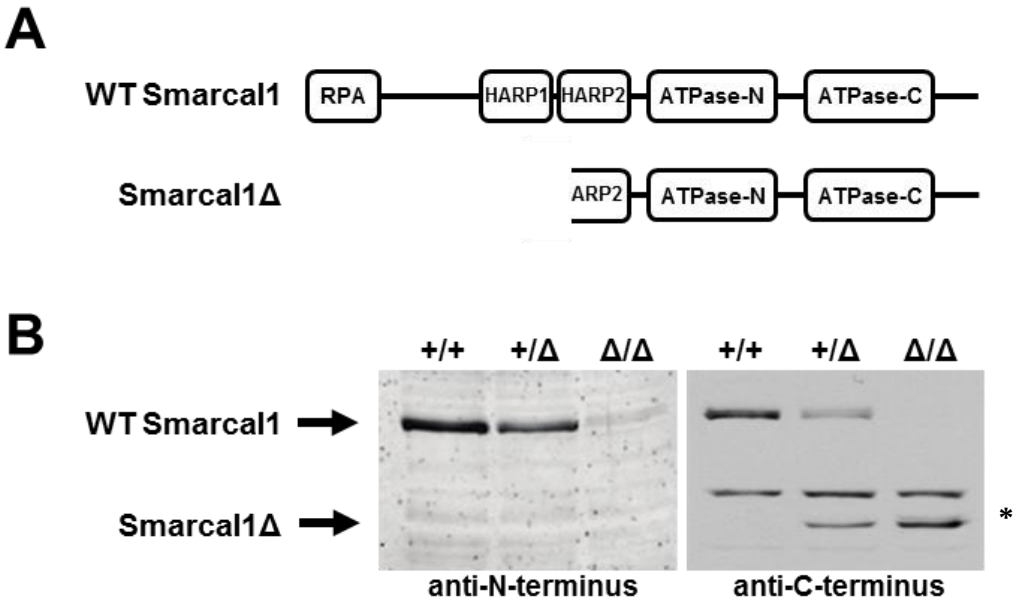

Figure 6. *Smarcal1Δ/Δ* mice express an N-terminal truncated Smarcal1 protein. A) Schematic of wild-type (WT) and N-terminal truncated Smarcal1 (Smarcal1Δ) with functional domains indicated. B) Whole cell lysates from mouse embryonic fibroblasts (MEFs) of the indicated genotypes were Western blotted with antibodies against the N- or C-terminus of Smarcal1. Asterisk denotes location of a non-specific band.

To determine whether Smarcal1Δ retained any functions of the wild-type protein, we performed *in vitro* analyses. Previous studies demonstrated that wild-type Smarcal1 is localized to stalled replication forks through its interaction with RPA (Bansbach et al. 2009, Ciccia et al. 2009, Postow et al. 2009, Yuan et al. 2009, Yusufzai et al. 2009). To assess if Smarcal1Δ, which lacks its RPA binding domain, can localize to stalled replication forks, U2OS cells were transfected with vectors encoding a fusion protein of GFP and either SMARCAL1Δ or wild-type SMARCAL1. GFP foci corresponding to SMARCAL1 localization to stalled replication forks induced by hydroxyurea were observed in cells expressing wild-type GFP-SMARCAL1 (Figure 7). In contrast, only diffuse GFP (no GFP foci) was present in cells expressing GFP-SMARCAL1Δ (Figure 7). These results indicate Smarcal1Δ is unable to localize to sites of replication stress.

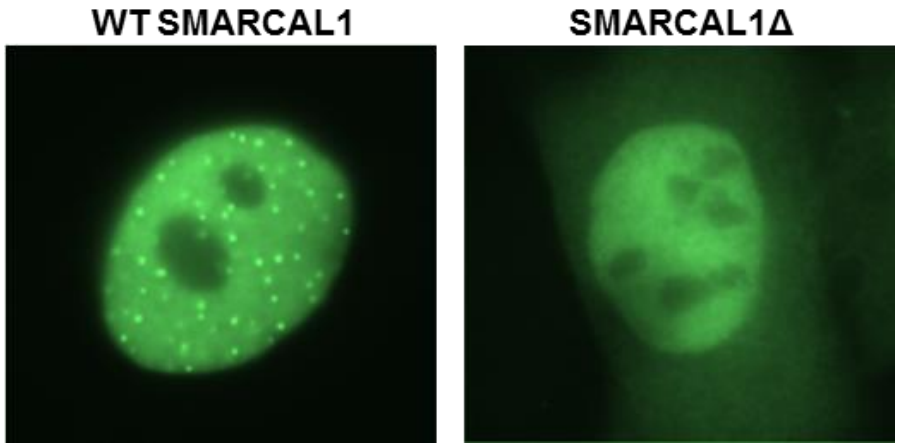

Figure 7. Smarcal1Δ is unable to localize to sites of replication stress. Representative images of U2OS cells expressing wild-type (WT) GFP-SMARCAL1 or GFP-SMARCAL1Δ following HU treatment.

Smarcal1 promotes genome stability through its fork regression and remodeling activities, which require its HARP2 domain (Betous et al. 2012, Betous et al. 2013, Couch et al. 2013). To measure this activity of Smarcal1Δ and to determine whether this truncated protein can exert any dominant negative effects against wild-type Smarcal1, fork reversal assays were performed (Figure 8A). Substrates with a leading-strand gap were incubated with increasing concentrations of SMARCAL1Δ alone or in the presence of wild-type SMARCAL1 (Figure 8B). Fork regression was not observed in reactions containing SMARCAL1Δ alone, whereas wild-type SMARCAL1 induced fork regression regardless of the concentration of SMARCAL1Δ (Figure 8B). Thus, the N-terminal truncated Smarcal1 protein is functionally dead and appears to exert no dominant negative effects on wild-type Smarcal1.

Loss of *Smarcal1* delays gamma irradiation (IR)-induced T-cell lymphomagenesis

Repeated whole body, low-dose IR of young mice induces T-cell lymphoma development, reportedly through the accumulation of DNA mutations in a hematopoietic stem or progenitor cell (HSPC) or an early T-cell progenitor derived from an HSPC (Kaplan and Brown 1952, Kominami and Niwa 2006). Following each round of irradiation, HSPCs rapidly proliferate to repopulate the lymphoid compartments depleted by IR-induced apoptosis. The presence of IR-associated DNA damage, coupled with the proliferative burst that occurs after IR exposure, is expected to generate significant levels of replication stress in both cycling HSPCs and the HSPC-derived thymic progenitors repopulating the depleted thymus following each radiation cycle.

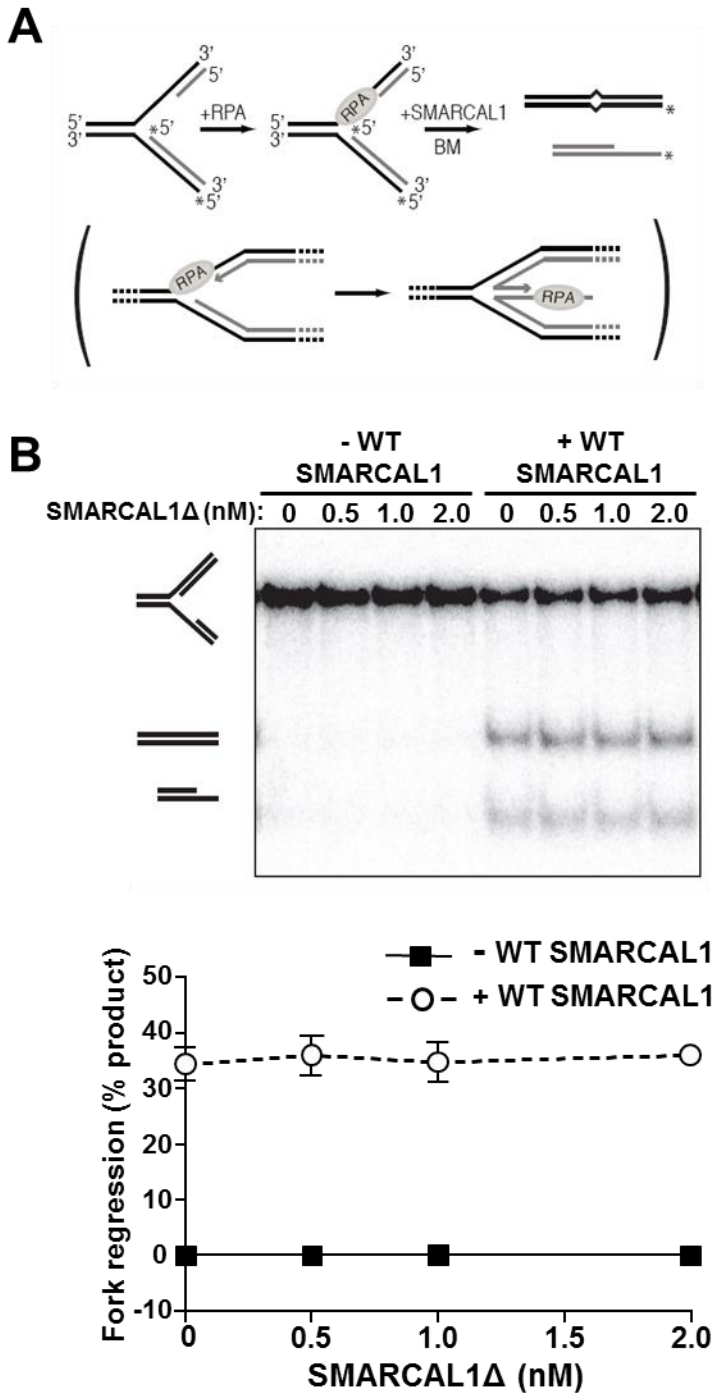

Figure 8. *Smarcal1Δ* in unable to drive fork regression *in vitro*. A) Schematic of the *in vitro* fork reversal assay with the *in vivo* physiological reaction shown in brackets. ^{32}P labeled strands are indicated with an asterisk. B) Fork reversal activity of increasing concentrations of SMARCAL1Δ was measured alone and in the presence of wild-type (WT) SMARCAL1. Native gel electrophoresis performed (top). Mean of fork regression quantification using phosphorimaging of three separate experiments is graphed (bottom); error bars are SEM.

To investigate the role of Smarcal1 in IR/replication stress-mediated T-cell lymphomagenesis, littermate-matched mice of all three *Smarcal1* genotypes were subjected to 4 weekly cycles of low-dose IR. Smarcal1 wild-type mice developed T-cell lymphomas at the expected rate with a mean survival of 143 days (Figure 9) (Kaplan and Brown 1952). However, *Smarcal1*$^{+/\Delta}$ and *Smarcal1*$^{\Delta/\Delta}$ mice had a delay in tumor onset and significantly increased overall survival (Figure 9, p=0.0399, log-rank test; mean survival 180 and 237 days respectively). Notably, 500 days after the last dose of IR, 23% of *Smarcal1*$^{+/\Delta}$ mice and 29% of *Smarcal1*$^{\Delta/\Delta}$ mice were still alive, whereas by 450 days, all of the *Smarcal1*$^{+/+}$ littermates had developed tumors and were sacrificed (Figure 9).

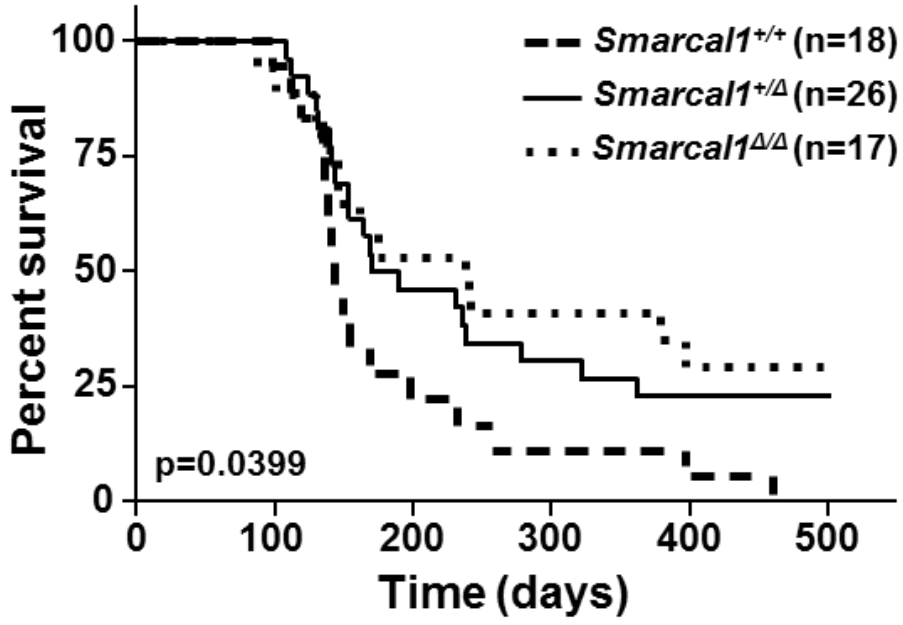

Figure 9. Loss of Smarcal1 inhibits radiation-induced T-cell lymphomagenesis. Kaplan-Meier survival curves of the indicated genotypes; overall p value denoted on graph; p=0.0307, +/+ vs. +/Δ and p=0.0217, +/+ vs. Δ/Δ; log-rank tests. Number of mice denoted by "n".

Genes that influence tumorigenesis can alter the rate of tumor development and/or the types of tumors that develop; therefore we also evaluated the tumor spectrum in the Smarcal1-deficient mice. As expected, most (89%) of the *Smarcal1*$^{+/+}$ mice developed T-cell lymphomas, while 11% developed benign adenomas (Figure 10). Surprisingly, T-cell lymphomas emerged in only 65% of the *Smarcal1*$^{+/\Delta}$ mice, whereas 12% developed sarcomas (Figures 10A, 10B). Leiomyosarcoma, hemangiosarcoma, and pleiomorphic sarcoma were observed in *Smarcal1*$^{+/\Delta}$ mice (Figure 10B). Two of these sarcomas occurred in relatively young mice (124 and 230 days old), indicating they did not emerge due to old-age. Only 59% of *Smarcal1*$^{\Delta/\Delta}$ mice developed T-cell lymphomas and an additional 12% had undetermined pathology (e.g., hind limb paralysis and death from unknown cause) (Figure 10A). The reduction in T-cell lymphoma frequency for both *Smarcal1*$^{+/\Delta}$ (24% reduction) and *Smarcal1*$^{\Delta/\Delta}$ mice (30% reduction) was significant ($p=0.0400$ +/+ vs. +/Δ and $p=0.0216$ +/+ vs. Δ/Δ, t-tests). The T-cell lymphomas that arose in all genotypes were Thy1.2 positive and also typically CD8+ or CD8+/CD4+ positive (Figure 10C).

Remarkably, 23% of *Smarcal1*$^{+/\Delta}$ and 29% of *Smarcal1*$^{\Delta/\Delta}$ mice never developed a tumor up to 500 days after the last dose of IR, whereas tumors were present in all *Smarcal1*$^{+/+}$ mice. This difference in tumor incidence was significant ($p=0.0194$ +/+ vs. +/Δ and $p=0.0089$ +/+ vs Δ/Δ, t-tests). Taken together, these data demonstrate that loss of Smarcal1 increased overall survival by inhibiting IR-induced T-cell lymphomagenesis and preventing tumor development altogether in a significant fraction of the mice. Our data also suggest that Smarcal1 haploinsufficiency influences tumor cell of origin, as several *Smarcal1*$^{+/\Delta}$ mice developed sarcomas, which are not typically associated with IR-induced tumorigenesis.

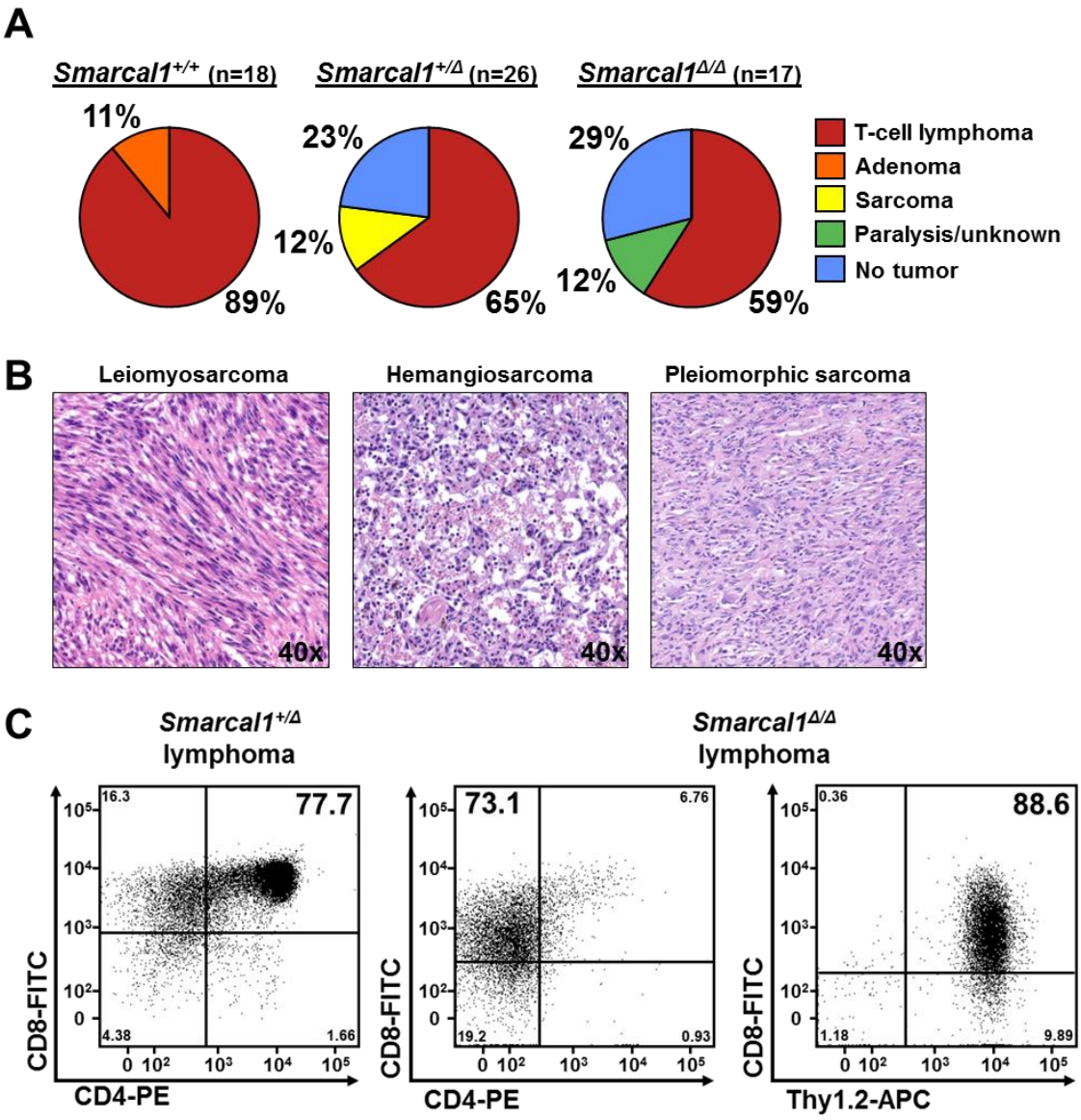

Figure 10. Smarcal1 loss alters the tumor spectrum that emerges in C57/Bl6 mice following repeated, low-dose IR. A) Tumor spectrum in the mice in Figure 9. B) Representative H&E images of the sarcomas that arose in *Smarcal1*$^{+/\Delta}$ mice. C) Representative dot plots of CD4+/CD8+ or CD8+/Thy1.2+ lymphomas that developed in the irradiated cohort of mice; genotype indicated.

Smarcal1-deficient thymocytes do not have an altered sensitivity to radiation

To gain insight into the biological mechanism behind the delay in tumor development observed in Smarcal1-deficient mice and since studies have disagreed on the requirements of Smarcal1 for the response to IR (Bansbach et al. 2009, Ciccia et al. 2009, Yuan et al. 2009, Keka et al. 2015), we first evaluated T-cell populations in the thymus in response to IR. We first assessed thymic T cells in unirradiated mice. There were similar percentages of CD4/CD8 double-positive (DP) and CD4 and CD8 single-positive (SP) T cells in Smarcal1-deficient mice compared to wild-type littermates (Figures 11A, 12A, 12B). There appeared to be reduced thymic cellularity in $Smarcal1^{+/\Delta}$ and $Smarcal1^{\Delta/\Delta}$ mice; however, the reductions in total numbers of DP and SP thymocytes were not statistically significant (Figures 11 B, 12A, 12B). Thus, loss of one or both alleles of *Smarcal1* does not significantly alter thymic T-cell numbers or the proportion of specific thymocyte populations under normal physiologic conditions.

Because differences in radiation sensitivity could alter the rate of tumorigenesis in mice, we evaluated thymocytes in littermates during the apoptosis phase induced by IR. Compared to unirradiated mice, all *Smarcal1* genotypes showed a reduction in the percentage of DP thymocytes of ~30% at 24 hours and ~60% at 48 hours after a single 1.75 Gy dose of IR (Figure 11A). The numbers of DP and SP thymocytes fell precipitously at each interval evaluated after IR (Figures 11A, 11C, 12A, 12B). Total DP numbers were reduced by >95% in all genotypes 48 hours post-IR, indicating a similar ablation of the thymic compartment for all mice (Figure 11C). Thymocyte apoptosis, as measured by

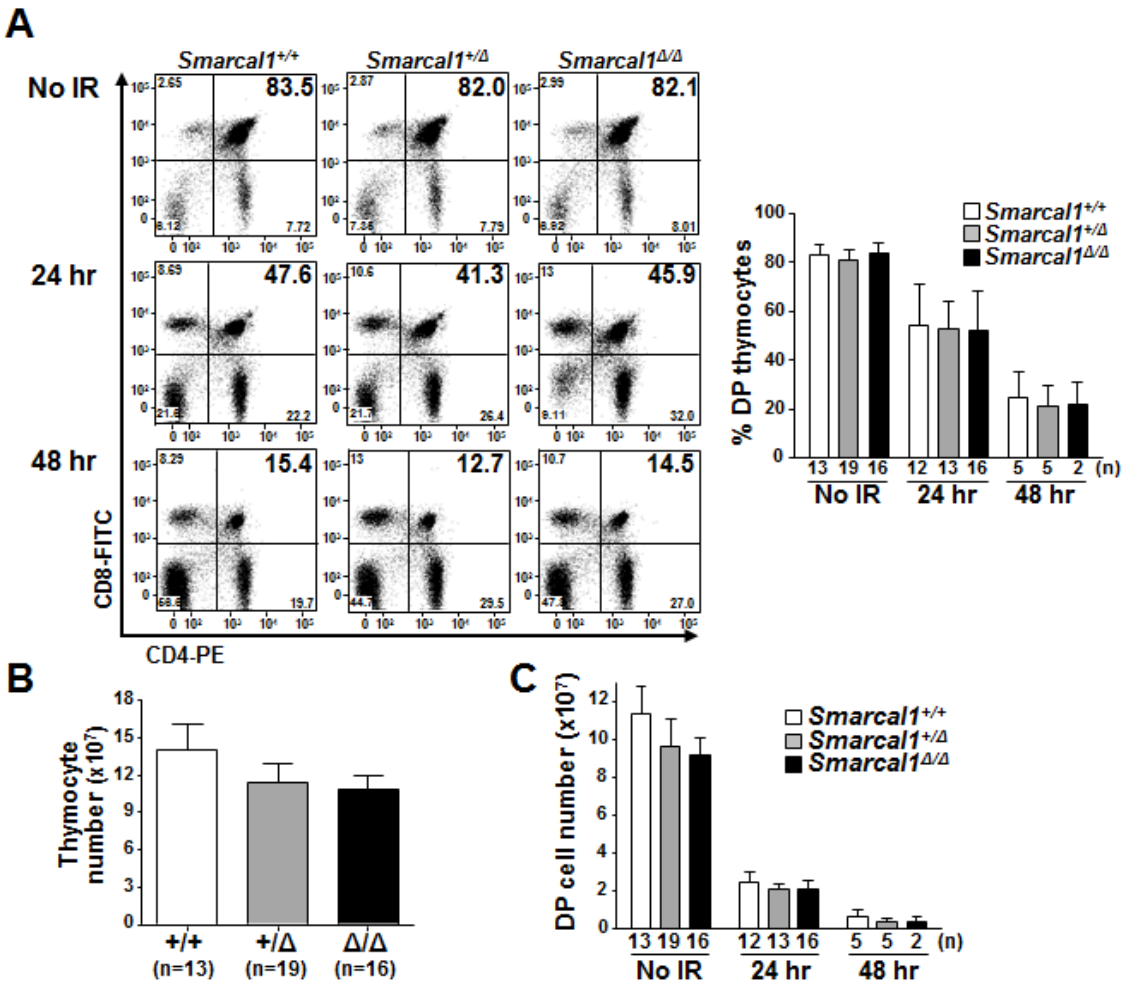

Figure 11. Smarcal1-deficiency does not alter DP thymocyte sensitivity to radiation. A) Representative dot plots of littermate matched thymic CD4/CD8 DP T cells without or at the indicated times following a single dose of 1.75 Gy of IR (left). Mean of data from seven independent experiments (right). B) Total thymic cellularity in unirradiated littermates. C) CD4/CD8 DP thymocytes not subjected to IR or at the indicated times after IR.

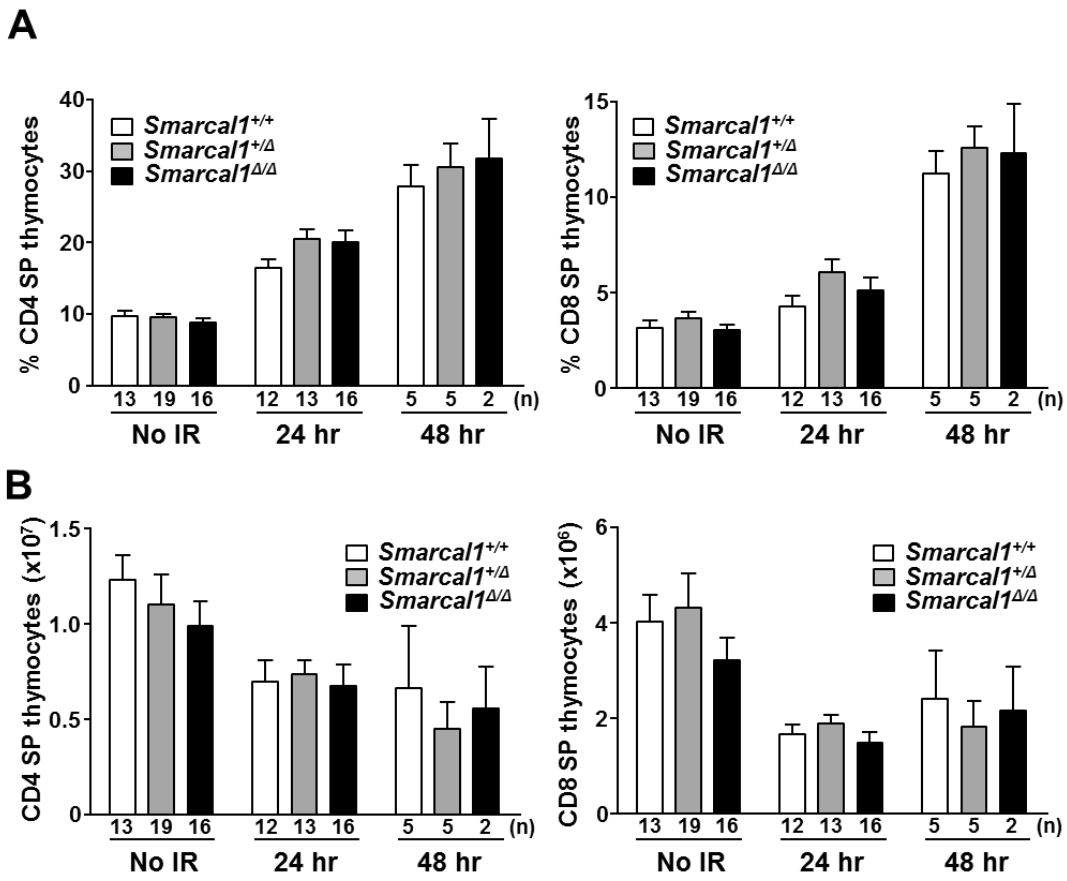

Figure 12. Radiation sensitivity is unchanged in Smarcal1-deficient SP thymocytes.
A, B) Percentages (A) or total numbers (B) of CD4 SP (left) or CD8 SP (right) thymocytes without irradiation or at the indicated intervals following IR. Data are the mean of seven independent experiments with littermates; error bars are SEM. The number of mice denoted by n.

cleaved caspase 3/7 and Annexin V, was analogous between all *Smarcal1* genotypes 24 hours after IR (Figure 13A, 13B). Similarly, thymocytes from littermates of all genotypes showed comparable amounts of phosphorylated histone H2AX (γH2AX), a marker of DNA breaks 24 hours post-IR (Figure 13C). Therefore, a deficiency in Smarcal1 does not appear to alter sensitivity to IR.

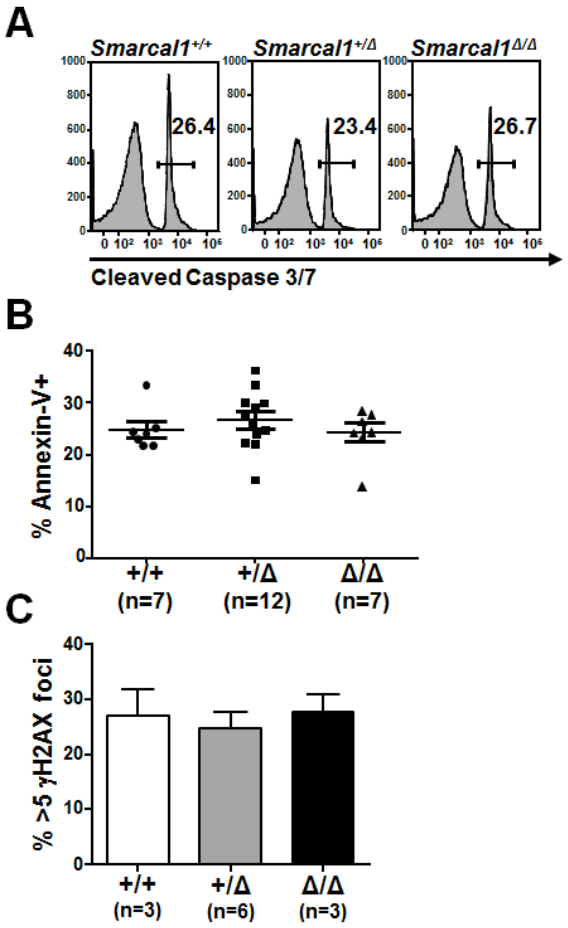

Figure 13. Smarcal1 loss does not affect apoptosis in thymocytes after low-dose IR. Thymi harvested 24 hours after littermates were subjected to a single dose of 1.75 Gy of IR. Cleaved caspase 3/7 activity (A) and Annexin V positivity (B) in thymocytes measured by flow cytometry. C) Immunofluorescence for γH2AX was quantified; thymocytes with >5 γH2AX foci graphed.

To determine if a loss of Smarcal1 would impact the cell cycle arrest that occurs upon radiation exposure, BrdU incorporation was measured in DP thymocytes. All *Smarcal1* genotypes showed a similar percentage of BrdU positive DP thymocytes in the absence of IR (Figure 14). Twenty-four hours after IR, DP thymocytes in littermates of all genotypes had <1% BrdU incorporation (Figure 14), demonstrating that Smarcal1-deficient cells have an intact and functioning DNA-damage induced cell cycle arrest response. Therefore, the early response to IR-induced DNA damage that results in cell cycle arrest and apoptosis is not altered with loss of Smarcal1 and likely does not contribute to the observed delay in T-cell lymphoma development in the *Smarcal1*$^{+/\Delta}$ and *Smarcal1*$^{\Delta/\Delta}$ mice.

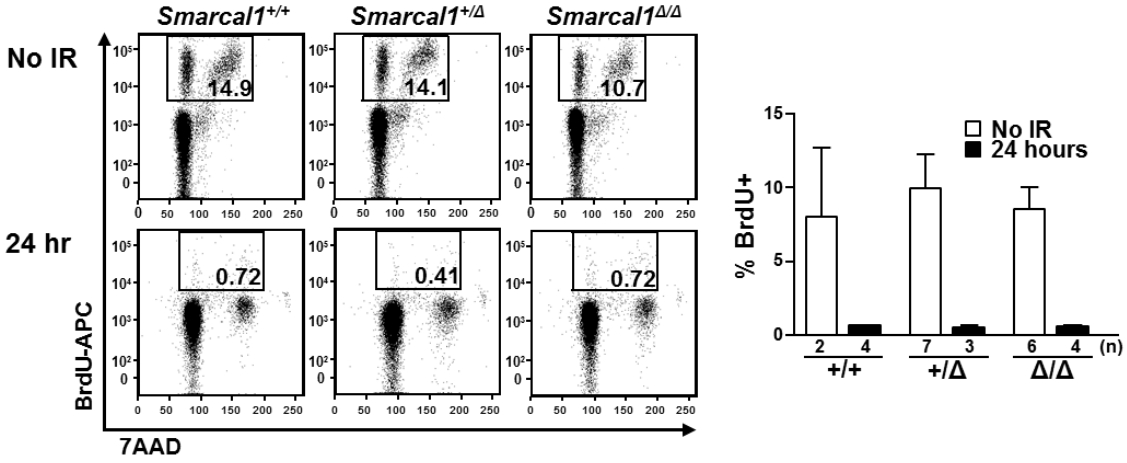

Figure 14. IR-induced cell cycle arrest is intact in Smarcal1-deficient thymocytes. Representative dot plots of BrdU incorporation in thymocytes from littermates without IR and 24 hours after IR (left). Mean BrdU incorporation of three independent litters. Error bars represent SEM; n denotes the number of mice (right).

***Smarcal1*-deficient mice have reduced numbers of T cells during the proliferative response to IR**

Following whole-body IR, thymocytes undergo apoptosis within 48 hours (Figure 13), requiring a replicative burst of HSPCs to generate precursor T cells that then proliferate and differentiate to repopulate the thymus, which is observed 72 hours after IR (Labi et al. 2010). To evaluate whether Smarcal1-deficient thymocytes are impaired during this proliferative burst, we evaluated thymocyte populations 72 hours following IR. Compared to wild-type littermates, there was a significant decrease in both the percentage and total number of DP T cells and a reduction in the number of SP thymocytes in *Smarcal1*$^{+/\Delta}$ and *Smarcal1*$^{\Delta/\Delta}$ mice (Figure 15). These data indicate Smarcal1-deficient mice have an impaired ability to repopulate the thymus after IR.

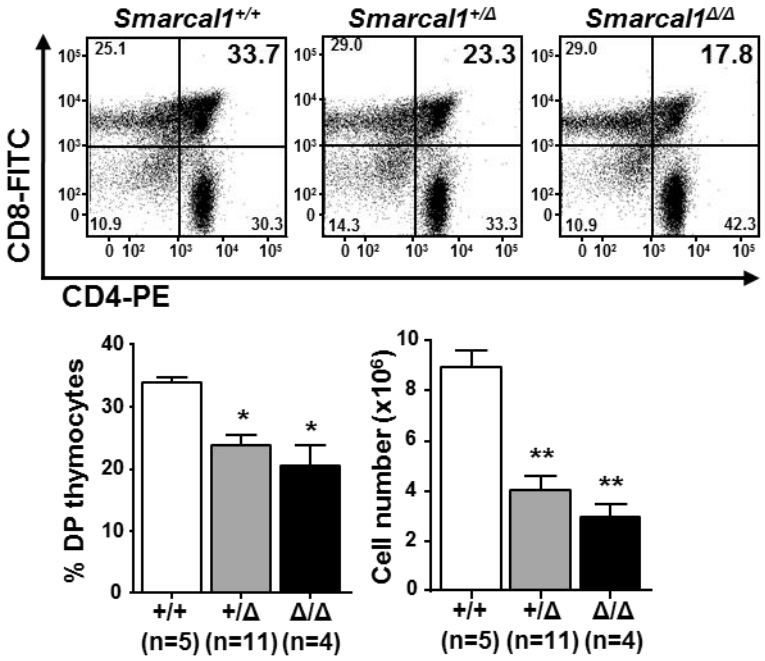

Figure 15. Loss of *Smarcal1* increases thymocyte sensitivity to replication stress. Thymi from littermates were harvested 72 hours after a single dose of 1.75 Gy IR. Representative dot plots of thymocytes (top), and mean percentage of DP thymocytes from three independent experiments (bottom left). Mean total DP thymocytes from each genotype from three independent experiments (bottom right). Error bars are SEM; A, *p<0.01, **p<0.001; one-way ANOVA.

To investigate the possible explanations for the decrease in thymocytes detected in mice lacking Smarcal1 during the proliferative burst, we first measured BrdU incorporation. At 72 hours post-IR, all *Smarcal1* genotypes showed an analogous percentage (~40%) of DP thymocytes had incorporated BrdU (Figure 16). This suggested there were similar numbers of thymocytes cycling in Smarcal1-deficient mice as in wild-type littermates. We then evaluated DNA damage and apoptosis, both of which are consequences of unresolved replications stress. As compared to wild-type thymocytes, there were significantly increased numbers of γH2AX foci (Figure 17A) and DNA breaks (Figure 17B) in *Smarcal1*$^{+/\Delta}$ and *Smarcal1*$^{\Delta/\Delta}$ littermates, 72 hours after IR. Additionally, Smarcal1-deficient thymocytes had elevated levels of apoptosis, as measured by cleaved caspase 3/7 activity (Figure 17C). Thus, thymocytes lacking one or both alleles of *Smarcal1* have increased DNA damage and apoptosis during a time of rapid proliferation, indicating increased sensitivity to replication stress. These data also suggest the decreased ability to respond to replication stress caused from the proliferative burst following IR likely contributes to the delay in T-cell lymphomagenesis in Smarcal1-deficient mice.

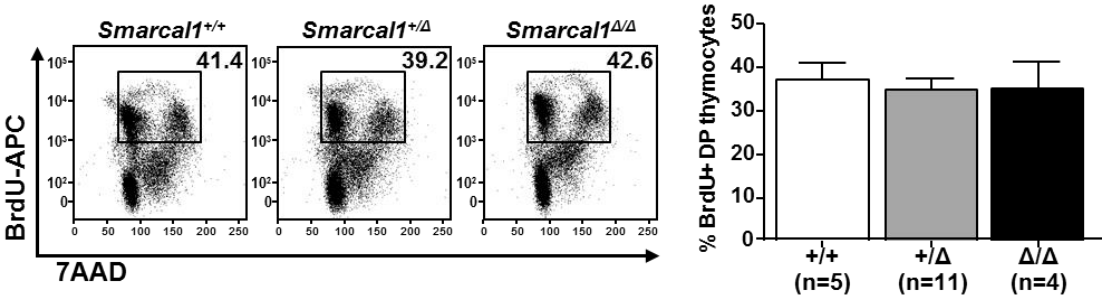

Figure 16. BrdU incorporation is unchanged in Smarcal1-deficient thymocytes during forced proliferation induced by IR. Representative dot plots of BrdU incorporation and DNA content (7AAD) of thymocytes 72 hours after IR treatment (left). Mean of three independent experiments (right).

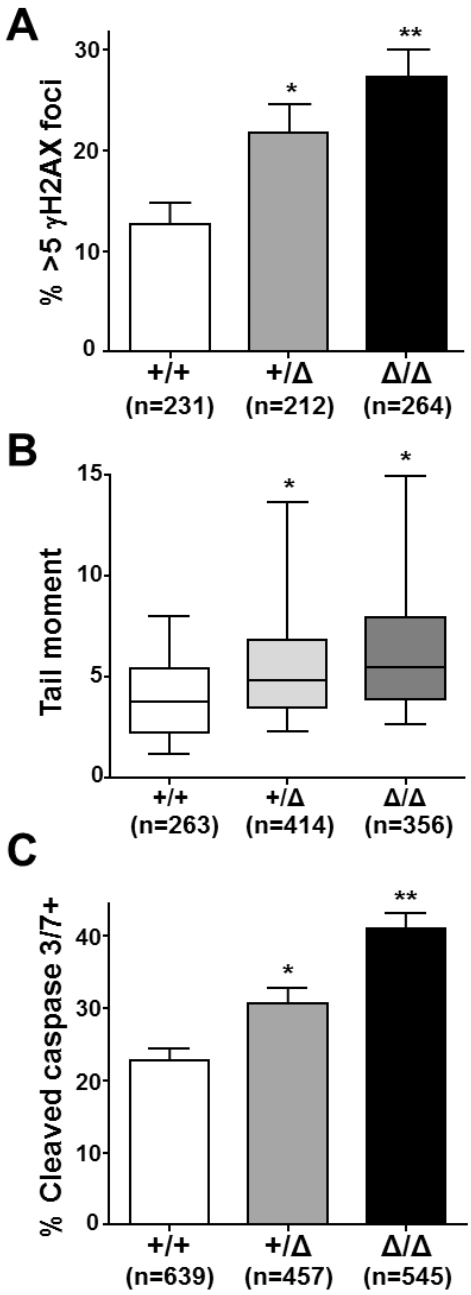

Figure 17. Forced proliferation induces DNA damage and apoptosis in Smarcal1-deficient thymocytes. A) Quantification of thymocytes with >5 γH2AX foci 72 hours after IR from 4 +/+, 3 +/Δ, and 4 Δ/Δ mice from two litters; n denotes the number of cells analyzed. B) Box-and-whisker plots of tail moments from neutral comet assays of thymocytes isolated from mice of the indicated genotypes. 3 +/+, 5 +/Δ and 4 Δ/Δ mice from two separate litters were evaluated. Boxes are the 25th and 75th percentiles, whiskers are 10th and 90th percentiles, and the lines are the medians. The number of cells evaluated is indicated by n. C) Mean percentage of cleaved caspase 3/7-positive thymocytes from a representative litter (3 +/+, 2 +/Δ, and 2 Δ/Δ) of 2 independent experiments; the number of cells analyzed is indicated by n. Error bars are SEM; A, *$p<0.05$, **$p<0.0001$; B, *$p<0.001$; C, *$p<0.01$, **$p<0.0001$; one-way ANOVA.

Loss of *Smarcal1* results in decreased HSPCs following IR

Because Smarcal1-deficient mice showed defects in repopulating the thymus after IR and thymic progenitor cells are derived from HSPCs that have been driven out of quiescence (Labi et al. 2010, Michalak et al. 2010), we assessed HSPC populations. We identified the HSPC-enriched LSK population (lineage-, cKit+, Sca1+), which we further refined into multi-potent progenitors (MPPs; lineage-, cKit+, Sca1+, CD48+, CD150-) and long-term hematopoietic stem cells (LT-HSCs; lineage-, cKit+, Sca1+, CD48-, CD150+) (Figure 18A) (Kiel et al. 2005, Yilmaz et al. 2006). To determine whether there were differences in HSPCs in unstressed Smarcal1-deficient mice, we evaluated unirradiated mice. There were similar numbers of LSKs, MPPs, and LT-HSCs in Smarcal1-deficient mice compared to wild-type littermates (Figure 19A, 19B). The percentages of these HSPC populations were also analogous between genotypes (Figure 18B, 18C).

To determine whether loss of Smarcal1 affected rapidly cycling HSPCs, we evaluated HSPC populations at intervals following IR when HSPCs are induced to proliferate. Compared to wild-type littermates, there was a significant decrease (~30%) in each of the LSK, MPP, and LT-HSC populations in *Smarcal1*$^{+/\Delta}$ and *Smarcal1*$^{\Delta/\Delta}$ mice 24 hours after IR (Figures 18C, 19A, 19B). At 72 hours after IR, there was a ~40%, ~55%, and ~30% reduction in the LSK, MPP, and LT-HSC populations, respectively, in the *Smarcal1*$^{+/\Delta}$ mice compared to wild-type littermates (Figures 19A, 19B). Similarly, the *Smarcal1*$^{\Delta/\Delta}$ mice showed a ~60%, ~65% and ~40% decrease, respectively, in these same populations compared to wild-type mice (Figure 19A, 19B). Therefore, the decreased numbers of HSPCs likely contributes to the delay in repopulation of the thymus and

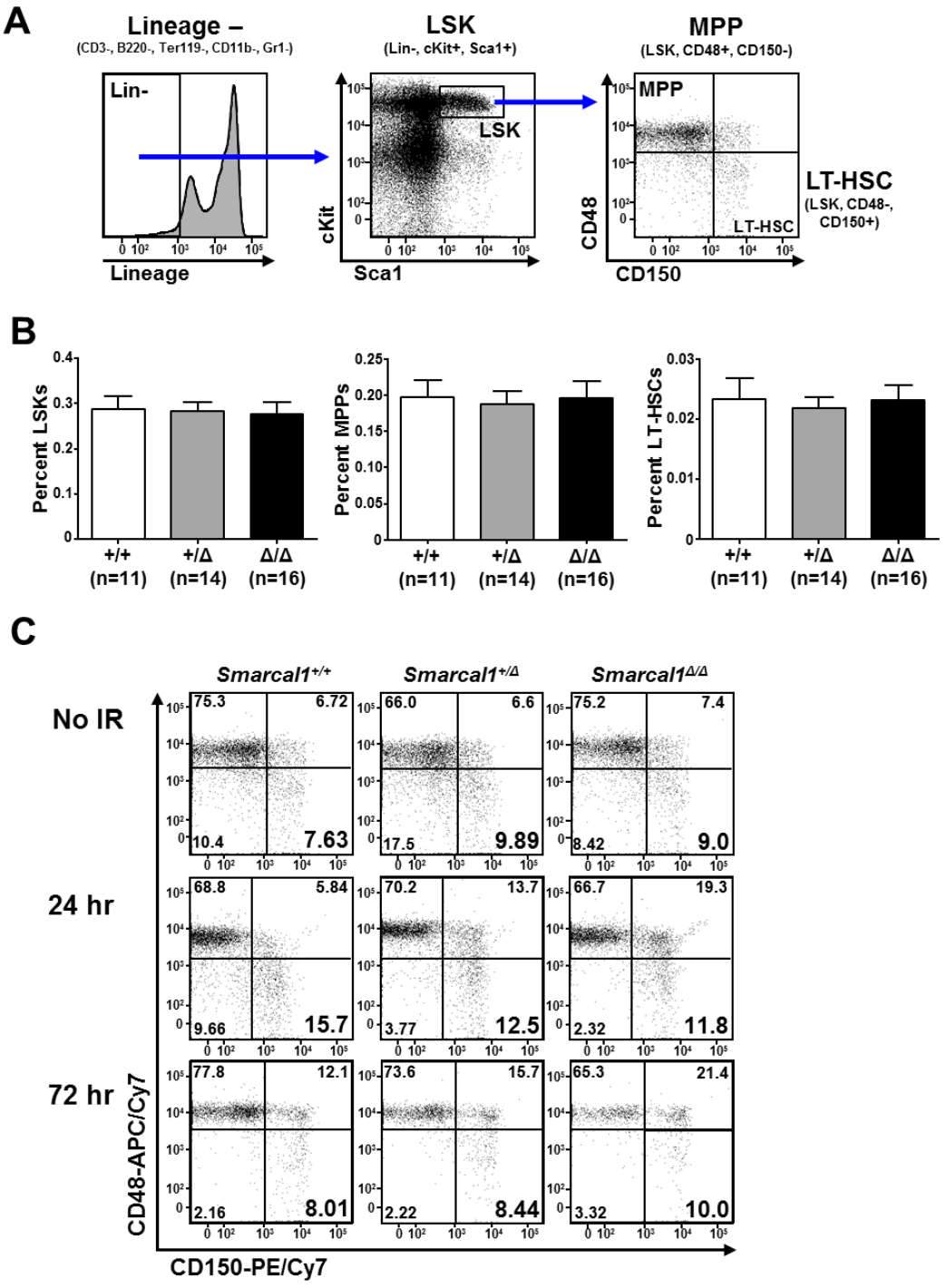

Figure 18. *Smarcal1* loss does not affect HSPC populations in unstressed mice. A) Schematic demonstrating the gating used to identify specific HSPC populations. B) Percentage of LSKs, MPPs, and LT-HSCs in the bone marrow of unirradiated littermates. Error bars are SEM; data from 5 independent experiments. C) Representative dot plots of MPPs and LT-HSCs from littermates not subjected to IR or analyzed at the indicated time after IR.

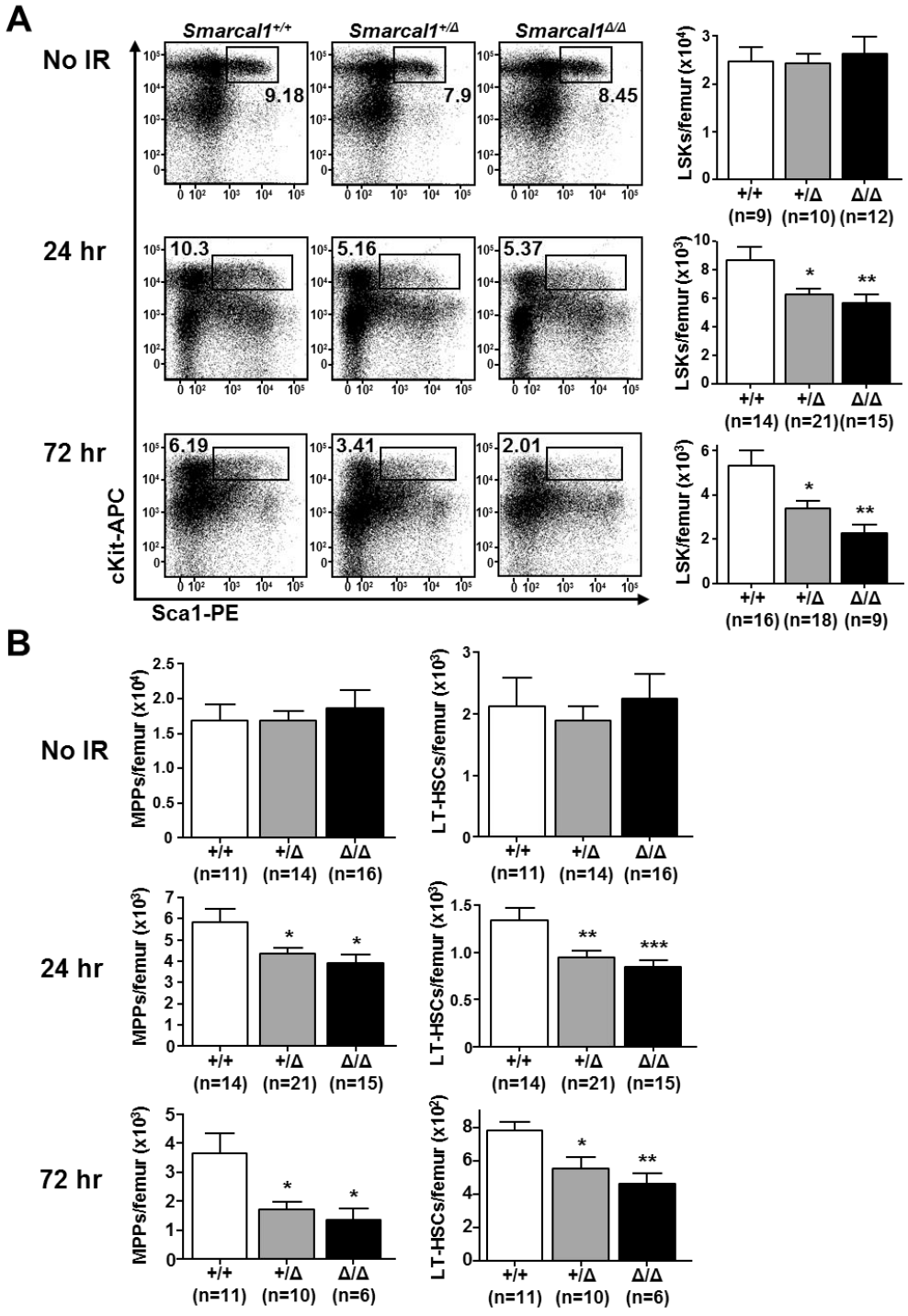

Figure 19. Reduced HSPCs in mice lacking one or both alleles of *Smarcal1* following forced proliferation. Bone marrow harvested from littermates unirradiated or at the indicated interval after IR. A) Representative dot plots of LSKs (left). Mean total LSKs per femur (right). B) Mean total MPPs (left) and LT-HSCs (right) at the indicated intervals following IR. Data are from six independent experiments. Error bars are SEM; A, *p<0.05, **p<0.01; B, *p<0.05, **p<0.01, ***p<0.001; one-way ANOVA.

the inhibition of lymphomagenesis observed in Smarcal1-deficient mice as there are fewer HSPCs with the potential to undergo transformation.

To determine if the HSPC reduction observed in Smarcal1-deficient mice was due to differences in proliferation rates and/or increased sensitivity to replication stress, we measured BrdU incorporation in the LSK compartment at intervals following whole-body IR. At 24 hours post-IR, we observed an analogous significant increase in BrdU positive LSKs, MPPs and LT-HSCs in all *Smarcal1* genotypes compared to unirradiated mice, and the percentage of BrdU positive cells remained elevated above steady-state in all genotypes 72 hours post IR (Figure 20).

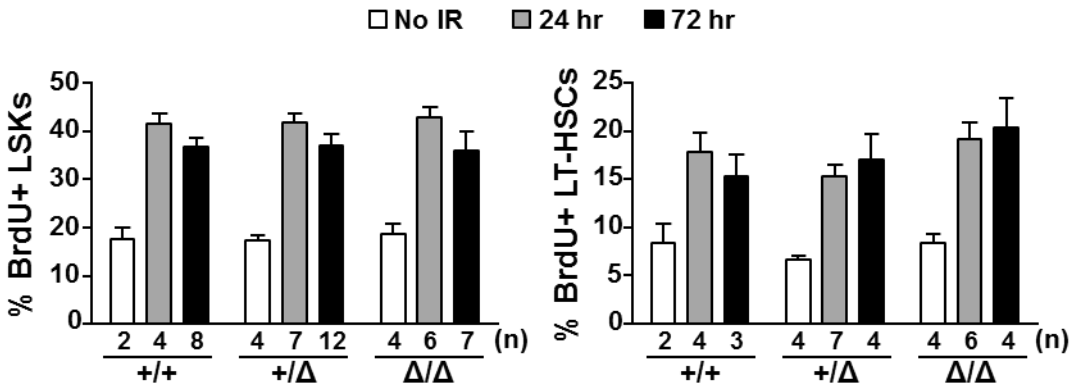

Figure 20. *Smarcal1*-deficient LSKs and LT-HSCs incorporate BrdU at rates similar to wild-type cells following IR. Bone marrow harvested from littermates unirradiated or at the indicated interval after IR. Mean percentage of BrdU positive LSKs (left) or LT-HSCs (right).

Since *Smarcal1*$^{+/\Delta}$ and *Smarcal1*$^{\Delta/\Delta}$ LSKs appeared to be proliferating at equal rates to wild-type Smarcal1 LSKs in response to IR, we measured DNA damage and apoptosis in bone marrow cells during this forced proliferative stress. Both *Smarcal1*$^{+/\Delta}$ and *Smarcal1*$^{\Delta/\Delta}$ bone marrow cells had significantly increased numbers of cells with γH2AX

foci (Figure 21A) and elevated amounts of broken DNA (Figure 21B) 24 hours after IR. Subsequently, Smarcal1-deficient bone marrow showed increased numbers of apoptotic cells as detected by cleaved caspase 3/7 activity 72 hours following IR (Figure 21C). These data indicate Smarcal1 is required to respond to proliferative stress in HSPCs, and loss of one or both alleles of *Smarcal1* increases HSPC susceptibility to DNA breakage caused by replication stress, which results in HSPC apoptosis.

Smarcal1-deficient HSPCs are more sensitive to replication stress

To further examine HSPC function and sensitivity to replication stress in Smarcal1-deficient mice, we utilized stimuli other than IR to induce *in vivo* replication stress. We subjected a cohort of *Smarcal1*$^{+/+}$, *Smarcal1*$^{+/\Delta}$ and *Smarcal1*$^{\Delta/\Delta}$ littermates to repeated injections of 5-fluorouracil (5-FU), a pyrimidine analogue that kills cycling hematopoietic cells, and thereby drives a burst of HSPC proliferation (Cheng et al. 2000). Compared to wild-type littermates, Smarcal1-deficient mice showed significantly reduced survival when challenged with repeated replication stress from 5-FU (Figure 22A; p=0.0194, log-rank test). Thirty-five days after the first 5-FU injection, 52% of wild-type mice were still alive, whereas only 5% of *Smarcal1*$^{+/\Delta}$ and 16% of *Smarcal1*$^{\Delta/\Delta}$ mice were alive. These data show bone marrow cells lacking one or both alleles of *Smarcal1* have increased sensitivity to a form of repeated, acute replication stress distinct from IR, demonstrating a requirement of Smarcal1 to respond to multiple forms of replication stress.

To directly compare the functionality of Smarcal1-deficient HSPCs in response to forced proliferation, we performed competitive bone marrow transplants. Littermate

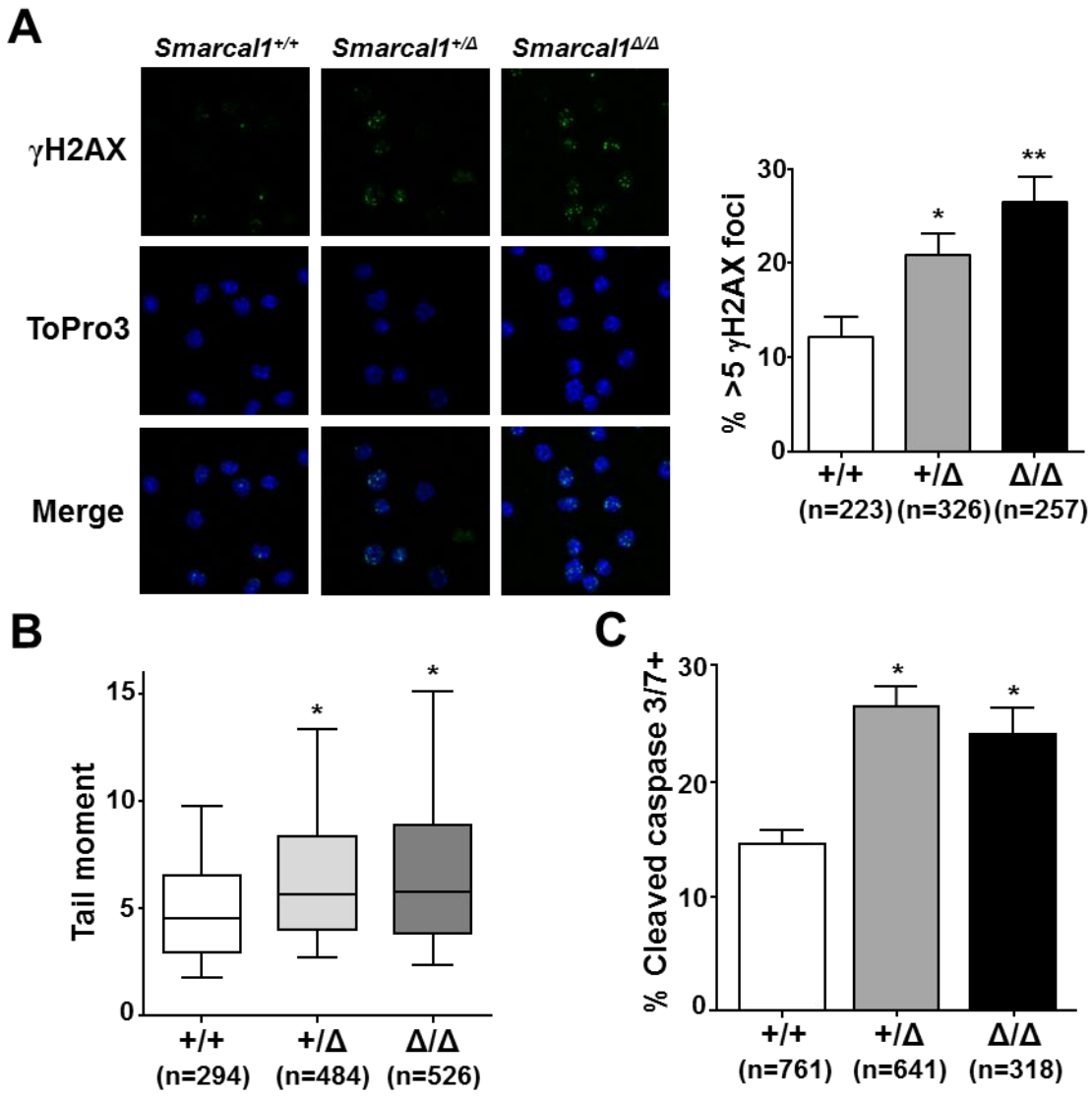

Figure 21. *Smarcal1*-deficient HSPCs have increased sensitivity to replication stress. A) Representative images of γH2AX immunofluorescence of bone marrow cells from littermates of the indicated genotypes (left) Quantification of bone marrow cells with >5 γH2AX foci 24 hours after IR from 3 +/+, 4 +/Δ, and 3 Δ/Δ littermates from two litters; n denotes the number of individual cells analyzed. B) Box-and-whisker plots of tail moments from neutral comet assays of bone marrow cells isolated from littermates of the indicated genotypes 24 hours after IR. Boxes are the 25th and 75th percentiles, whiskers are 10th and 90th percentiles, and the lines are the medians. The number of individual cells analyzed is indicated by n from 3 +/+, 5 +/Δ, and 3 Δ/Δ littermates from two litters. C) Mean percentage of cleaved caspase 3/7-positive bone marrow cells from a representative litter (3 +/+, 2 +/Δ, and 2 Δ/Δ); 2 independent experiments; the number of cells analyzed is indicated by n. Error bars are SEM; B, *p<0.05, **p<0.001; C, *p<0.001; D, *p<0.0001, one-way ANOVA.

donor CD45.2 *Smarcal1*$^{+/+}$, *Smarcal1*$^{+/\Delta}$, and *Smarcal1*$^{\Delta/\Delta}$ bone marrow cells were injected with CD45.1 wild-type bone marrow cells (1:1 ratio) into lethally irradiated CD45.1 recipients. Four weeks post-transplant, and at each of the subsequent analyses, we detected a significant decrease in the percentage of CD45.2 peripheral leukocytes in recipient mice that received *Smarcal1*$^{+/\Delta}$ or *Smarcal1*$^{\Delta/\Delta}$ bone marrow compared to mice that received wild-type bone marrow (Figure 22B). By week 16, mice that received wild-type bone marrow had ~43% of circulating CD45.2 expressing leukocytes, whereas only ~32% of circulating leukocytes expressed CD45.2 in mice that received *Smarcal1*$^{+/\Delta}$ or *Smarcal1*$^{\Delta/\Delta}$ bone marrow (Figure 22B).

We also evaluated CD45.2 expression in thymocytes and bone marrow cells in recipient mice at sacrifice (16 weeks post-transplant). Compared to mice that received wild-type bone marrow, we observed a decrease in the percentage of CD45.2 positive cells in the DP and SP T-cell compartments within the thymus of mice that received *Smarcal1*$^{+/\Delta}$ or *Smarcal1*$^{\Delta/\Delta}$ bone marrow (Figure 22C). Moreover, analysis of bone marrow revealed a significant reduction in the number of CD45.2 positive total bone marrow cells in mice that received Smarcal1-deficient cells with decreases in the numbers of LSKs and MPPs (Figure 22D). There was not a significant reduction in the less proliferative LT-HSCs in mice that received Smarcal1-deficient bone marrow (Figure 22D). Therefore, these data demonstrate Smarcal1-deficient HSPCs are less functionally fit relative to wild-type HSPCs when challenged to repopulate the hematopoietic system, providing further evidence that Smarcal1 is required by HSPCs to mediate a normal response to replication stress.

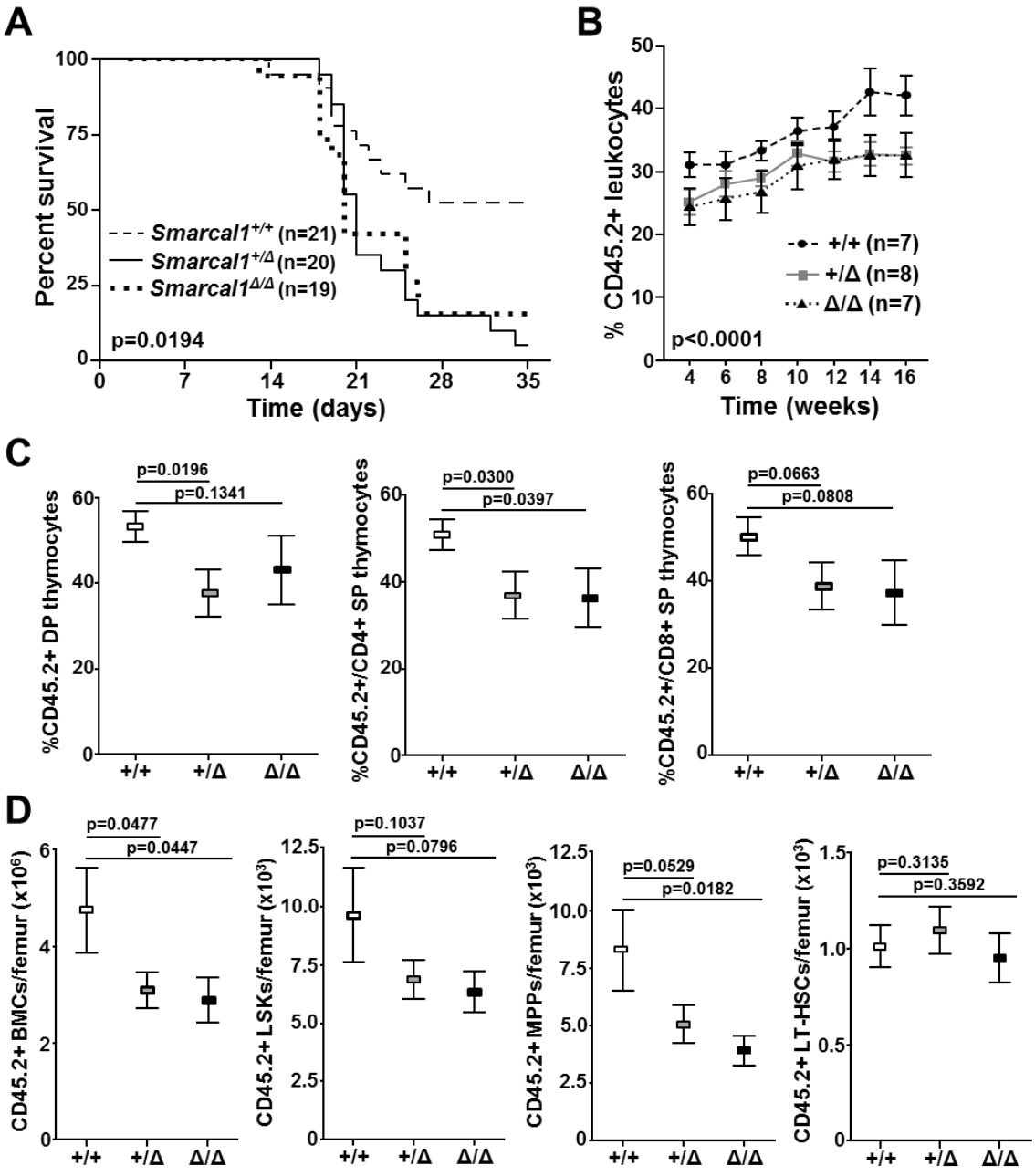

Figure 22. Loss of *Smarcal1* impairs HSPC function. A) Kaplan-Meier survival curves of the indicated genotypes following 5-FU injections every week for 5 weeks beginning at time 0; number of mice denoted by n; overall p value denoted on graph; p=0.0031 +/+ vs. +/Δ and p=0.0203 +/+ vs. Δ/Δ, log-rank tests. B) Mean percentage of CD45.2+ peripheral leukocytes in mice of the indicated genotype were determined at intervals following competitive bone marrow transplantation; n denotes the number of mice; error bars are SEM; p value determined by a two way ANOVA. C) Quantification of the percentage of CD45.2+ DP and SP thymocytes 16 weeks post-transplant. D) Quantification of the total numbers of CD45.2+ BMCs, LSKs, MPPs and LT-HSCs 16 weeks post-transplant. Error bars are SEM from n=7 *Smarcal1*$^{+/+}$, n=8 *Smarcal1*$^{+/\Delta}$, and n=7 *Smarcal1*$^{\Delta/\Delta}$ mice for C and D; p values for C and D indicated.

Discussion

Biochemical and cellular analysis of Smarcal1 has shown it is activated by DNA replication stress and recruited to stalled replication forks. There Smarcal1 facilitates the completion of DNA synthesis by catalyzing fork remodeling, which is thought to promote genome stability (Bansbach et al. 2009, Ciccia et al. 2009, Postow et al. 2009, Yuan et al. 2009, Yusufzai et al. 2009, Betous et al. 2012, Betous et al. 2013, Couch et al. 2013). However, the *in vivo* significance of these findings, particularly in relationship to the development of malignancies and SIOD, the disease associated with mutant Smarcal1, remained unresolved. Our data show that functional Smarcal1 is necessary for cellular viability during acute replication stress in hematopoietic cells. A lack of even one allele of *Smarcal1* was sufficient to confer sensitivity to multiple forms of replication stress in hematopoietic cells, leading to increased DNA damage and apoptosis. However, our data also unexpectedly revealed that being able to respond properly to replication stress contributes to tumorigenesis. Loss of one or both alleles of *Smarcal1* significantly delayed DNA-damage induced T-cell lymphomagenesis and prevented lymphoma development altogether in a quarter of the mice. Thus, a Smarcal1 deficiency protected mice from IR-induced lymphomagenesis, indicating that a disabled replication stress response could shield against DNA replication stress-induced tumorigenesis.

IR-induced T-cell lymphomagenesis is reportedly due to the combined effects of acquired mutations in an HSPC, resulting in its cellular transformation, and the induced proliferation of this cell from signals that indicate lymphoid compartments need to be repopulated (Kominami and Niwa 2006). Reducing HSPC proliferation by blocking lymphocyte apoptosis after IR inhibited lymphoma development, demonstrating the critical

role of the apoptotic response during IR-mediated lymphomagenesis (Labi et al. 2010, Michalak et al. 2010). Our data show that more DNA breaks and apoptosis occur in lymphocytes and bone marrow cells that have lost Smarcal1 during the proliferative, repopulation phase of the IR response, but that Smarcal1-deficient hematopoietic cells are as equally sensitive to the immediate apoptotic effects of IR as wild-type hematopoietic cells. These results are in contrast to data with other cell systems (shRNA and knockout chicken cell lines) that indicate a reduction or loss of Smarcal1 increases radiosensitivity (Ciccia et al. 2009, Keka et al. 2015). Our results indicate Smarcal1 does not contribute to gamma radiation sensitivity, but it is necessary for the proliferation that ensues as a consequence of the IR. Our data show the replication stress response that occurs due to HSPCs being forced out of quiescence necessitates Smarcal1 be functional to aid in the repair and restart of replication forks. Similarly, as they attempt to repopulate the thymus, HSPC-derived precursor T cells also experience proliferative stress that needs functional Smarcal1 to survive. With a haploinsufficiency or loss of both alleles of *Smarcal1*, both mature and precursor hematopoietic cells default to apoptosis from the replication stress due to the increased amount of unrepaired DNA damage. This leads to reduced pools of hematopoietic cells in the thymus and the bone marrow with mutated DNA. In support of this concept, we observed a significant reduction in the number of Smarcal1-deficient DP thymocytes and HSPCs 72 hours after IR compared to wild-type littermates. Additionally, Smarcal1 binding to RPA is reported necessary to facilitate the repair of double-strand DNA breaks (Keka et al. 2015). Therefore, although the initial apoptotic response of lymphocytes is required for IR-induced T-cell lymphomagenesis (Labi et al. 2010,

Michalak et al. 2010), the clearance of proliferating progenitors with damaged DNA also conferred protection against lymphoma development.

Although sarcomas arise from mesenchymal cells, the actual cell of origin of sarcomas is controversial. It is hypothesized that a sarcoma arises from a mesenchymal stem or progenitor cell that has the potential to differentiate into osteoblasts, chondroblasts, and adipocytes (Nombela-Arrieta et al. 2011). Sarcoma development is extremely rare in the IR model we used; yet, 12% of the *Smarcal1* heterozygous mice developed sarcomas rather than T-cell lymphomas in response to IR. This was not because these 12% lived longer than the rest of the cohort and developed sarcoma due to age. Instead, these data suggest that a Smarcal1 haploinsufficiency conferred an increased susceptibility to the development of sarcoma from IR. If a mesenchymal stem or progenitor cell is the cell of origin of sarcomas, a reduced ability of these cells to respond to replication stress during a time of increased mesenchymal cell development (weeks 4-8 of mouse life) occurred preferentially in the heterozygous mice and resulted in the acquisition of mutations that allowed it to transform and not die. It is unclear why the *Smarcal1*$^{\Delta/\Delta}$ mice also did not develop sarcomas, but suggests that complete loss of functional Smarcal1 conferred protection against sarcoma development. Future investigations are needed to determine the reasons for the development of sarcomas in *Smarcal1* heterozygous mice.

While our results have demonstrated a critical function for Smarcal1 during tumor development, our findings also have significant implications for other fields of research and particularly, SIOD patients. For example, hematopoietic cell proliferation and replication stress occurs in response to multiple stimuli, including infection, injury, and aging (Cheshier et al. 2007, Adams et al. 2015, Walter et al. 2015). Physiological stimuli

that drive hematopoietic stem cells out of quiescence lead to the accrual of DNA damage, apoptosis, and stem cell attrition (Baldridge et al. 2010, Flach et al. 2014, Alvarez et al. 2015, Walter et al. 2015). Over time, proliferative stress results in DNA damage and stem cell loss or dysfunction. Our data reveal that Smarcal1 is critical for normal HSPC function in response to multiple forms of proliferative stimuli (IR, 5-FU, and competitive transplantation). With each of these stimuli, Smarcal1-deficient HSPCs were unable to respond as well as wild-type HSPCs and this resulted in reduced numbers of HSPCs, leading to reduced tumorigenesis, diminished cell expansion, and cell death.

Our data also provide a significant increase in understanding of the pathophysiology of SIOD. SIOD patients with homozygous mutations in *SMARCAL1* are characterized by a severe, progressive immunodeficiency and increased rates of infection (Spranger et al. 1991, Boerkoel et al. 2002). Our data demonstrate a lack of Smarcal1 in rapidly cycling hematopoietic cells results in elevated DNA damage and loss of hematopoietic cells. When responding to infection, lymphocytes rapidly proliferate, which likely leads to increased replication stress and elevated lymphocyte apoptosis in SIOD patients. HSPCs would then need to proliferate to repopulate the lymphocyte compartments, resulting in elevated HSPC replication stress and apoptosis, which leads to a further decrease in lymphocytes and increased susceptibility to infection. Therefore, a reduced ability to repopulate lymphocyte compartments following normal childhood infections may explain the progressive lymphopenia observed in SIOD patients (Spranger et al. 1991, Boerkoel et al. 2002). Additionally, a recent report suggests that T cells in SIOD patients may have decreased ability to proliferate due to reduced expression of the IL-7α receptor (Sanyal et al. 2015). We determined that neither circulating nor pre-cursor

thymic T cells in Smarcal1-deficient mice had a decrease in IL-7α receptor (data not shown). However, they did have defects in their ability to respond to replication stress and repopulate the thymus.

Therefore, our data significantly increase our understanding of the function of Smarcal1 in replication stress *in vivo* by revealing its requirement for mediating replication stress to protect from hematopoietic cell loss. Our results also indicate that inhibiting DNA replication can provide a protective function against tumorigenesis caused from replication stress. Finally, our data likely reveal the biological mechanism behind the lymphoid deficiencies of SIOD patients as being an HSPC defect.

CHAPTER III

DNA REPLICATION FORK REMODELING PROTEINS ARE ESSENTIAL FOR RESOLVING ONCOGENE-INDUCED REPLICATION STRESS

This chapter from:

Puccetti, M.V.; Adams, C.M.; Kushinsky, S.; and Eischen, C.M. In preparation. 2018.

Introduction

Oncogenes, such as the transcription factor MYC, induce DNA replication stress by stimulating premature S-phase entry and origin firing, causing transcriptional interference with the replisome, and modifying cellular metabolism (Kotsantis et al. 2018). To suppress oncogene-induced replication stress, which is believed to drive genomic instability and transformation (Bartkova et al. 2006, Di Micco et al. 2006, Halazonetis et al. 2008), cells activate a replication stress response to prevent replication fork collapse and facilitate DNA synthesis. Several classes of proteins are known to interact with damaged replisomes and facilitate fork repair and restart. However, this process is highly complex and the molecular details of this process remain unresolved. Moreover, the contribution of specific replication stress response proteins in resolving different types of replication stress, including oncogenic stress, remains poorly understood.

Smarcal1 and Zranb3 are closely related, specialized DNA translocases (Yusufzai and Kadonaga 2008, Yusufzai and Kadonaga 2010). Although both proteins function similarly *in vitro* by binding DNA at replication forks, re-annealing excessive single-

stranded DNA (ssDNA), and promoting fork regression and remodeling (Bansbach et al. 2009, Ciccia et al. 2009, Postow et al. 2009, Yuan et al. 2009, Yusufzai et al. 2009, Ciccia et al. 2012, Yuan et al. 2012), differences in their function are beginning to be elucidated. For example, Smarcal1 and Zranb3 are recruited to stalled forks through different protein interactions (Bansbach et al. 2009, Ciccia et al. 2009, Postow et al. 2009, Yuan et al. 2009, Yusufzai et al. 2009, Ciccia et al. 2012, Yuan et al. 2012) and have different substrate preferences (Betous et al. 2013). Smarcal1 also resolves replication stress at telomeres (Poole et al. 2015, Cox et al. 2016), whereas Zranb3 has unique endonuclease activity of undefined significance (Weston et al. 2012). It is currently unknown whether Smarcal1 and Zranb3 can compensate for one another to stabilize replication forks *in vivo* under physiological conditions, such as when Myc is dysregulated in primary cells prior to cancer development.

Using a murine model where Myc is overexpressed in B lymphocytes causing B-cell lymphomagenesis (Eμ-*myc* transgenic) (Adams et al. 1985), we observed profound differences in survival between Eμ-*myc* mice that were *Smarcal1*-deficient or *Zranb3*-deficient. We determined these differences were due to alterations in DNA damage, apoptosis, proliferation and the gene dosage of *Smarcal1* or *Zranb3*. Thus, this study establishes that there are unique, non-redundant biological functions of both Smarcal1 and Zranb3 in responding to Myc-induced replication stress *in vivo*. More importantly, our data show gene dosage of fork remodeling proteins strongly influences oncogene-induced tumor development phenotypes and establishes a novel role of these two proteins in resolving an endogenous source of replication stress.

Materials and methods

Mice

C57Bl/6 *Smarcal1*$^{+/\Delta}$ mice were previously provided by Dr. Cornelius Boerkoel (University of British Columbia). C57Bl/6 *Zranb3*$^{+/-}$ founder mice were purchased from Texas Institute of Genomic Medicine (TIGM, Fort Worth, TX). *Zranb3*$^{+/-}$ mice were generated from murine embryonic stem cells retrovirally transduced with a vector encoding an exon gene trapping cassette. A unique gene trapping insertion was in intron 8 of the *Zranb3* gene locus (Figure 25). For survival studies, mice were monitored and sacrificed upon signs of tumor development and/or illness. The Smarcal1 Eμ-*myc* survival study was completed between 2010 and 2013. The Zranb3 Eμ-*myc* survival study was completed between 2015 and 2017. All spleens and bone marrow evaluated were removed from mice prior to any lymphoma development. All experiments were performed with male and female littermate-matched mice. All mouse studies were reviewed and approved by the Institutional Animal Care and Use Committee at either Vanderbilt University or Thomas Jefferson University.

Cell culture and retroviral infection

Bone marrow-derived pro-B-cell cultures were generated by culturing bone marrow cells in RPM1 1640 with 20% FBS, 2 mM glutamine, 55 μM β-mercaptoethanol, penicillin/streptomycin and 10 ng/mL interleukin-7. Cells were continually grown on a stromal feeder layer derived from the bone marrow stroma of the same mouse. pro-B cells were retrovirally transduced with MSCV-MycER-IRES-GFP as previously described (Adams et al. 2016).

B-cell purification

Spleens or bone marrow from mice were processed into single cell suspensions. B cells were enriched by incubating cells with a biotinylated B-cell enrichment antibody cocktail (CD43, CD4, Ter-119) followed by incubation with streptavidin magnetic particles and magnetically negatively selected according to the manufacturer's instructions (BD Biosciences, San Jose, CA).

DNA fiber labeling

Purified bone marrow B cells or bone marrow-derived pro-B cells were pulse labeled with 25 μM IdU (5-Iodo-2'-deoxyuridine; MilliporeSigma, Burlington, MA) for 20 or 30 minutes respectively, washed with 1X DPBS, and labeled with 250 μM CldU (5-Chloro-2'-deoxyuridine; MilliporeSigma, Burlington, MA) for 20 or 30 minutes, respectively. Cells were lysed with DNA spreading buffer (0.5% SDS, 200 mM Tris-HCl (pH 7.4), 50 mM EDTA) for 6 minutes and DNA spread across frosted microscope slides. Slides were air dried for 40 minutes and fixed with 3:1 methanol/acetic acid for 2 minutes at room temperature. DNA was denatured by submerging slides in 2.5N HCl for 80 minutes at room temperature. Slides were blocked for 1 hour at room temperature with blocking buffer (10% normal goat serum, 0.1% Triton-X 100 in 1X DPBS) and incubated with rat anti-CldU antibody diluted 1:50 in blocking buffer for 1 hour at room temperature (Abcam; Cambridge, MA). After washing 3X with 1X DPBS, slides were incubated with AlexaFluor-488 goat anti-rat secondary antibody diluted 1:200 in blocking buffer (Invitrogen; Waltham, MA) for 30 minutes at room temperature. Slides were then washed 3X in 1X DPBS and incubated with mouse anti-IdU antibody (BD Biosciences; San Jose,

CA) diluted 1:50 in blocking buffer for 1 hour at room temperature. Slides were washed 3X with 1X DPBS and incubated with Cy3 goat anti-mouse secondary antibody diluted 1:200 in blocking buffer (Invitrogen; Waltham, MA) for 30 minutes at room temperature. All antibody incubations were done in a humidified chamber. Slides were washed 3X with 1X DPBS, air dried for 20 minutes and mounted with ProLong Gold (Invitrogen; Waltham, MA) and allowed to cure overnight. Images were captured on a Nikon Eclipse N*i* using a 100X oil objective (Nikon; Melville, NY) and fibers were measured and analyzed using ImageJ.

Flow cytometry analysis of immunophenotype and BrdU incorporation

Bone marrow or spleens were harvested from mice and processed into single cell suspensions. Immunophenotyping was performed using fluorochrome linked antibodies specific for IgM (Southern Biotech; Birmingham, AL), IgD (Southern Biotech; Birmingham, AL), CD19 (Southern Biotech; Birmingham, AL), B220 (Southern Biotech; Birmingham, AL), CD43 (BD Biosciences; San Jose, CA), CD4 (eBiosciences; Waltham, MA), Ly6A/E (BD Biosciences; San Jose, CA), and CD34 (BD Biosciences; San Jose, CA). For BrdU experiments, mice were intraperitoneally injected with 1 mg BrdU and sacrificed after 16 hours. BrdU incorporation was measured according to the manufacturer's instructions (BD Biosciences, San Jose, CA). All samples were evaluated on an LSRII or BD Fortessa instrument (BD Biosciences, San Jose, CA) and analyzed using FlowJo (FlowJo; Ashland, OR).

Western blotting

Whole cell lysates were Western blotted using antibodies specific for Zranb3 (Bethyl Laboratories; Montgomery, TX), cleaved caspase 3 (Cell Signaling; Danvers, MA), Myc (MilliporeSigma; Burlington, MA) and β-actin (MilliporeSigma; Burlington, MA) as previously described (Zindy et al. 1998, Alt et al. 2005). The Smarcal1 antibody was provided by Dr. David Cortez (Vanderbilt University).

Immunofluorescence

Quantification of γH2AX foci was performed as previously described (Puccetti et al. 2017). Images were captured on a Nikon C2 or A1R confocal microscope (Nikon; Melville, NY) and analyzed using ImageJ. All samples were blinded for analysis and a minimum of 100 cells were analyzed per experiment.

Neutral comet assays

Neutral comet assays were performed on pro-B cells or purified splenic B cells as previously described (Alt et al. 2005, Bouska et al. 2008). Images were captured on a Nikon Eclipse N*i* (Nikon, Japan) with a 10X objective. All samples were blinded and a minimum of 75 cells per sample were scored.

Statistics

Statistical analysis was determined using long-rank tests (Figure 26), one-way ANOVA with a Bonferroni post-test (Figures 28, 30C, 30D 31C, 31D 33, 34C, 35C, 35D 40, 41), Student's t-test (Figures 23B, 24, 43, 44), chi-square test (Figures 23D, 30A, 30B, 31A,

31B, 34A, 34B, 35A, 35B). All calculations were performed using GraphPad Prism 6 software (GraphPad Software; La Jolla, CA).

Results

Myc overexpression generates replication stress in primary B cells.

Myc dysregulation is a source of endogenous DNA replication stress that is thought to contribute to tumorigenesis (Vafa et al. 2002, Srinivasan et al. 2013, Maya-Mendoza et al. 2015, Rohban and Campaner 2015). Currently, there is a paucity of knowledge on endogenous replication stress and in particular, Myc-induced replication stress *in vivo* and the molecular consequences of Myc overexpression at the replication fork in primary cells. To evaluate the effects of a physiological source of DNA replication stress, Eμ-*myc* transgenic mice that overexpress Myc in B cells and develop B-cell lymphomas were utilized. We performed single-molecule DNA fiber analysis on purified B cells from Eμ-*myc* transgenic and littermate-matched wild-type mice (Figure 23A). Compared to wild-type B cells, Myc-overexpressing B cells showed a significant reduction in mean fiber length, which is indicative of impaired DNA replication (Figure 23B, 23C). Fibers that only incorporate the first nucleotide analog (IdU), but not second (CldU), represent stalled forks or terminated replication. The percentage of fibers from Eμ-*myc* B cell that only incorporated (IdU) was significantly increased compared to wild-type controls (Figure 23D). Thus, Myc overexpression results in fork stalling and impaired replication fork progression in primary murine B cells.

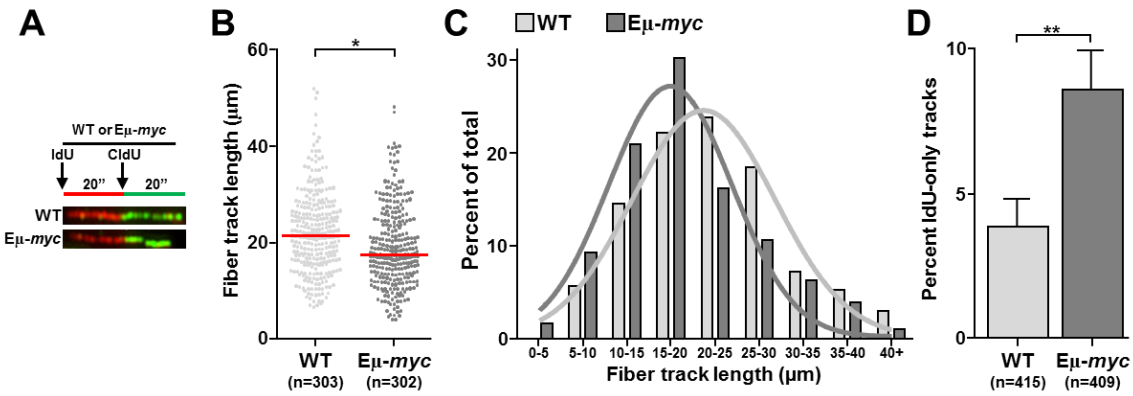

Figure 23. Myc overexpression in primary B cells impairs replication fork progression. B cells were purified from bone marrow of wild-type (WT) and Eμ-*myc* mice. A) Design of DNA fiber analysis and representative fiber images. B) Quantification of total fiber length from two independent experiments. C) Binned fiber length frequencies from (B). D) Quantification of fibers that only incorporated IdU from (B). Number (n) of fibers (B) or replication structures (D) evaluated indicated. Student's t-tests (B) or chi-square tests (D) determined significance, *p<0.0001, **p=0.0052.

Prolonged fork stalling can lead to replication fork collapse into double-stranded DNA (dsDNA) breaks (Zeman and Cimprich 2014), therefore, we evaluated DNA damage in B cells. Compared to wild-type littermates, Eμ-*myc* mice had a significantly higher percentage of B cells with γH2AX foci, a marker of dsDNA breaks (Figure 24). Therefore, our data show Myc overexpression generates replication stress and induces DNA damage in primary B lymphocytes prior to cellular transformation.

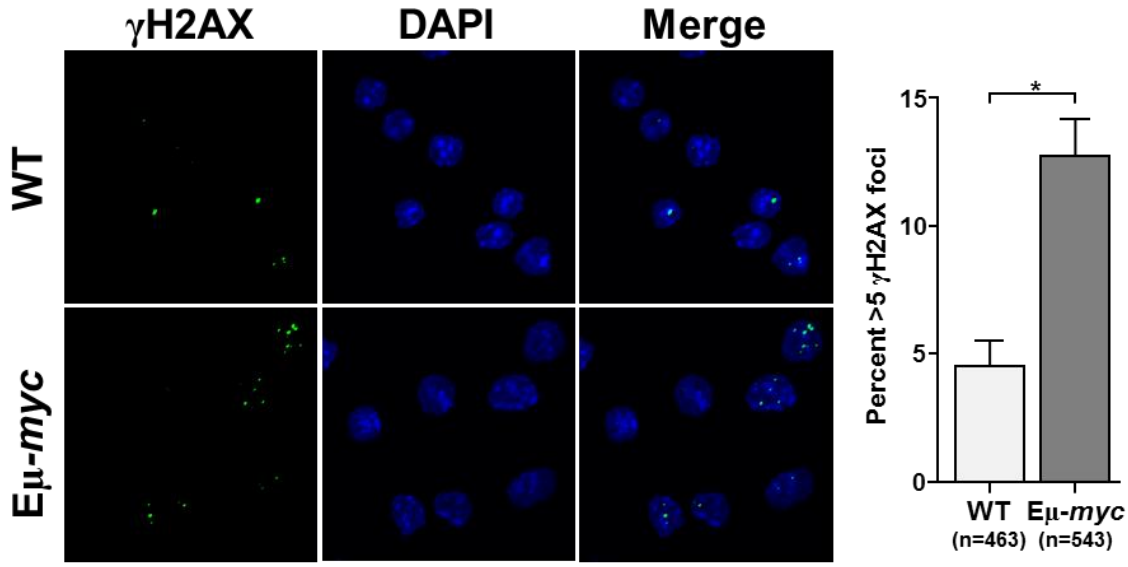

Figure 24. Primary B cells overexpressing Myc have increased levels of DNA damage. B cells were purified from bone marrow of wild-type (WT) and Eμ-*myc* mice. Representative images (left) and quantification (right) of B cells with >5 γH2AX foci. Student's t-tests determined significance, *p<0.0001.

Loss of *Smarcal1* or *Zranb3* significantly alters Myc-driven lymphoma development

Smarcal1 and Zranb3 have been implicated in resolving replication stress caused by drugs that induce fork stalling *in vitro* (Bansbach et al. 2009, Ciccia et al. 2009, Postow et al. 2009, Yuan et al. 2009, Ciccia et al. 2012, Yuan et al. 2012), but their role in responding to replication stress under physiological conditions is incompletely understood. To assess in an unbiased, biologically relevant system whether Smarcal1 and Zranb3 contribute to the biological response to Myc overexpression, we utilized mouse models. We previously reported *Smarcal1* loss-of-function mice, which express an N-terminal truncated, non-functional Smarcal1 protein, Smarcal1Δ (Puccetti et al. 2017). We obtained *Zranb3* knockout mice, which contain a gene trapping cassette in intron 8 of the *Zranb3* gene locus (Figure 25A). We confirmed these mice do not express Zranb3 protein (Figure 25B). Similar to *Smarcal1*-deficient mice, *Zranb3*$^{+/-}$ and *Zranb3*$^{-/-}$ mice displayed no overt abnormalities.

We crossed both the *Smarcal1Δ* and *Zranb3* knockout mice to Eμ-*myc* transgenic mice and monitored lymphoma development. Previously, using a γ-radiation replication stress-induced model of T-cell lymphomagenesis, we reported loss of one or two alleles of *Smarcal1* inhibited lymphomagenesis and increased overall survival (Puccetti et al. 2017). However, with Myc-induced replication stress, there was a significant acceleration in B-cell lymphoma development and decreased survival of *Smarcal1*$^{+/\Delta}$ Eμ-*myc* mice compared to *Smarcal1*$^{+/+}$ Eμ-*myc* mice (Figure 26A) with median survivals of 103 days and 125 days, respectively. Unexpectedly, *Smarcal1*$^{\Delta/\Delta}$ Eμ-*myc* mice showed no statistically significant difference in overall survival compared to wild-type Eμ-*myc* mice ($p=0.3224$) and had a median survival of 134 days. Additionally, *Smarcal1*$^{\Delta/\Delta}$ Eμ-*myc* mice

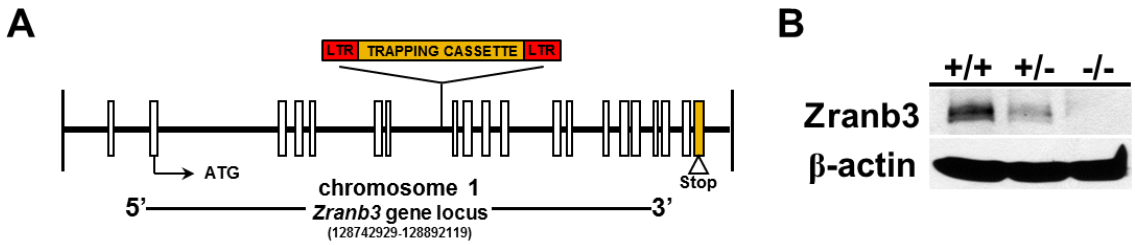

Figure 25. Generation of *Zranb3* knockout mice. A) Schematic showing the location of the gene trapping cassette introduced into the locus of the *Zranb3* gene in the *Zranb3* knockout mice. B) Whole cell lysates from mouse embryonic fibroblasts (MEFs) were Western blotted with an antibody against Zranb3.

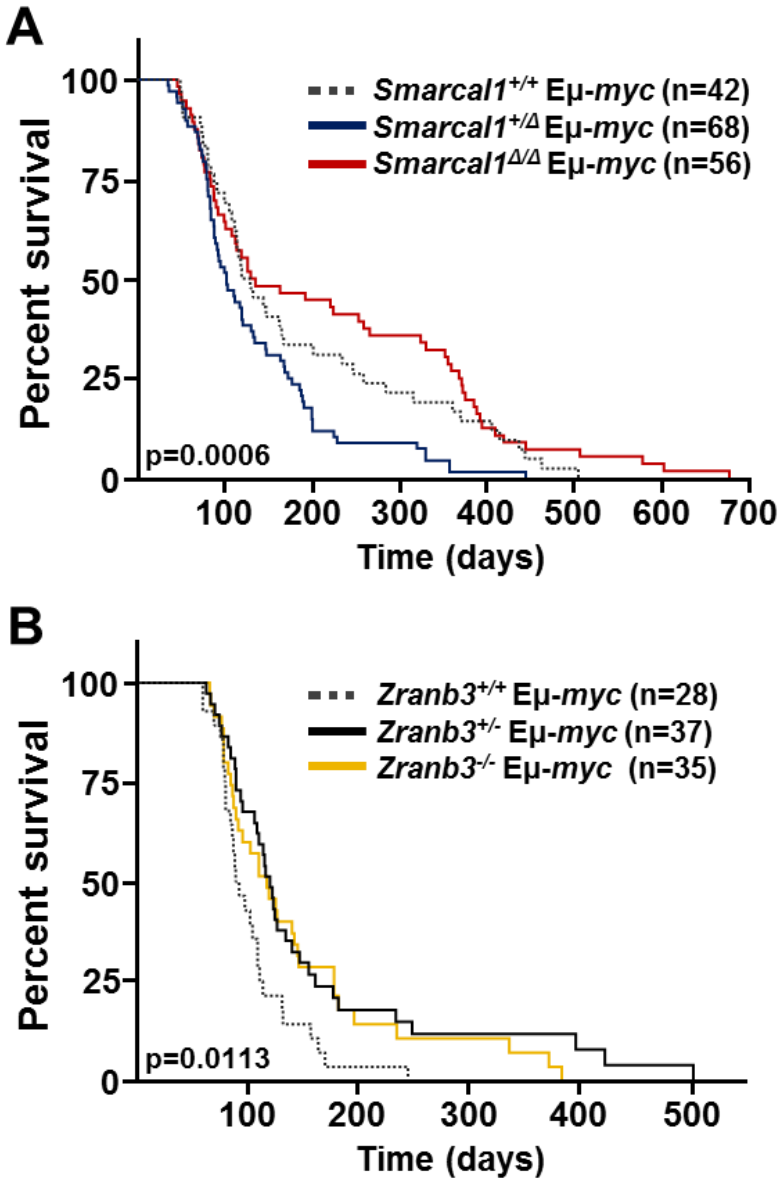

Figure 26. *Smarcal1*- or *Zranb3*-deficiency significantly alters Myc-induced lymphomagenesis. A, B) Kaplan-Meier survival curves of mice of the indicated genotypes. Overall *P* values in figures and p=0.0142 (+/+ vs. +/Δ), p=0.0002 (+/Δ vs. Δ/Δ), p=0.3224 (+/+ vs. Δ/Δ) (A), and p=0.0066 (+/+ vs. +/-), p=0.0199 (+/+ vs. -/-) (B), log-rank tests. Number (n) of mice indicated.

showed significantly increased survival compared to *Smarcal1*$^{+/\Delta}$ Eμ-*myc* mice (p=0.0002, Figure 26A), revealing a Smarcal1 gene dosage effect on Myc-induced lymphomagenesis.

For proteins thought to have very similar functions, evaluation of *Zranb3*-deficient Eμ-*myc* mice revealed surprisingly different results than those obtained from *Smarcal1*-deficient Eμ-*myc* mice. Specifically, lymphoma development was significantly inhibited in both *Zranb3*$^{+/-}$ and *Zranb3*$^{-/-}$ Eμ-*myc* mice compared to wild-type littermates (Figure 26B), with median survivals of 119, 116 and 90 days, respectively. Thus, our data demonstrate these closely related proteins have non-redundant functions in responding to Myc-induced replication stress.

Eμ-*myc* mice typically develop pre-B and/or B-cell lymphomas (Adams et al. 1985, Harris et al. 1988), but a subset of *Smarcal1*$^{+/\Delta}$, *Zranb3*$^{+/-}$, and *Zranb3*$^{-/-}$ Eμ-*myc* mice developed early progenitor B-cell lymphomas (Figure 27 and Table 1), suggesting Smarcal1 and Zranb3 loss influenced the tumor cell of origin. Taken together, our data indicate both Smarcal1 and Zranb3 are critical, non-redundant proteins in the cellular response to Myc-induced replication stress. Moreover, the expression levels of these proteins profoundly affect Myc-induced lymphoma development and survival.

Table 1. *Smarcal1*$^{+/\Delta}$ Eμ-*myc*, *Zranb3*$^{+/-}$ Eμ-*myc* and *Zranb3*$^{-/-}$ Eμ-*myc* mice develop pre-cursor B cell lymphomas.

Phenotype[a]	Smarcal1$^{+/+}$	Smarcal1$^{+/\Delta}$	Smarcal1$^{\Delta/\Delta}$	Zranb3$^{+/+}$	Zranb3$^{+/-}$	Zranb3$^{-/-}$
B220^{+} CD19^{+} IgM$^{+/-}$ IgD$^{+/-}$	9 (100%)	9 (81.8%)	8 (100%)	5 (100%)	9 (75.0%)	5 (83.3%)
B220^{+} CD19$^{+/-}$ IgM^{lo} Sca1$^{+/lo}$ CD34^{+} CD4^{+}	0 (0%)	2 (18.2%)	0 (0%)	0 (0%)	3 (25.0%)	1 (16.7%)

[a]determined by flow cytometry

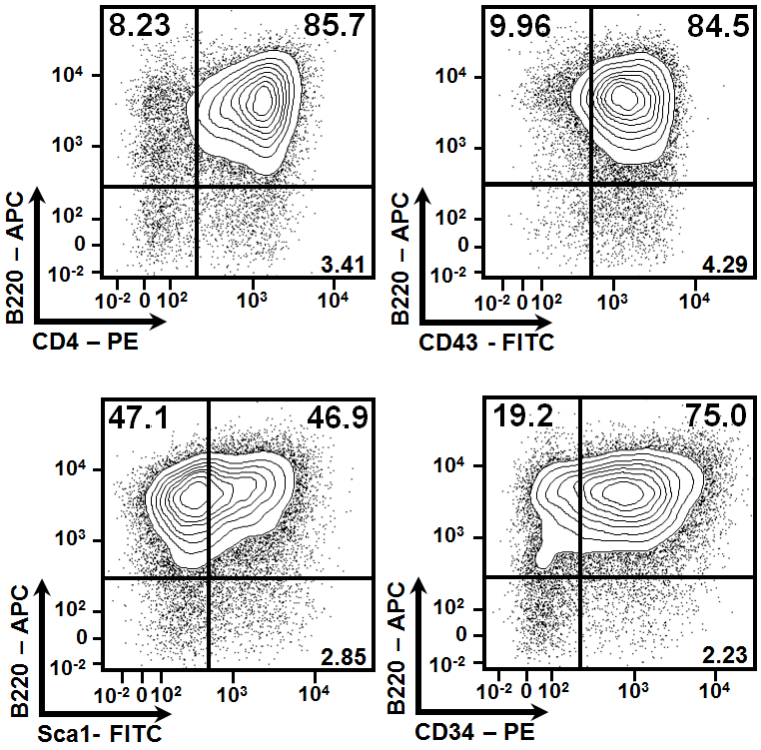

Figure 27. *Smarcal1*$^{+/\Delta}$, *Zranb3*$^{+/-}$, and *Zranb3*$^{-/-}$ Eμ-*myc* develop pre-cursor B-cell lymphomas. Representative contour plots (B-cell surface markers indicated) of lymphomas arising in a fraction of the *Smarcal1*$^{+/\Delta}$ Eμ-*myc*, *Zranb3*$^{+/-}$ Eμ-*myc* and *Zranb3*$^{-/-}$ Eμ-*myc* mice (also see Table 1).

***Smarcal1* loss results in replication fork collapse upon Myc overexpression.**

To begin to determine the cause(s) of the differences in survival of the *Smarcal1*- and *Zranb3*-deficient Eμ-*myc* mice, we first evaluated the consequences of Smarcal1 loss at the replication fork in Myc-overexpressing B cells. We performed single-molecule DNA fiber analysis on purified bone marrow B cells from *Smarcal1* Eμ-*myc* littermates (Figure 28A). Loss of both alleles of *Smarcal1* led to a significant reduction in mean fiber length compared to wild-type Eμ-*myc* B cells (Figures 28B, 28C). Loss of a single allele led to an intermediate, but statistically significant reduction in fiber length (Figures 28B, 28C). To independently validate these results, we retrovirally expressed a 4-hydroxytamoxifen (4-OHT)-inducible form of Myc, MycER, in pro-B cells from wild-type, $Smarcal1^{+/\Delta}$ and $Smarcal1^{\Delta/\Delta}$ mice. We performed fiber analysis in these cells 8 hours after activation of MycER with 4-OHT addition, a point when MycER has induced S-phase entry but prior to significant Myc-induced apoptosis (Figure 28D). With MycER induction, we also observed a significant reduction in mean fiber length in the $Smarcal1^{\Delta/\Delta}$ cells and an intermediate reduction in the $Smarcal1^{+/\Delta}$ cells (Figures 28E, 28F). The differences in fiber lengths were not due to *Smarcal1* loss alone, as non-transgenic B cells from $Smarcal1^{+/\Delta}$ and $Smarcal1^{\Delta/\Delta}$ mice and pro-B cells with non-active MycER, did not show changes in fiber length (Figure 29). Therefore, using two independent approaches with primary B cells, our results demonstrate loss of Smarcal1 function at replication forks in the presence of oncogene overexpression impairs DNA replication.

Since the fork reversal/remodeling function of Smarcal1 stabilizes stalled forks and promotes replication restart, we sought to determine whether fibers from *Smarcal1*-

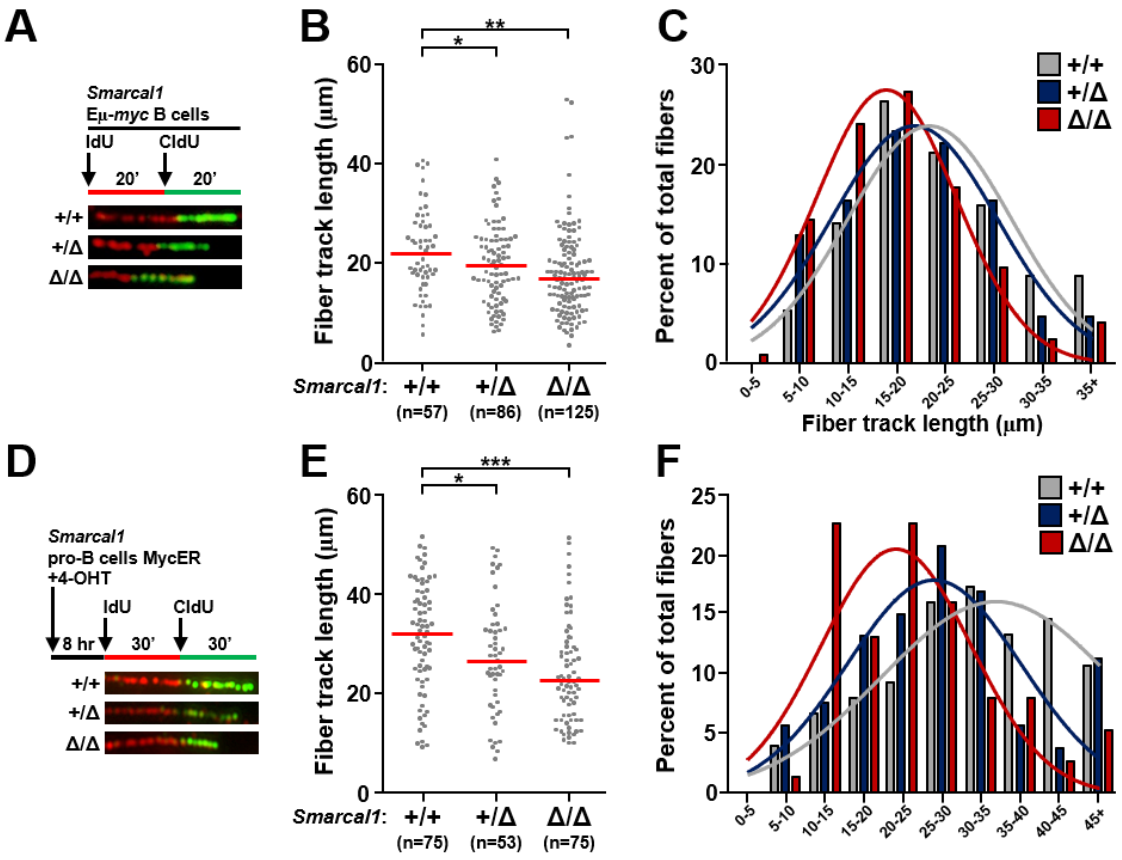

Figure 28. *Smarcal1*-deficiency further impairs replication fork progression in B cells with dysregulated Myc. B cells were purified from the bone marrow of *Smarcal1*$^{+/+}$ Eμ-*myc*, *Smarcal1*$^{+/Δ}$ Eμ-*myc*, and *Smarcal1*$^{Δ/Δ}$ Eμ-*myc* mice (A-C). Pro-B-cell cultures from *Smarcal1*$^{+/+}$, *Smarcal1*$^{+/Δ}$, and *Smarcal1*$^{Δ/Δ}$ mice expressing MycER with MycER activated by the addition of 4-hydroxytamoxifen (4-OHT) for 8 hours (D-F). (A, D) Design of DNA fiber labeling experiments and images of representative fiber tracks. B, E) Quantification of total fiber length in purified Eμ-*myc* B cells (B) or MycER pro-B cells (E). Experiments were performed twice, data from a representative experiment shown. C, F) Fiber length frequencies from (B) and (E), respectively. The total number (n) of fibers (B, E) is indicated. Lines are the median (B, E). One-way ANOVA with Bonferroni correction (B, E) determined significance, *p<0.05, **p<0.01, ***p<0.001.

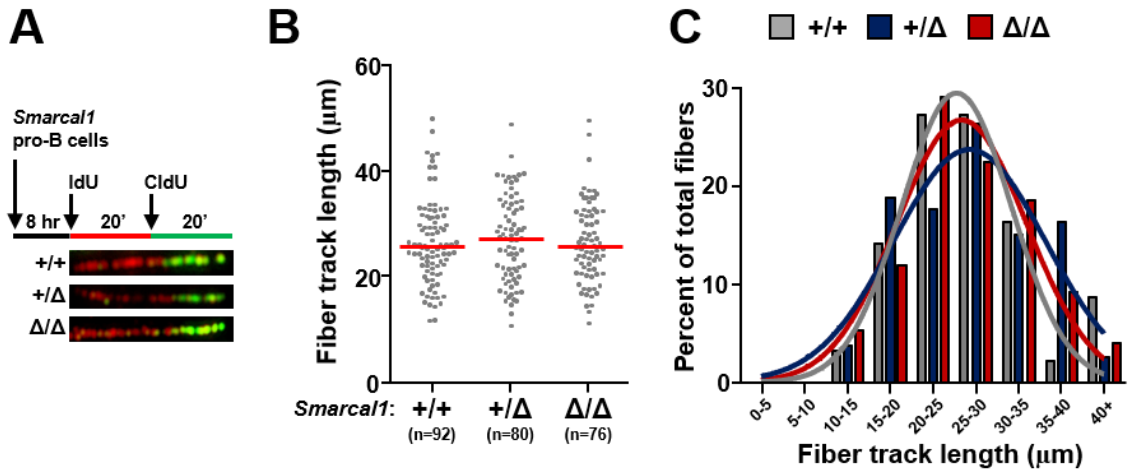

Figure 29. *Smarcal1* loss alone does not alter DNA replication fork kinetics in B cells. Bone marrow-derived pro-B cells of the indicated genotypes were retrovirally transduced with MSCV-MycER-IRES-GFP and treated with an ethanol vehicle control. A) Schematic outlining the DNA fiber labeling experiment and images of representative fiber tracks. B) Quantification of fiber track lengths from cells of the indicated genotypes. C) Fiber track length frequencies of the measurements in (B). The total number of fibers measured is indicated by "n" (B).

deficient cells displayed evidence of increased fork collapse. In both the *Smarcal1*$^{\Delta/\Delta}$ Eμ-*myc* bone marrow B cells and the *Smarcal1*$^{\Delta/\Delta}$ pro-B cells with activated MycER, there was a significant increase in the percentage of fibers that had only incorporated IdU (Figure 30A, 30B). Moderate differences were observed with loss of a single allele of *Smarcal1*, but it did not reach statistical significance. As an additional method to quantify fork stalling, we also evaluated the incidence of sister replication fork asymmetry by comparing the relative CldU track lengths of replication forks originating from the same origin. In both Eμ-*myc* and MycER activated *Smarcal1*$^{\Delta/\Delta}$ B cells, we observed increased rates of fork asymmetry (Figure 30C, 30D), supporting the conclusion that loss of Smarcal1 results in replication fork stalling when cells are subjected to oncogenic stress.

Stalled replication forks are a cause of DNA damage, as they can result in double-strand DNA breaks (Zeman and Cimprich 2014). Evaluation of DNA damage in Eμ-*myc* B cells and pro-B cells following MycER activation showed a significant increase in the percentage of cells with γH2AX foci and DNA breaks in *Smarcal1*-deficient B cells (Figure 31). Myc overexpression in B cells lacking both alleles of *Smarcal1* resulted in higher levels of DNA damage than loss of a single *Smarcal1* allele, which resulted in a small, but significant increase in B cells with damaged DNA (Figures 31). This phenotype was not due to *Smarcal1* loss alone, as non-transgenic B cells from *Smarcal1*$^{+/\Delta}$ and *Smarcal1*$^{\Delta/\Delta}$ mice and pro-B cells with non-active MycER, did not show increases in DNA damage (Figure 32). Thus, our data demonstrate Smarcal1 stabilizes replication forks in response to oncogene dysregulation in a gene dosage dependent manner with loss of a single *Smarcal1* allele resulting in a mild increase in DNA damage

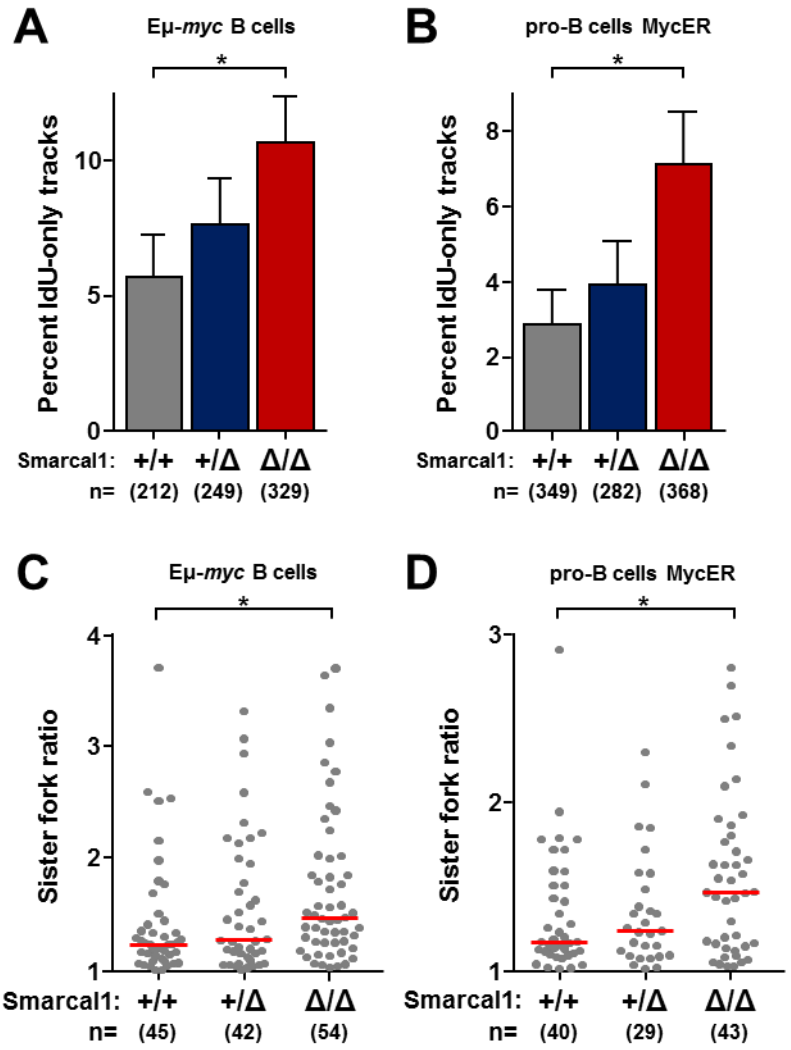

Figure 30. *Smarcal1*-deficient B cells have increased rates of replication fork collapse with Myc dysregulation. B cells were purified from the bone marrow of *Smarcal1*$^{+/+}$ Eμ-*myc*, *Smarcal1*$^{+/\Delta}$ Eμ-*myc*, and *Smarcal1*$^{\Delta/\Delta}$ Eμ-*myc* mice (A, C). Pro-B-cell cultures from *Smarcal1*$^{+/+}$, *Smarcal1*$^{+/\Delta}$, and *Smarcal1*$^{\Delta/\Delta}$ mice expressing MycER, with MycER activated by the addition of 4-hydroxytamoxifen (4-OHT) for 8 hours (B, D). A, B) Quantification of DNA fibers that only incorporated IdU from Eμ-*myc* B cells (A) or MycER pro-B cells (B). C, D) Ratios of measured CldU track lengths from left and right moving forks from the same replication origin from Eμ-*myc* B cells (C) or MycER pro-B cells (D). The total number (n) of replication structures analyzed is indicated. All data are from 2-3 independent experiments. A, B) Error bars are SEM. C, D) Median is the red line. Chi-square test (A, B) or one-way ANOVA with Bonferroni correction (C, D) determined significance, *p<0.05.

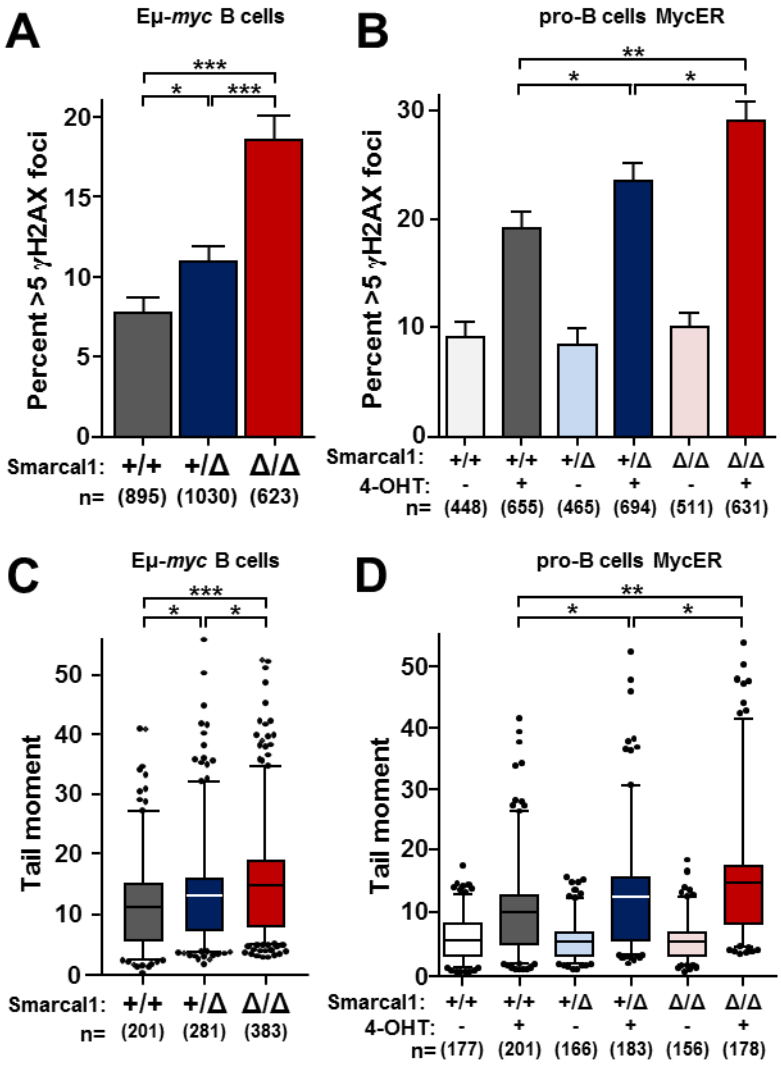

Figure 31. *Smarcal1*-deficient B cells overexpressing Myc have increased DNA damage. B cells were purified from the spleen of *Smarcal1*$^{+/+}$ Eμ-*myc*, *Smarcal1*$^{+/Δ}$ Eμ-*myc*, and *Smarcal1*$^{Δ/Δ}$ Eμ-*myc* mice (A, C). Pro-B-cell cultures from *Smarcal1*$^{+/+}$, *Smarcal1*$^{+/Δ}$, and *Smarcal1*$^{Δ/Δ}$ mice expressing MycER (B, D) with MycER activated by the addition of 4-hydroxytamoxifen (4-OHT) for 8 hours. A, B) Quantification of Eμ-*myc* B cells (A) or MycER pro-B cells (B) with >5 γH2AX foci. C, D) Box-and-whisker plots of tail moments of Eμ-*myc* B cells (C) or MycER pro-B cells (D). The total number (n) of cells analyzed is indicated. All data are from 2-3 independent experiments. A, B) Error bars are SEM. C, D) Line is the median, boxes are the 25th and 75th percentiles, whiskers are the 5th and 95th percentiles. Chi-square test (A, B) or one-way ANOVA with Bonferroni correction (C, D) determined significance, *p<0.05, **p<0.001, ***p<0.0001.

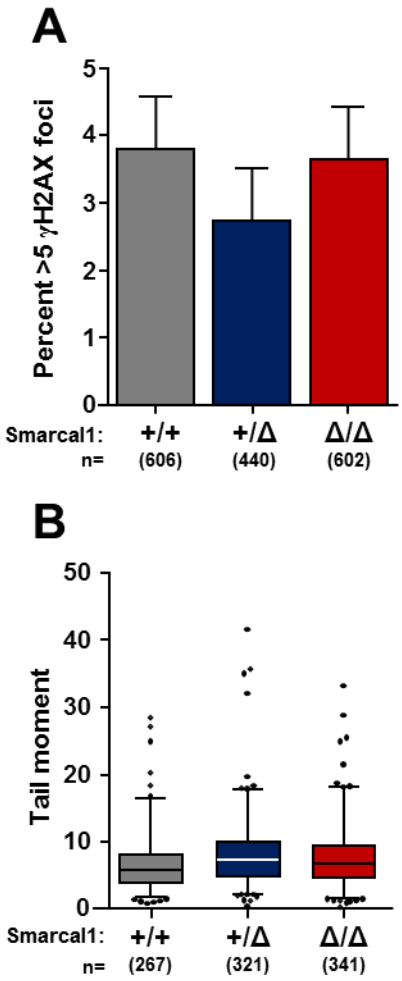

Figure 32. DNA damage is not increased in primary B cells lacking *Smarcal1* alone. B cells were purified from the spleens of *Smarcal1*$^{+/+}$, *Smarcal1*$^{+/\Delta}$, and *Smarcal1*$^{\Delta/\Delta}$ mice. A) Quantification of cells with >5 γH2AX foci. B) Box-and-whisker blots of tail moments. A) Error bars are SEM. B) Line is the median, boxes are the 25th and 75th percentiles, whiskers are the 5th and 95th percentiles. Number of cells analyzed is indicated by "n".

and fork collapse, whereas complete loss of *Smarcal1* leads to a higher rate of DNA damage and fork collapse.

Zranb3 is required for replication fork stability when Myc is overexpressed.

To determine the effects of *Zranb3* loss on replication fork stability in B cells with Myc dysregulation, we evaluated DNA fiber length, fork collapse, and DNA damage. Utilizing both Eμ-*myc* bone marrow B cells and MycER expressing pro-B cells, we observed a significant reduction in mean DNA fiber length in both *Zranb3*$^{+/-}$ and *Zranb3*$^{-/-}$ cells with Myc overexpression or MycER activation (Figure 33). Evaluation of B cells for evidence of fork collapse showed significantly higher rates of IdU-only fibers and asymmetric sister forks in the *Zranb3*$^{+/-}$ and *Zranb3*$^{-/-}$ Eμ-*myc* B cells and MycER activated pro-B cells compared to wild-type controls (Figure 34). However, unlike with Smarcal1, loss of a single allele of *Zranb3* induced the same levels of fork stalling as that in *Zranb3*$^{-/-}$ B cells, indicating a loss of both *Zranb3*alleles was no more deleterious to forks than a *Zranb3* haploinsufficiency. The increase in fork collapse in *Zranb3*-deficient B cells correlated with significantly increased rates of γH2AX foci and DNA breaks in both the Eμ-*myc* bone marrow B cells and MycER activated pro-B cells (Figure 35). The differences on DNA fiber length, fork collapse, and DNA damage/breaks with loss of Zranb3 were dependent upon Myc overexpression, as B cells lacking one or both alleles of *Zranb3* with normal levels of Myc did not show differences with wild-type controls (Figures 36, 37). Together, these data demonstrate Zranb3 is crucial for maintaining replication fork stability in the presence of dysregulated Myc. Moreover, unlike *Smarcal1*, loss of a single allele of *Zranb3* results in a phenotype

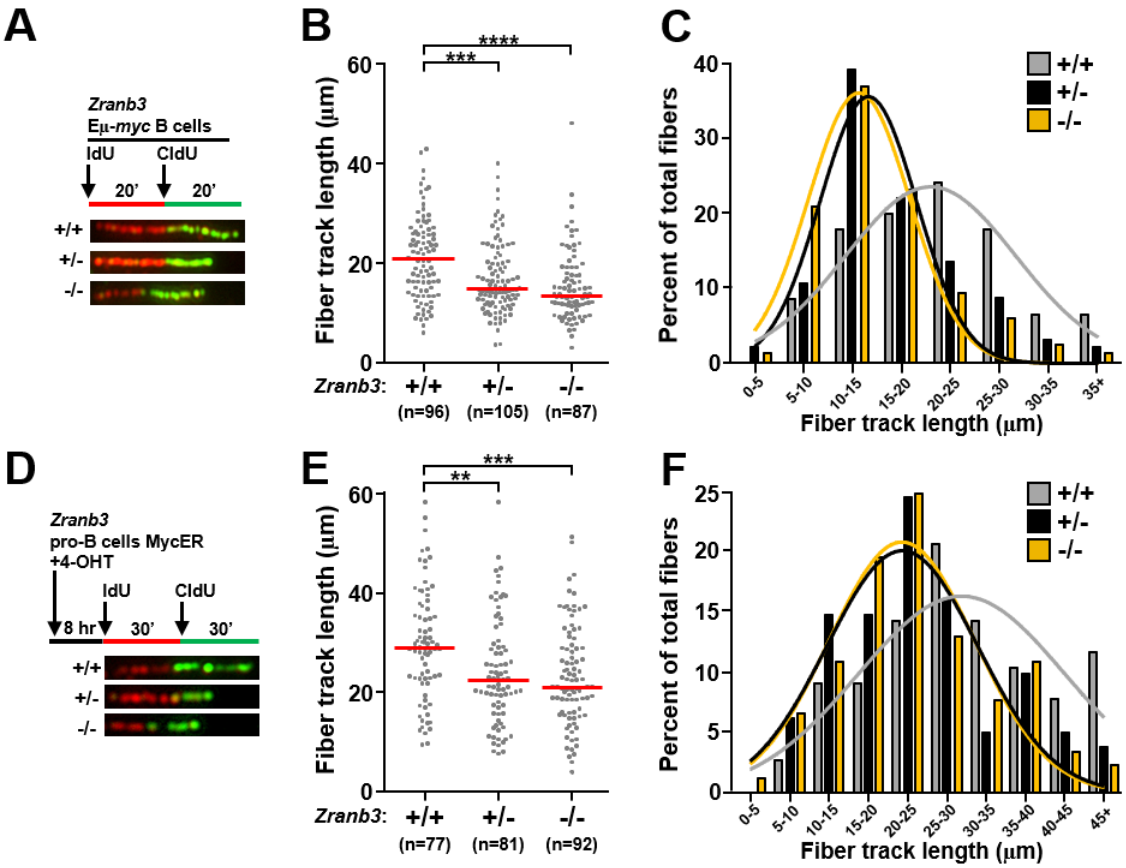

Figure 33. Replication fork progression is significantly impaired with Myc dysregulation and *Zranb3* loss. B cells were purified from the bone marrow of *Zranb3*$^{+/+}$ Eμ-*myc*, *Zranb3*$^{+/-}$ Eμ-*myc* and Zranb3$^{-/-}$ Eμ-*myc* mice (A-C). Pro-B cells from *Zranb3*$^{+/+}$, *Zranb3*$^{+/-}$ and *Zranb3*$^{-/-}$ mice expressing MycER (D-F) with MycER activated by the addition of 4-hydroxytamoxifen (4-OHT) for 8 hours. (A, D) Design of DNA fiber labeling experiments and images of representative fiber tracks. B, E) Quantification of total fiber length in purified Eμ-*myc* B cells (B) or pro-B cells (E). Experiments were performed twice, data from a representative experiment shown. C, F) Fiber length frequencies from (B) and (E), respectively. The total number (n) of fibers (B, E) is indicated. Lines are median (B, E). One-way ANOVA with Bonferroni correction (B, E) determined significance, **p<0.001, ***p<0.0001.

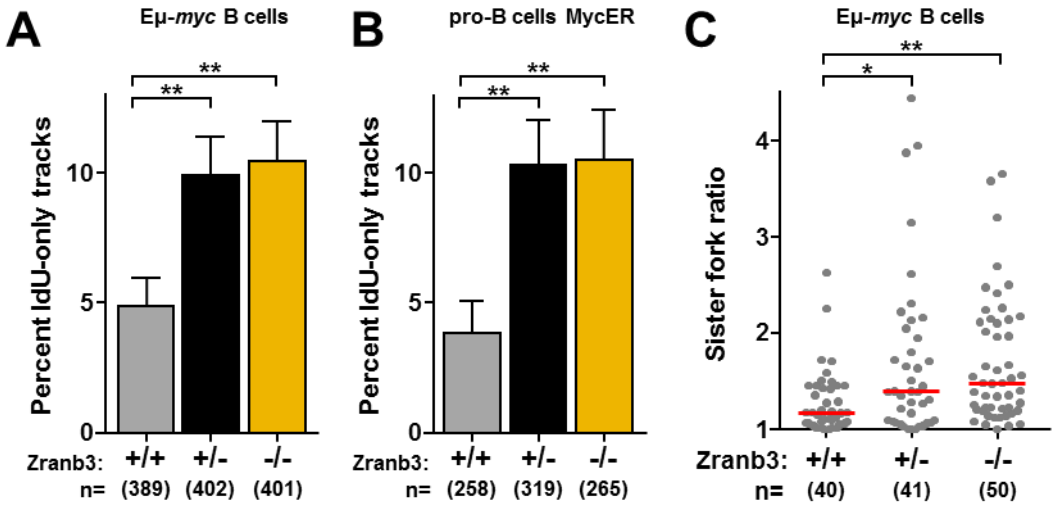

Figure 34. *Zranb3*-deficient B cells have increased rates of replication fork collapse with Myc overexpression. B cells were purified from the bone marrow of *Zranb3*$^{+/+}$ Eμ-*myc*, *Zranb3*$^{+/-}$ Eμ-*myc* and Zranb3$^{-/-}$ Eμ-*myc* mice (A, B). Pro-B cells from *Zranb3*$^{+/+}$, *Zranb3*$^{+/-}$ and *Zranb3*$^{-/-}$ mice expressing MycER (C) with MycER activated by the addition of 4-hydroxytamoxifen (4-OHT) for 8 hours. A, B) Quantification of DNA fibers that only incorporated IdU from Eμ-*myc* B cells (A) or pro-B cells (B). C) Ratios of measured CldU track lengths from left and right moving forks from the same replication origin from Eμ-*myc* B cells. The total number of replication structures analyzed is indicated. All data are from 2-3 independent experiments. A, B) Error bars are SEM. C) Median is plotted. Chi-square test (A, B) or one-way ANOVA with Bonferroni correction determined significance, *p<0.05, **p<0.01.

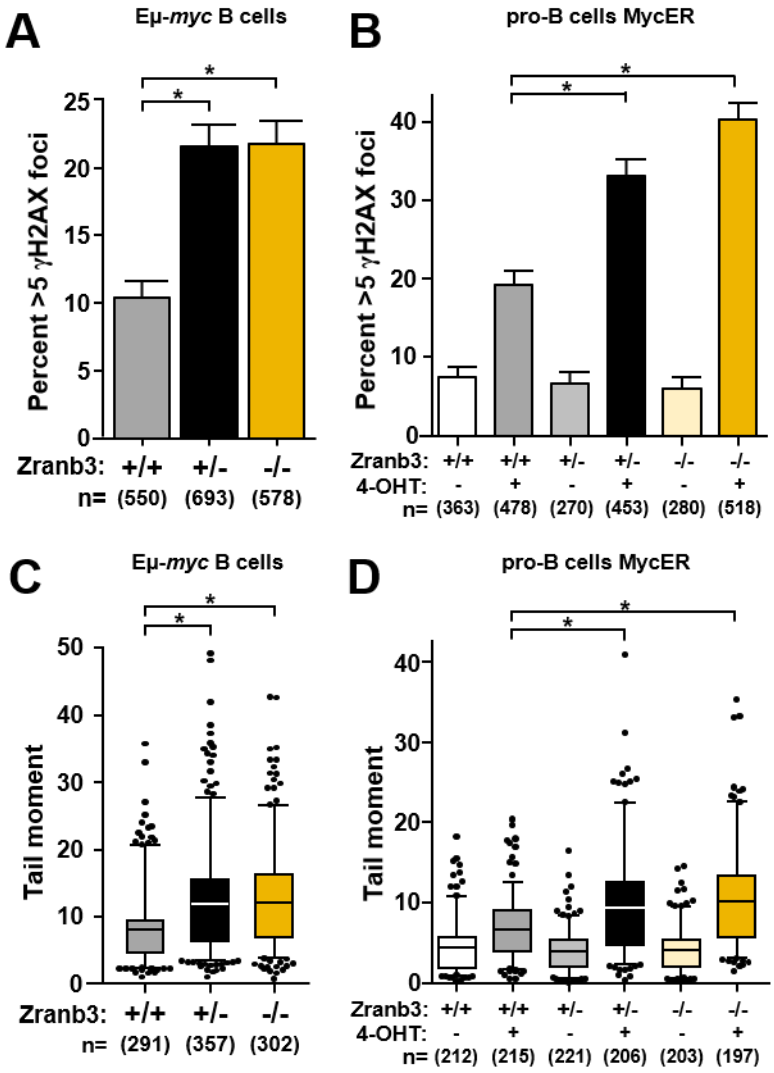

Figure 35. *Zranb3*-deficient B cells overexpressing Myc have increased DNA damage. B cells were purified from the bone marrow of *Zranb3*$^{+/+}$ Eμ-*myc*, *Zranb3*$^{+/-}$ Eμ-*myc* and Zranb3$^{-/-}$ Eμ-*myc* mice (A, B). Pro-B cells from *Zranb3*$^{+/+}$, *Zranb3*$^{+/-}$ and *Zranb3*$^{-/-}$ mice expressing MycER (C, D) with MycER activated by the addition of 4-hydroxytamoxifen (4-OHT) for 8 hours. A, B) Quantification of Eμ-*myc* B cells (A) or pro-B cells (B) with >5 γH2AX foci. C, D) Box-and-whisker plots of tail moments of Eμ-*myc* B cells (C) or pro-B cells (D). The total number (n) of cells analyzed is indicated. All data are from 2-3 independent experiments. A, B) Error bars are SEM. C, D) Line is the median, boxes are the 25th and 75th percentiles, whiskers are the 5th and 95th percentiles. Chi-square test (A, B) or one-way ANOVA with Bonferroni correction determined significance (C, D), *p<0.0001.

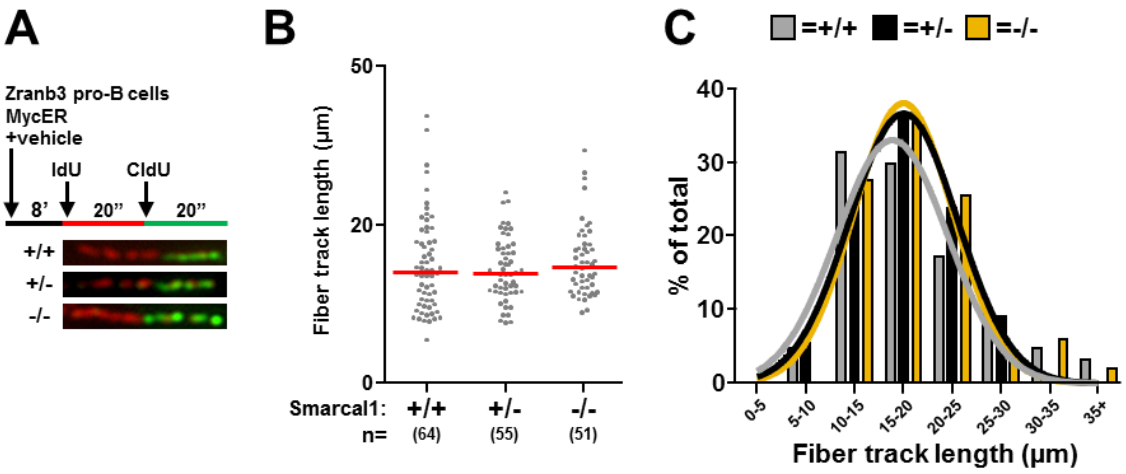

Figure 36. *Zranb3* loss alone does not alter DNA replication fork kinetics in B cells. Bone marrow derived pro-B cells of the indicated genotypes were retrovirally transduced with MSCV-MycER-IRES-GFP and treated with an ethanol vehicle control. **A)** Schematic outlining the DNA fiber labeling experiment and images of representative fiber tracks. **B)** Quantification of fiber track lengths from cells of the indicated genotypes. **C)** Fiber track length frequencies of the measurements in (B). The total number of fibers measured (B) is indicated by "n". Data from one of two representative experiments.

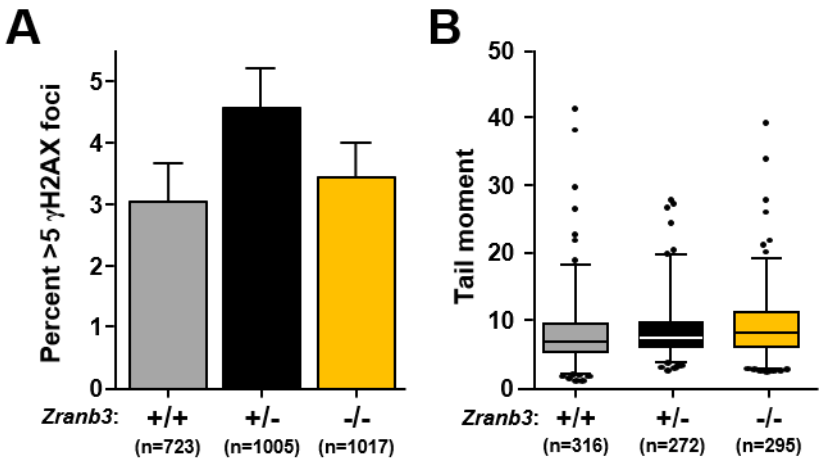

Figure 37. Loss of Zranb3 alone does not alter DNA damage levels in primary B cells . B cells were purified from the spleens of *Zranb3*$^{+/+}$, *Zranb3*$^{+/-}$, and *Zranb3*$^{-/-}$ mice. A) Quantification of cells with >5 γH2AX foci. B) Box-and-whisker blots of tail moments. A) Error bars are SEM. B) Line is the median, boxes are the 25th and 75th percentiles, whiskers are the 5th and 95th percentiles. Number of cells analyzed is indicated by "n".

equally as severe as the *Zranb3*-null phenotype, suggesting a more critical biological function for Zranb3 in resolving oncogene-induced replication stress. Furthermore, these data demonstrate that Smarcal1 and Zranb3 are unable to biologically compensate for one another, as loss of either protein led to significant replication defects.

***Smarcal1*- and *Zranb3*-deficient Eμ-*myc* mice have reduced numbers of B cells.**

To determine the biological consequences of elevated fork collapse and DNA breaks due to Myc overexpression in *Smarcal1*-deficient and *Zranb3*-deficient B cells, we evaluated B-cell populations in mice from both colonies. We first evaluated hematopoietic organs in 6-8 week-old, non-Eμ-*myc* transgenic mice to determine if loss of either protein alone altered B-cell populations *in vivo*. *Smarcal1*-deficient and *Zranb3*-deficient mice displayed no overt B-cell phenotypes in either the spleen or the bone marrow. Total splenic cellularity was unchanged between all genotypes (Figure 38A) as was the total percentages of B cells in the spleen (Figure 38B). Moreover, loss of *Zranb3* or *Smarcal1* did not affect the ability of bone marrow cells to differentiate into pro-B cells *ex vitro* (Figure 39). Although it is reported that Smarcal1 loss results in a mild reduction in B cells in mice (Baradaran-Heravi et al. 2012), our data indicate loss of either Smarcal1 or Zranb3 alone is insufficient to alter B-cell development or total B-cell numbers in young, unstressed animals.

Since *Smarcal1*-deficient and *Zranb3*-deficient Eμ-*myc* mice had altered rates of B-cell lymphoma development and their loss leads to replication fork collapse and the accumulation of DNA damage in primary B cells, we questioned whether Myc overexpression in *Smarcal1*-deficient and *Zranb3*-deficient mice would result in changes

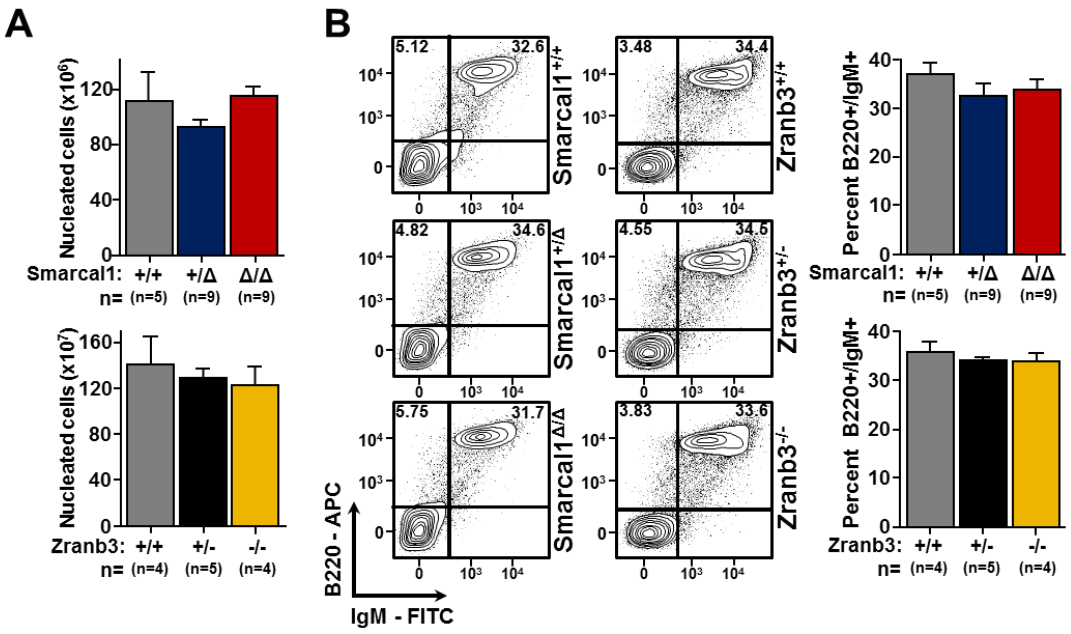

Figure 38. *Smarcal1*- or *Zranb3*-deficiency alone does not alter B-cell populations in the spleen. A) Quantification of total nucleated cells in the spleens of young, *Smarcal1*$^{+/+}$, *Smarcal1*$^{+/\Delta}$, *Smarcal1*$^{\Delta/\Delta}$, *Zranb3*$^{+/+}$, *Zranb3*$^{+/-}$ and *Zranb3*$^{-/-}$ mice. B) Representative contour plots of B-cell populations in the spleen (left) and quantification of the total percentage of immature/mature (B220+/IgM+) B cells in the spleens of the animals in (A).

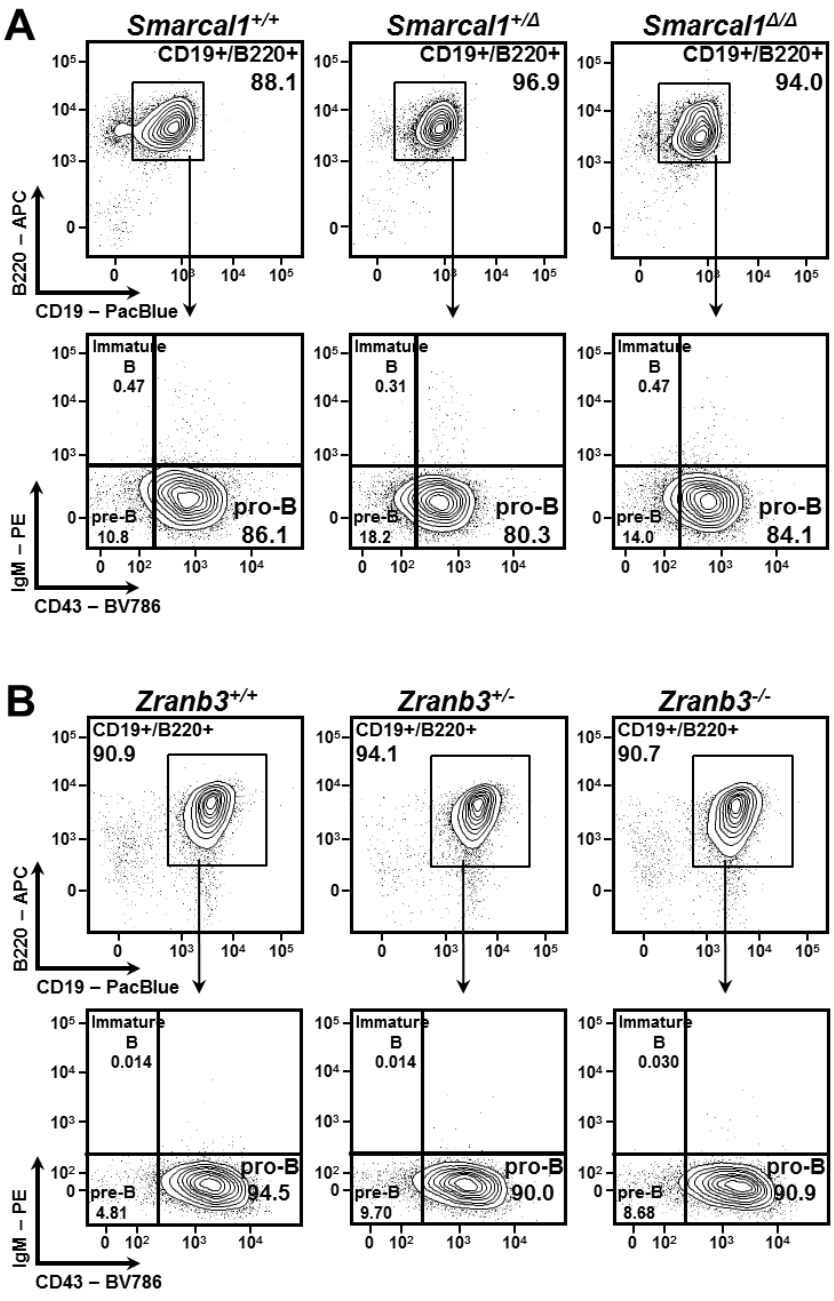

Figure 39. Loss of *Smarcal1* or *Zranb3* alone does not affect B-cell differentiation into pro-B cells *ex-vivo*. Representative contour plots of specific B cell populations from bone marrow-derived pro-B-cell cultures derived from mice from the *Smarcal1*(A) and *Zranb3* (B) colonies.

in B cell numbers. *Smarcal1*$^{+/\Delta}$ and *Smarcal1*$^{\Delta/\Delta}$ Eμ-*myc* mice did not show any difference in total splenocyte numbers (Figures 40A) but *Smarcal1*$^{\Delta/\Delta}$ Eμ-*myc* mice had approximately one-third fewer mature B cells when compared to either *Smarcal1*$^{+/\Delta}$ and wild-type Eμ-*myc* littermates (Figure 40B). In contrast, *Zranb3*$^{+/-}$ and *Zranb3*$^{-/-}$ Eμ-*myc* mice had an approximately 10-20% reduction in total splenic cellularity (Figure 40A), and also showed a significant reduction in total splenic mature B cells compared to wild-type controls (Figure 40B). Taken together, these data suggest complete loss of *Smarcal1* and loss of only a single allele of *Zranb3* is sufficient to sensitize B cells to Myc overexpression, which results in a reduction in total B splenocytes that is not observed in wild-type or *Smarcal1*$^{+/\Delta}$ littermates.

***Smarcal1*- and *Zranb3*-deficient Eμ-*myc* mice have altered B-cell proliferation.**

We next assessed whether the reductions in B cells in *Smarcal1*$^{\Delta/\Delta}$, *Zranb3*$^{+/-}$ and *Zranb3*$^{-/-}$ Eμ-*myc* mice were due to alterations in proliferation and/or sensitivity to Myc-induced apoptosis. Splenic B cells from *Smarcal1*$^{\Delta/\Delta}$ Eμ-*myc* mice incorporated significantly less BrdU compared to wild-type and *Smarcal1*$^{+/\Delta}$ Eμ-*myc* littermates, which were statistically identical (Figure 41). Notably, neither *Zranb3*$^{+/-}$ Eμ-*myc* nor *Zranb3*$^{-/-}$ Eμ-*myc* B cells had differences in cell cycle or BrdU incorporation compared to wild-type Eμ-*myc* littermates (Figure 41). Lack of either *Smarcal1* or *Zranb3* alone without Myc overexpression had no effect on B-cell proliferation or BrdU incorporation (Figure 42). Thus, loss of both alleles of *Smarcal1* leads to reduced proliferation rates in B cells overexpressing Myc; however this does not occur with loss of single allele of *Smarcal1* or one or both copies of *Zranb3* or in the absence of Myc overexpression.

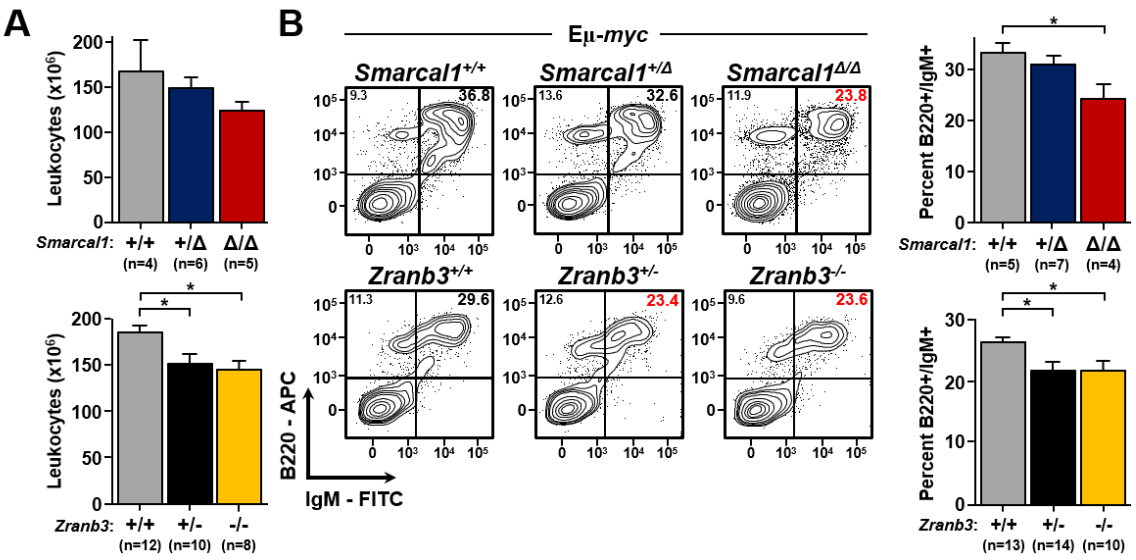

Figure 40. *Smarcal1*$^{\Delta/\Delta}$ Eμ-*myc*, *Zranb3*$^{+/-}$ Eμ-*myc* and *Zranb3*$^{-/-}$ Eμ-*myc* mice have reduced numbers of B cells in the spleen. A) Quantification of total nucleated cells in the spleens of Eμ-*myc* mice of the indicated genotypes. B) Representative contour plots (left) and quantification (right) of mature (IgM+/B220+) B cells from Eμ-*myc* littermates of the indicated genotypes. All data from three independent litters. A, B) Error bars are SEM. Significance determined with one-way ANOVA with Bonferroni correction. *p<0.05.

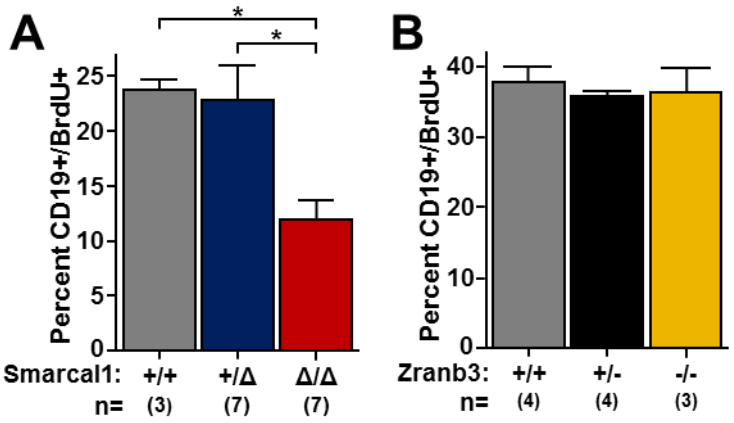

Figure 41. Loss of *Smarcal1* reduces B-cell proliferation, whereas loss of *Zranb3* does not when Myc is overexpressed. Quantification of CD19+/BrdU+ Eμ-*myc* splenocytes of the indicated genotypes. Total number (n) of mice indicated. Error bars are S.E.M; *p<0.05, one-way ANOVA with Bonferroni post-test (A, B).

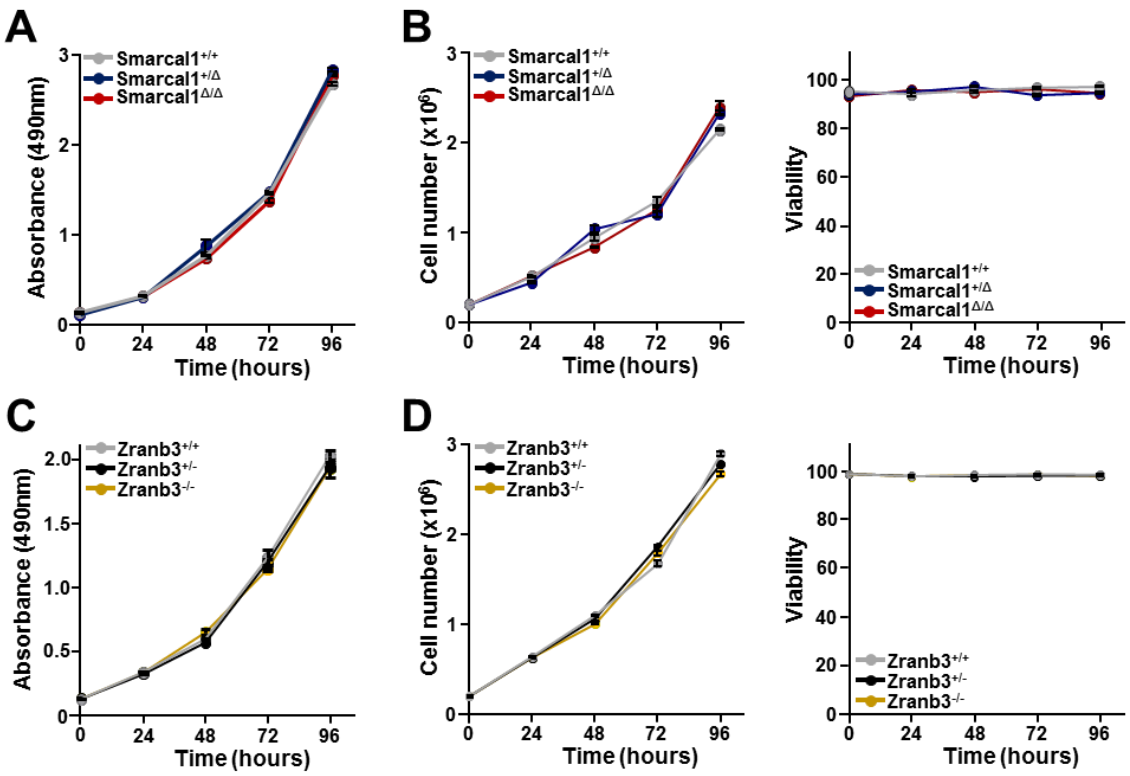

Figure 42. Loss of *Smarcal1* or *Zranb3* does not affect pro-B-cell growth. pro-B-cell cultures were derived from bone marrow from mice of the indicated genotype. A, C) MTS growth assays were performed at the listed intervals. C, D) Trypan blue dye exclusion was used to determine total cell viable cell number (left) and total viability (right) at 24 hour intervals.

Loss of *Smarcal1* or *Zranb3* sensitizes B cells to Myc-induced apoptosis.

Myc overexpression induces a robust apoptotic response that serves as a barrier to transformation (Tansey 2014). Since we detected reductions in B cell numbers and increased DNA breaks in Eμ-*myc* mice deficient in *Smarcal1* or *Zranb3*, we evaluated apoptosis. Because apoptotic B cells *in vivo* are cleared quickly and death can be caused independent of Myc, we utilized our MycER inducible system in pro-B cells *ex vivo*. Following the addition of 4-OHT to activate MycER, there was a significant decrease of cell expansion (Figure 43A), cell number (Figure 43B), and viability (Figure 43B) of *Smarcal1*$^{+/\Delta}$ and *Smarcal1*$^{\Delta/\Delta}$ B cells relative to wild-type B cells. There were also increased numbers of B cells lacking Smarcal1 that were Annexin-V positive (Figure 43C), that contained sub-G1 DNA (Figure 43D), and that showed increased cleaved caspase 3 (Figure 43E) following MycER activation. The effects on *Smarcal1*$^{+/\Delta}$ B cells were intermediate between wild-type and null B cells, indicating a degree of retained Smarcal1 function in the heterozygous mice. Loss of one or both alleles of *Zranb3* also lead to significantly decreased cell expansion (Figure 44A), cell number (Figure 44B) and viability (Figure 44B) following MycER activation. *Zranb3*-deficiency also resulted in increased Annexin-V (Figure 44C), sub-G1 DNA (Figure 44D), and cleaved caspase 3 (Figure 44E) with MycER activation. However, in contrast to *Smarcal1*, the effects of MycER activation in *Zranb3*$^{+/-}$ B cells were similar to that in *Zranb3*$^{-/-}$ B cells. These results provide additional evidence that loss of a single *Zranb3* allele is profoundly deleterious to B cells overexpressing Myc, whereas Myc overexpressing B cells are impacted, but can tolerate loss of one allele of *Smarcal1* providing fertile ground for transformation.

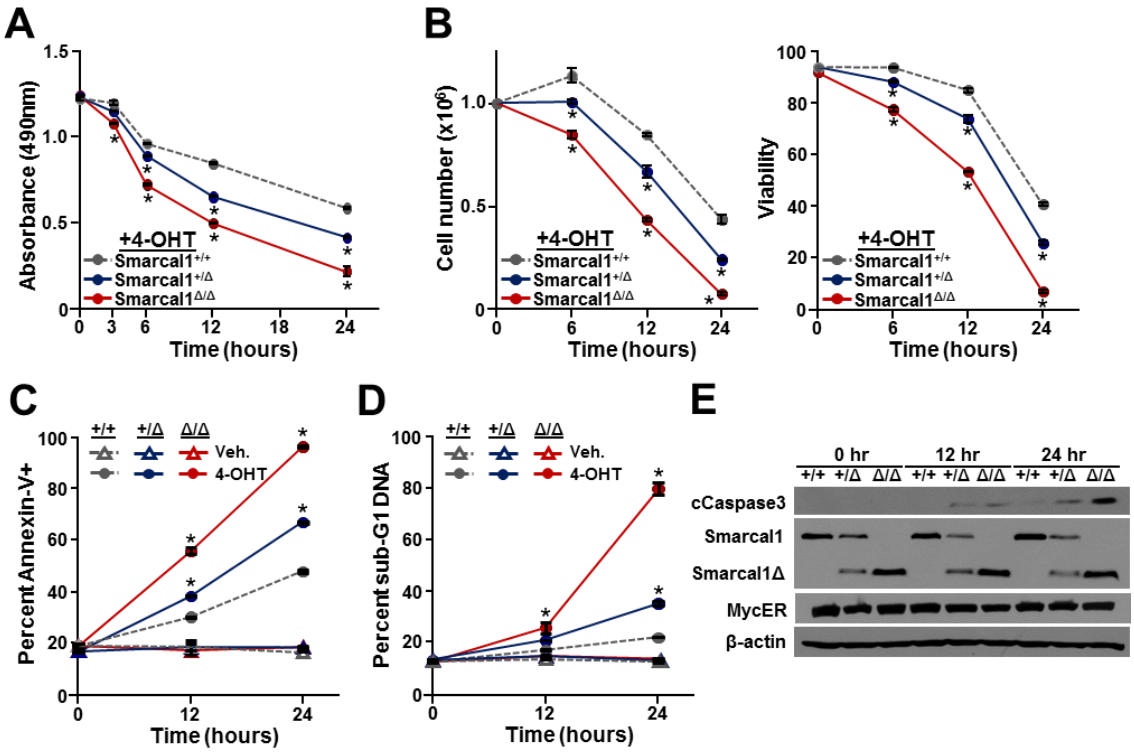

Figure 43. Loss of *Smarcal1* sensitizes B cells to Myc-induced apoptosis. pro-B cells expressing MycER of the indicated genotypes. MycER was activated with 4-hydroxytamoxifen (4-OHT) at intervals. MTS assays (A), cell numbers (B), viability (B), Annexin-V positivity (C), and sub-G1 DNA content (D). E) Western blots of whole cell lysates for the indicated proteins at intervals after activation of Myc with 4-OHT. Student's t-tests determined significance, *p<0.0001.

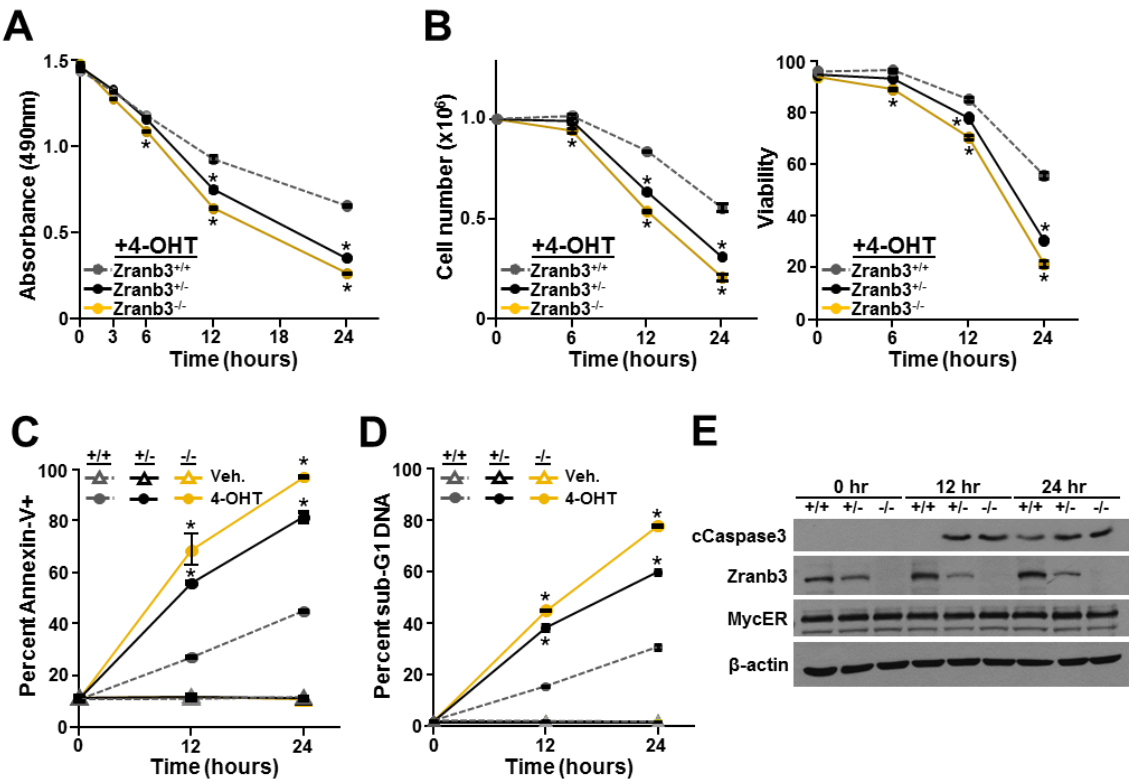

Figure 44. Loss of *Zranb3* sensitizes B cells to Myc-induced apoptosis. pro-B cells expressing MycER of the indicated genotypes. MycER was activated with 4-hydroxytamoxifen (4-OHT) at intervals. MTS assays (A), cell numbers (B), viability (B), Annexin-V positivity (C), and sub-G1 DNA content (D). E) Western blots of whole cell lysates for the indicated proteins at intervals after activation of Myc with 4-OHT. Student's t-tests determined significance, *p<0.0001.

Discussion

The DNA replication stress response is an essential mechanism employed by cells to protect replication forks and facilitate successful DNA synthesis (Zeman and Cimprich 2014). However, while significant strides have been made in understanding the biochemical aspects of this process, the biological importance and consequences of specific proteins that mediate fork reversal, particularly in physiological conditions, remained unknown. In this study utilizing primary cells and mouse models, our data reveal that both Smarcal1 and Zranb3 function are essential and non-redundant for mediating DNA replication stress and stabilizing replication forks during oncogene dysregulation. Biologically, we demonstrate in mice the consequence of loss of either *Smarcal1* or *Zranb3* profoundly altered Myc-driven B-cell lymphomagenesis and revealed that gene dosage had a significant impact. Zranb3 haploinsufficiency inhibited Myc-induced lymphoma development and increased survival, whereas loss of one *Smarcal1* allele accelerated lymphomagenesis and decreased survival. Our data reveal levels of Smarcal1 and Zranb3 have unique roles in stabilizing forks and preventing DNA breaks and apoptosis during oncogenic replication stress *in vivo* that was not previously known.

Prior to this current study, no endogenous sources of replication stress had been reported to require Zranb3 and telomeric sequences were the only known source of endogenous stress requiring Smarcal1 (Poole et al. 2015, Cox et al. 2016). Here, we identified Myc overexpression as an endogenous source of replication stress that required both Smarcal1 and Zranb3 for replication fork stability. Notably, our data indicate their functions are not redundant in that neither Smarcal1 nor Zranb3 could compensate for the other when one was lost in the presence of Myc overexpression. Moreover, the different

biological results that were observed in Eμ-*myc* mice and B cells from them with a *Smarcal1*-deficiency compared to a *Zranb3*-deficiency indicate distinct functions for these proteins in resolving the type of replication stress caused by an oncogene. Thus, while loss of Smarcal1 or Zranb3 negatively impacted fork stability during oncogenic stress, they appear to have unique biological functions in promoting fork repair and restart during this particular type of stress.

Our results significantly increase understanding of the mechanisms and the proteins required for fork stability during oncogene-induced replication stress. Previously, the role of the Wrn helicase was assessed in Eμ-*myc* mice (Moser et al. 2012). Wrn functions to resolve unfavorable structures upstream from the replication fork, whereas Smarcal1 and Zranb3 drive fork regression and restoration (Grandori et al. 2003, Poole and Cortez 2017). Similar to our results with the *Zranb3* knockout Eμ-*myc* mice, Eμ-*myc* mice with hypomorphic *Wrn* alleles also had a delayed lymphoma (Moser et al. 2012). Interestingly, cell cycle progression appears to contribute to these phenotypes. Zranb3 loss in Eμ-*myc* mice did not alter the cell cycle of the B cells, but did delay lymphomagenesis due to increased B-cell apoptosis. *Smarcal1*-null Eμ-*myc* mice showed significantly reduced numbers of B cells in S-phase and increased B-cell apoptosis, but no alteration in lymphoma development. Thus, future studies will be important to discern the specific biological differences that require multiple fork protection factors that are expressed in eukaryotic cells and determine the different effects their loss has on biological processes like cell cycle progression and apoptosis.

In addition, our data reveal there are different requirements for Smarcal1, and likely Zranb3, with different replication stresses *in vivo*. Specifically, we previously reported that

loss of one or both alleles of *Smarcal1* inhibited gamma radiation-induced DNA replication stress-mediated T cell lymphoma development. In this model, radiation stimulates massive hematopoietic stem and progenitor cell proliferation and acutely generates high levels of replication stress in these cells, resulting in T-cell lymphomagenesis. In contrast, Eμ-*myc* mice have a constitutively increased level of Myc (3-4 fold) in B cells causing chronic replication stress in B cells, resulting in an acceleration of B-cell lymphomagenesis with loss of one *Smarcal1* allele and no change in the rate of B-cell lymphoma development in *Smarcal1*-null Eμ-*myc* mice. In the radiation model, loss of a single allele of *Smarcal1* was sufficient to confer profound sensitivity to hematopoietic cells to this acute replication stress, causing significant apoptosis of cycling cells. However, with Myc overexpression, *Smarcal1* haploinsufficiency resulted in a mild cellular phenotype in B cells with a modest increase in apoptosis and small changes in replication fork stability and DNA damage, which resulted in increased B-cell transformation. The significant differences in the biological effects in these two studies uncover DNA replication stress-specific and/or cellular context-dependent functions of Smarcal1. Future studies investigating the differences between acute and chronic replication stress would further define the precise physiological settings that require Smarcal1 for fork protection and begin to characterize the differences Zranb3 has in relationship to Smarcal1.

Overall, our data significantly advance understanding of the closely related proteins, Smarcal1 and Zranb3, by revealing their essential, non-redundant *in vivo* function in replication fork stability during oncogenic stress and their contribution to Myc-driven tumor development. In addition, our data suggest that Smarcal1 and Zranb3 are likely to be important in other Myc-driven malignancies, and could provide a therapeutic

opportunity. Since Myc is dysregulated in at least 70% of human cancers, they may also rely on Smarcal1 and Zranb3 to stabilize forks and complete replication. Developing inhibitors against these proteins, particularly Zranb3, could provide therapeutic benefit. Additionally, they may be useful in synthetic lethal approaches combined with replication-stress inducing drugs to target Myc-driven malignancies. Further studies investigating these approaches are needed to determine the therapeutic efficacy of drugging fork remodelers in Myc-driven cancers.

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

Ensuring accurate and complete genome duplication in the face of the numerous replication obstacles that occur during S-phase is an essential facet of DNA replication. DNA replication stress is a complex cellular event and has significant implications for genomic stability, cellular viability and human disease. Since its identification as a driver of genomic instability and tumorigenesis (Bartkova et al. 2006, Di Micco et al. 2006, Halazonetis et al. 2008, Gaillard et al. 2015), replication stress has been intimately linked to cancer as both an initiator of transformation and potential therapeutic target in transformed cells. However, the cellular replication stress response is incredibly complex and relies upon dozens of proteins to sense, signal, coordinate and implement the biological response to replication stress. The physical protection of the replication fork itself is a critical component of the replication stress response as fork collapse can lead to the under-replication of DNA as well as genomic instability via the propensity of stalled forks to be processed into genotoxic dsDNA breaks. Eukaryotic cells express a multitude of proteins which function to stabilize and repair stalled replication forks and prevent replication fork collapse (Zeman and Cimprich 2014). However, our understanding of the biological mechanisms of how these proteins function, and their roles in tumorigenesis, remains incomplete.

During my thesis, I evaluated two closely related replication stress response proteins, Smarcal1 and Zranb3. These homologous proteins catalyze similar biochemical

reactions *in vitro*, however little was known regarding their biological functions under physiological conditions *in vivo*. Through the use of mouse models, I performed the first evaluations of these proteins *in vivo* and characterized their contribution to two types of murine lymphoma.

First, through the use of an irradiation model of T-cell lymphomagenesis, I determined that Smarcal1 is a critical factor required for hematopoietic cell viability during forced proliferation. Both T cells and HSPCs lacking one or both alleles of *Smarcal1* were highly sensitive to radiation-induced replication stress and underwent apoptosis during the recovery phase from radiation due to increased DNA damage. This protected mice from lymphoma development, as this reduction in HSPCs decreased the pool of cells with the potential to undergo transformation, thus leading to delayed lymphoma onset and increased overall survival (Puccetti et al. 2017).

I further evaluated both Smarcal1 and Zranb3 in a Myc-induced model of B-cell lymphomagenesis. This study was the first to directly compare these homologous proteins *in vivo* using an endogenous source of replication stress. My data revealed mice and primary cells require both Smarcal1 and Zranb3 to resolve Myc-induced replication stress and that these proteins function to stabilize replication forks in a non-redundant manner. Moreover, deficiencies in either protein had distinct biological effects as haploinsufficiency of *Smarcal1* accelerated Myc-driven tumor development while *Zranb3* haploinsufficiency inhibited tumor development. Moreover, loss of *Smarcal1* did not affect B-cell lymphomagenesis, while *Zranb3* loss inhibited it. Thus, these data identified a novel source of endogenous replication stress that requires both Smarcal1 and Zranb3 and

demonstrated that both proteins function to stabilize and repair replication forks in a non-redundant manner.

Below I will discuss how my data increases our knowledge of the replication stress response and furthers our understanding of its role in tumorigenesis. I will also describe the biological implications of my data and future directions for additional studies concerning Smarcal1 and Zranb3.

Smarcal1 loss inhibits radiation-induced T-cell lymphomagenesis

The repeated exposure of young mice to low-dose, whole body radiation induces T-cell lymphoma development (Kaplan and Brown 1952). Previous studies have suggested the tumor cell of origin in this model is an HSPC and tumor formation occurs when radiation-induced depletion of peripheral lymphocytes serves as a biological stimulus to drive HSPCs out of quiescence to rapidly repopulate the depleted lymphoid organs (Kominami and Niwa 2006). During this process, an HSPC harboring IR-induced mutations undergoes transformation leading to lymphoma development. Evidence of the HSPC cell-of-origin hypothesis is provided by experiments where shielding of the femora during irradiation and blocking of thymocyte apoptosis (which prevents HSPC proliferation/repopulation) inhibits lymphoma formation (Sado et al. 1991, Kominami and Niwa 2006, Labi et al. 2010, Michalak et al. 2010). Moreover, implantation of an unirradiated thymus into the kidney capsule of irradiated mice leads to lymphoma formation in the thymic graft, suggesting the neoplastic cell of origin originates elsewhere and then migrates to the thymus (Kaplan et al. 1956, Humblet et al. 1989).

Since physiologic stresses that induce HSPC proliferation are a known source of replication stress (Flach et al. 2014, Alvarez et al. 2015, Walter et al. 2015, Flach and Milyavsky 2018), we hypothesized that low-dose radiation exposure would generate significant replication stress in cycling HSPCs. Indeed, as described in Chapter II, we observed DNA damage and apoptosis in wild-type HSPCs during the repopulation of the thymus. However, specific replication fork protection factors had never been studied in this context and it was unknown whether fork remodeling proteins contributed to fork protection during radiation-induced replication stress in HSPCs. Since Smarcal1 had been identified as a novel protein involved in fork repair and restart, we questioned whether Smarcal1 contributed to radiation-induced T-cell lymphoma development.

Interestingly, we observed significant inhibition in lymphomagenesis in mice lacking one or both alleles of *Smarcal1* (Puccetti et al. 2017). We determined this was due to elevated rates of DNA damage and apoptosis of cycling HSPCs and thymic progenitors. These data were the first in identifying a biological role of Smarcal1 in hematopoietic cells *in vivo* while also showing, for the first time, that its absence can alter tumor development phenotypes. Prior to the publishing of this work, replication stress sensitivity had only been identified in *Smarcal1*-deficient, cancer cell lines *in vitro*. We demonstrated when HSPCs are forced to proliferate *in vivo*, Smarcal1 is required to prevent DNA damage and cell death.

Moreover, our data strengthen the hypothesis that HSPCs are the cell of origin in radiation-induced T-cell lymphomagenesis. While previous studies had modulated HSPC biology during the response to radiation (i.e. by preventing IR-induced mutations via femoral shielding and by blocking their exit from quiescence by inhibiting thymocyte

apoptosis), we observed increased apoptosis of HSPCs lacking one or both alleles of *Smarcal1* themselves during forced proliferation. We determined this was not due to alterations in initial radiation sensitivity or changes in proliferation rates, but rather was a consequence of increased rates of DNA damage in cycling, *Smarcal1*-deficient HSPCs. Thus, our data help further the understanding of proteins required for fork protection in hematopoietic cells while also providing additional understanding of the pathogenesis of radiation-induced lymphoma development.

Our findings also have significant implications for understanding the pathophysiology of SIOD. Progressive immunodeficiency is a hallmark of SIOD and severe, recurrent infections are a significant cause of death in SIOD patients (see Chapter I). Our data provide a possible explanation for the physiologic mechanism of immune cell loss in SIOD patients. With *Smarcal1*-deficiency, repeatedly forcing HSPCs to proliferate with radiation exposure resulted in stem cell attrition (Puccetti et al. 2017). In SIOD patients, we propose that routine childhood infections induce immune cell and HSPC proliferation as part of the normal physiologic response to infection. This induces replication stress which likely requires SMARCAL1 for resolution. Lack of SMARCAL1 function would then lead to increased fork collapse, DNA damage and immune cell apoptosis. This deficiency would result in a further stimulus for HSPC proliferation to replenish these depleted cells, leading to additional HSPC replication stress and cell death. Thus, like with repeated rounds of radiation exposure in mice, recurring infections in SIOD patients may lead to a negative feedback-loop-like process where continual immune cell proliferation results in cell death and a stimulus for additional HSPC proliferation, which leads to further cell attrition, immunodeficiency and susceptibility to infection. While the

potential biological parallel between radiation exposure and recurrent infection offers an enticing explanation for the physiological mechanism of immunodeficiency in SIOD patients, further experimentation is required to confirm the validity of this hypothesis.

In addition to radiation, a plethora of other stimuli are able to induce HSPC proliferation including anemia caused by serial bleeding and inflammatory and pro-proliferative cytokines (Cheshier et al. 2007, Essers et al. 2009, Baldridge et al. 2010, Takizawa et al. 2011, Itkin et al. 2012, An et al. 2015, Guryanova et al. 2016). The subsequent exit from quiescence caused by these stimuli induces HSPC replication stress (Walter et al. 2015). While our data show that Smarcal1 is required to resolve HSPC replication stress caused by radiation exposure, it also begs the question whether this phenotype is a result of a specific characteristic(s) of radiation or whether Smarcal1 is a general fork protection factor in HSPCs and resolves replication stress induced by other sources as well. Fork collapse and impaired replication have been observed in *SMARCAL1*-deficient cancer cell lines *in vitro* upon treatment with HU and other drugs (Bansbach et al. 2009, Ciccia et al. 2009, Postow et al. 2009, Yuan et al. 2009, Yusufzai et al. 2009, Walter et al. 2015), which provides some evidence that SMARCAL1 is required for mediating replication stress caused by different exogenous sources *in vitro*. Moreover, our data show that in addition to radiation, 5-FU also impairs HSPC biology *in vivo*. However, these experiments all rely upon exogenous stimuli that are not a part of normal cellular physiology and may not be representative of the types of replication stress that contribute to human disease. Thus, it is of critical importance to identify the endogenous biological sources of replication stress, such as oncogenic stress, that may require Smarcal1 and to determine whether or not they have specific effects in different cell populations.

While this study shows that loss of *Smarcal1* has a protective effect against tumor development, it is important to put our data in the context of the published literature. Proteins at the replication fork have complex biological interactions with one another and losses of different combinations of proteins can influence cellular phenotypes. For instance, two recent publications have identified a link between SMARCAL1 and BRCA1/2. Upon replication stress and SMARCAL1-mediated fork reversal, BRCA1 and BRCA2 function in conjunction with the RAD51 recombinase to stabilize the reversed fork and protect it from nucleolytic cleavage (Kolinjivadi et al. 2017, Taglialatela et al. 2017). In BRCA1- or BRCA2-deficient cells, SMARCAL1-mediated fork reversal results in DNA damage and genomic instability caused by MRE11-induced fork degradation, as forks are no longer protected by RAD51. However, depletion of SMARCAL1 rescues this phenotype, restores fork stability and prevents DNA damage. Moreover, cells deficient in both BRCA1/2-and SMARCAL1 actually show *increased* cell survival upon treatment with replication stress-inducing drugs compared to knockdown of either protein alone.

Thus, in some instances, like radiation-induced lymphomagenesis, *Smarcal1* loss leads to DNA damage and cell death as cells are reliant upon its fork remodeling functions to survive replication stress. In other contexts however, such as with *BRCA1* or *BRCA2* loss, SMARCAL1-deficiency actually promotes genome stability and cell survival. Thus, the contributions of SMARCAL1 to tumor development are likely complex and context dependent. Future studies are needed to further elucidate the biological interactions of SMARCAL1 with other proteins involved in replication fork protection and how these interactions influence fork stability and tumor development.

The contribution of replication fork remodeling proteins to Myc-driven lymphomagenesis

The radiation-induced model of T-cell lymphomagenesis is an acute model of replication stress, where exposure to an exogenous stimulus elicits acute cellular responses. While replication stress can certainly be an acute event, as it is with the addition of drugs like HU, it can also occur as a more chronic pressure in physiological settings. Chronic replication stress is associated with oncogene activation, which has been proposed to be a driver of genomic instability and transformation. Specifically, a model has emerged where oncogene dysregulation occurs early in the process of neoplastic transformation. This in turn generates significant replication stress, DNA damage and genomic instability. Cells respond to this stress by undergoing apoptosis or senescence, which functions as a tumor suppressive barrier. This response is mainly mediated through the p53 pathway and results in significant pressure to inactivate p53 in order bypass the apoptotic or senescent response that is inhibiting tumorigenesis (Bartkova et al. 2006, Di Micco et al. 2006, Halazonetis et al. 2008). However, there is still much to be known regarding specific proteins and their contribution to this barrier.

In order to determine if fork remodeling proteins are involved in the cellular response to chronic replication stress, we examined the contribution of *Smarcal1* and *Zranb3* to Myc-mediated lymphomagenesis. As described in Chapter III, we determined that both Smarcal1 and Zranb3 function to stabilize replication forks during Myc overexpression and do so in a non-redundant fashion. Moreover, we observed gene dosage effects on overall survival with loss of either protein. Interestingly, loss of a single allele of *Smarcal1* accelerated tumor development whereas complete loss of *Smarcal1* did not

affect overall survival. Conversely, loss of even a single allele of *Zranb3* inhibited Myc-driven lymphoma development. Thus, these divergent survival phenotypes suggest both proteins have unique, non-redundant roles in mediating fork protection during oncogene-induced replication stress.

It is interesting to compare the results of the Smarcal1-Myc experiments with those of the radiation-induced study. While recognizing the differences both in cell type and mechanism of replication stress induction, it is intriguing that *Smarcal1* loss had profoundly different effects on tumor development in both of these model systems. Our data provide some evidence that either the source, duration (acute vs. chronic) or a combination of both affect how Smarcal1 protects the replication fork during replication stress.

The phenotypes observed in the heterozygous mice are particularly interesting. In the radiation model, loss of single allele of *Smarcal1* was sufficient to confer sensitivity to both HSPCs and developing thymocytes to acute replication stress. These cells underwent apoptosis due to elevated levels of fork collapse and DNA damage. However, with Myc overexpression, loss of a single allele of *Smarcal1* resulted in a mild cellular phenotype. We only observed slight changes in replication fork stability and DNA damage in Myc overexpressing, *Smarcal1*$^{+/\Delta}$ primary cells as well as reduced sensitivity to Myc-induced apoptosis relative to *Smarcal1*-null cells. *Smarcal1* haploinsufficiency therefore accelerated tumor development, which we postulate to be due to slight increases in DNA damage/fork collapse that facilitate transformation but are not significant enough to induce cell cycle arrest or apoptosis. Thus, *Smarcal1* gene dosage appears to have dramatic effects on tumor formation depending on the specific cellular context. In the future, it would be of

interest to determine what types of phenotypes occur with *Smarcal1* loss and other sources of replication stress and whether some of these phenotypes are due to the specific sources of replication stress themselves or are due to length of time the tumor cells of origin are subjected to replicative stress (or both). Moreover, future studies with the *Zranb3* knockout mice utilizing an acute source of replication stress, such as radiation, are critical for determining whether different types of replication stress alter tumor formation in *Zranb3*-deficient mice.

Moreover, our data contribute significantly to understanding the mechanisms of Myc-mediated replication stress and transformation. While multiple studies have shown that Myc overexpression induces DNA damage and replication stress *in vitro*, our data establish the consequences of Myc overexpression on replication fork stability and progression in hematopoietic cells *in vivo*. The effects of Myc on replication and replication stress are complex and likely require multiple factors for resolution and eventual S-phase progression. For instance, the ATR/Chk1 pathway is activated in cells overexpressing Myc and inhibition of ATR or Chk1 is synthetically lethal in MYC-overexpressing cancer cells (Hoglund et al. 2011, Murga et al. 2011, Cottini et al. 2015, Sanjiv et al. 2016). Moreover, other fork protection factors, like the WRN helicase, have been implicated in the resolution of Myc-induced replication stress in epithelial cells. WRN resolves unfavorable DNA structures upstream of the replication fork and is transcriptionally upregulated by MYC (Grandori et al. 2003, Robinson et al. 2009). Eμ-*myc* mice expressing a hypomorphic *Wrn* allele, $Wrn^{\Delta hel}$, showed substantially delayed lymphoma development due to unresolved Myc-induced replication stress and the activation of senescence in B cells. Moreover,

carcinoma cell lines overexpressing MYC and xenografted into mice were sensitive to WRN knockdown and showed decreased cell growth (Moser et al. 2012).

Our data identify two additional factors that are involved in fork protection upon Myc-induced replication stress. Complete loss of both *Smarcal1* and *Zranb3* led to increased levels of replication fork collapse, DNA damage and apoptosis. Loss of even a single allele of *Zranb3* was sufficient to induce these phenotypes in Myc-overexpressing B cells. This naturally raises the question when either (or both) of these proteins could serve as therapeutic targets in Myc-driven malignancies.

In human cancers, SMARCAL1 is rarely mutated or deleted and its expression is unchanged at the mRNA level in human lymphomas compared to normal controls (data not shown). However, Western blotting performed in our lab on whole cell lysate from Myc-driven murine and human lymphoma cell lines shows Smarcal1 is overexpressed at the protein level (data not shown). Thus, it is possible that SMARCAL1 may be required for cancer cells to mediate the elevated levels of replication stress associated with transformation and oncogene dysregulation. Moreover, while *ZRANB3* appears to be deleted in a subset of endometrial cancers (Lawrence et al. 2014), analysis of mRNA expression in lymphomas shows no change in its expression (data not shown). Whether its expression changes at the protein level in these cancers is unknown.

Nevertheless, our data suggest that inhibition of either protein alone may be sufficient to induce apoptosis in Myc-overexpressing tumor cells. However, since cancer cells have additional changes associated with transformation compared to the normal cells utilized in our experiments, this idea requires experimental validation. Moreover, our data suggest ZRANB3 may be a more efficacious therapeutic target, as even *Zranb3*

haploinsufficiency was sufficient to induce high levels of apoptosis in Myc-overexpressing B cells. Also, while complete *Smarcal1* loss did lead to high levels of apoptosis and could possibly kill Myc-driven cancer cells, *Smarcal1* haploinsufficiency accelerated tumor development in our studies. This would possibly deter the clinical targeting of SMARCAL1 as incomplete inhibition could lead to only a partial response in the tumor cells themselves and could actually promote cancer formation in other tissues.

However, our data suggest it would be worthwhile to investigate the therapeutic potential of targeting ZRANB3 in Myc-driven B-cell lymphomas either as a single agent or in combination with other replication stress inducing drugs. It would be interesting to determine if ZRANB3 inhibition was synthetically lethal with other DNA repair/replication defects, perhaps pathways involved in the repair of dsDNA breaks that occur upon replication fork collapse. Moreover, it is possible that silencing ZRANB3 in combination with other proteins involved the Myc-induced replication stress response, such as WRN, would be synergistic and more effective at tumor cell killing. Future studies establishing relationships between ZRANB3 and other fork protection factors, as well as Myc-driven tumor cell reliance on ZRANB3, are needed to explore these possibilities.

Future directions

While my thesis work has begun to provide insight into the biological functions of Smarcal1 and Zranb3, particularly in regards to lymphomagenesis and hematopoietic cell biology, there is much to be discerned about their physiologic functions. Here, I propose some future studies that may help elucidate additional biological functions of these related fork remodeling proteins.

Molecular mechanisms of fork protection

While SMARCAL1 and ZRANB3 are able to re-anneal single stranded DNA and promote fork repair and restart through fork regression and translocase functions, there is emerging evidence that each protein has unique non-redundant activities. In addition to the biochemical differences described in Chapter I, previous studies have shown that depletion of SMARCAL1 and ZRANB3 together has an additive effect when cell lines are treated with replication stress-inducing drugs (Ciccia et al. 2012, Yuan et al. 2012). Moreover, the data presented in Chapter III demonstrated for the first time that Smarcal1 and Zranb3 both are required for resolving oncogene-induced replication stress and do so in a non-redundant fashion. However, several questions remain regarding the elucidation of the unique biological functions of each protein.

First, while our data identifies Myc-induced replication stress as an endogenous source of replication stress that is mediated by Smarcal1 and Zranb3 independently, it is possible that these proteins, and other fork remodelers, could still have a degree of functional redundancy in other contexts. The sources of endogenous replication stress and the specific requirements of different replication fork protection factors are still poorly understood. For example, the exact factors required for fork protection during stalling caused by different types of replication stress, such as nucleotide depletion, oncogene overexpression, secondary DNA structures, damaged bases or transcriptional interference is still relatively unknown. Further studies investigating specific types of replication stress and the functions of SMARCAL1 and ZRANB3, and other fork remodeling proteins, are essential to understand the exact molecular mechanisms of fork protection during replication stress.

Moreover, while we utilized *Myc* as a source of oncogenic stress, not all oncogenes behave the same upon overexpression and most have a degree of phenotypic specificity in regards to DNA replication and fork stability. Dozens of oncogenes have been assessed for their effects on DNA replication and there has been a wide array of reported phenotypes related to cell cycle progression, origin firing, fork speed, DNA damage formation, cellular metabolism, ATM/ATR signaling and transcriptional interference, depending on the specific oncogene (Kotsantis et al. 2018). Most oncogenes will exhibit some shared cellular phenotypes with others, while also differing to various degrees. As a brief example, both CYCLIN E and MYC overexpression leads to transcriptional interference at a subset of origins that fire only upon oncogene overexpression. This leads to a similar pattern of DNA damage and fork collapse in both CYCLIN E and MYC overexpressing cells (Macheret and Halazonetis 2018). However, MYC overexpression also upregulates key enzymes involved in nucleotide biosynthesis, which results in increased dNTP pools and improved cell growth and replication progression (see Chapter I). CYCLIN E does not affect nucleotide biosynthesis, and its overexpression leads to dNTP depletion and fork stalling (Bester et al. 2011). Thus, the exact mechanisms of replication stress induced by oncogene overexpression differ and may require different factors for fork protection depending on the specific cellular context. While Smarcal1 and Zranb3 may resolve Myc-induced replication stress non-redundantly, this may not be necessarily true in regards to other sources of oncogenic stress. In the future, experiments utilizing different oncogenes would be useful for further delineating the roles of fork remodeling proteins in resolving oncogene-induced replication stress.

Hematopoiesis

Replication stress in HSPCs is a significant cause of impaired stem cell function and attrition. This is exacerbated by stimuli that induce HSPC proliferation including anemia and inflammation (see Chapter I). In humans, HSPC-defects have been seen in patients harboring mutations in proteins involved in the cellular response to DNA damage and/or DNA replication. For instance, Fanconi anemia (FA) is a rare bone-marrow failure syndrome caused by mutations in FA family member genes. These proteins have diverse functions in cellular processes including inter-strand crosslink repair, homologous recombination and replication fork protection. Greater than 90% of these patients develop bone marrow failure by age 40 and many also develop myelogenous leukemia (Ceccaldi et al. 2016). Moreover, patients with Seckel syndrome (ATR mutations) can present with pan-cytopenia while immunodeficiency is a hallmark of SIOD. Thus, replication stress is a direct contributor to HSPC dysfunction in humans. However, the exact functions of fork remodeling proteins in normal hematopoiesis have not been investigated to date.

In Chapter II, we showed that *Smarcal1*$^{+/\Delta}$ and *Smarcal1*$^{\Delta/\Delta}$ mice do not have any abnormalities in HSPCs in a normal, unstressed state. However, these animals are housed in a temperature controlled, pathogen-free environment with unlimited access to food and water, which greatly differs from the environmental exposures experienced by wild mice and humans. It would be important to determine if any HSPC defects emerge in mice subjected to stresses similar to those in their natural environment. For instance, it is possible that *Smarcal1*-deficient animals are unable to mount a normal immune response to pathogens that normally infect wild mice as cell proliferation and expansion is a normal component of the inflammatory response. By infecting these animals with bacterial or viral

agents, we could test the efficacy of the immune response in *Smarcal1*-deficient animals while also determining if *Smarcal1*-deficient HSPCs are able to maintain normal levels of hematopoietic cells during stressful conditions.

Moreover, it is unknown whether *ZRANB3* has any effects on HSPC biology. As noted previously in this section, it will be important to challenge *Zranb3*-deficient mice with acute sources of replication stress, such as radiation, to determine if it has a function in facilitating cellular viability during HSPC replication stress. Moreover, completing a thorough analysis of the HSPC compartments, as we did with the *Smarcal1*-deficeint mice, would provide insight into any innate HSPC defects that might occur upon loss of *Zranb3*. Additional experiments, such as serial and competitive HSPC transplantation assays, could provide further evidence of the involvement of fork remodeling proteins in HSPC biology.

Replication stress and aging

Replication stress and the functional decline of HSPCs is a consequence of normal aging in mice (Flach et al. 2014). Moreover, in humans, mutations in multiple replication stress response genes, including *WRN* (Werner syndrome) and *BLM* (Bloom syndrome), are associated with pre-mature aging disorders. However, the connection between fork remodeling proteins, such as SMARCAL1 and ZRANB3, and aging has not been explored at this point.

We have generated data that suggest both proteins have a function in protecting replication forks in hematopoietic cells in mice. However, in both studies, all experiments were performed using young animals (6-8 weeks of age), well before the appearance of any potential age-related phenotypes in the bone marrow. In addition to evaluating HSPC

function in response to multiple stressors (as described above), aging both the *Smarcal1*- and *Zranb3*-deficient mice may reveal evidence of bone marrow/HSPC dysfunction. Since hematopoietic stem and progenitor cells are a (relatively) highly proliferative cell population, it is possible that the multiple rounds of replication these cells undergo during the lives of these animals could require fork reversal to maintain cellular viability. I would hypothesize that loss of *Smarcal1* or *Zranb3* could lead to increased rates of stem cell attrition or alterations in specific progenitor populations, depending on the expression and requirements of each individual protein in specific HSPC compartments. Additionally, it would be interesting to see if *Smarcal1* and *Zranb3* also had functions in other highly proliferative stem cell populations, such as stem cells located within the intestinal epithelium.

Cancer and SIOD

While a few studies have reported incidences of cancer in SIOD patients (Baradaran-Heravi et al. 2012, Carroll et al. 2013), the link between *SMARCAL1* mutations and tumorigenesis in humans is unclear. In our studies in mice, the effects of *Smarcal1* loss on tumor development phenotypes appear to depend on the cell type, source and duration of replication stress. In a model of acute replication stress, loss of *Smarcal1* protected against radiation-induced T-cell lymphoma development due to apoptosis of pre-neoplastic HSPCs. Moreover, a subset of *Smarcal1*$^{+/\Delta}$ mice developed sarcomas, which are not typically associated with radiation-induced cancer models in mice. Alternatively, in a model of chronic replication stress where Myc is overexpressed in B cells, loss of a single allele of *Smarcal1* accelerated Myc-driven B-cell lymphoma development and loss of both

copies did not affect overall survival. Thus, the relationship between *Smarcal1* and tumor development is likely complex and context dependent.

However, our data show that loss of a single allele of *Smarcal1* promotes tumor development in the context of Myc overexpression; and this raises several interesting questions in relation to SIOD and cancer. Since some SIOD patients have missense mutations which vary in retained SMARCAL1 function (Betous et al. 2012, Carroll et al. 2013, Carroll et al. 2014), it is possible that their susceptibility to cancer may depend on the specific mutation and degree of SMARCAL1 functionality retained. Moreover, patients with non-sense or severely non-functional SMARCAL1 mutations could possibly be protected against cancer development, similarly to the *Smarcal1*-deficient mice in the radiation induced T cell models. However, since most SIOD patients do not survive into adulthood, detecting alterations in cancer susceptibility may be challenging.

However, our data raise important questions about tumor susceptibility in the parents of SIOD patients or in other people with heterozygous SMARCAL1 mutations. Since loss of a single copy of *Smarcal1* in the context of Myc overexpression greatly accelerated lymphoma development in mice, it is possible that *SMARCAL1* heterozygous humans have increased susceptibility to cancer. It would be interesting to gather epidemiological data on people harboring heterozygous *SMARCAL1* mutations to determine if there are differences in cancer rates relative to the general population. If so, *SMARCAL1* could serve as a potential biomarker in a subset of people and could be used to identify candidates for early cancer screenings.

Concluding remarks

In conclusion, my dissertation research has advanced our understanding of the biological significance of the replication stress response in hematopoietic cells and provided significant evidence of the importance of replication fork reversal in lymphomagenesis. Specifically, I showed that loss of *Smarcal1* significantly inhibited radiation-induced T-cell lymphoma development. This was due to increased levels of DNA damage and apoptosis in cycling HSPCs and thymic progenitor cells, which are likely the cell of origin which undergoes transformation in these animals. Moreover, I showed that the biological effects of fork remodeling protein loss differ depending on the source of replication stress. In a model of Myc-driven B-cell lymphomagenesis, I determined that *Smarcal1* haploinsufficiency accelerated tumor development while complete *Smarcal1* loss did not affect overall survival. Moreover, loss of a single allele of *Zranb3* was sufficient to induce DNA damage and apoptosis in Myc-overexpressing B cells and lymphoma development was inhibited in both *Zranb3*$^{+/-}$ and *Zranb3*$^{-/-}$ Eμ-*myc* mice. Overall, these data have significant implications for understanding the specific biological roles of replication fork remodeling proteins and enhances our understanding of how they influence replication-stress induced lymphoma development. However, many outstanding questions still remain and future studies should address outstanding questions related to the biology of these proteins.

REFERENCES

Adams, C. M., S. W. Hiebert and C. M. Eischen (2016). "Myc Induces miRNA-Mediated Apoptosis in Response to HDAC Inhibition in Hematologic Malignancies." Cancer Res **76**(3): 736-748.

Adams, J. M., A. W. Harris, C. A. Pinkert, L. M. Corcoran, W. S. Alexander, S. Cory, R. D. Palmiter, et al. (1985). "The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice." Nature **318**(6046): 533-538.

Adams, P. D., H. Jasper and K. L. Rudolph (2015). "Aging-Induced Stem Cell Mutations as Drivers for Disease and Cancer." Cell Stem Cell **16**(6): 601-612.

Allevato, M., E. Bolotin, M. Grossman, D. Mane-Padros, F. M. Sladek and E. Martinez (2017). "Sequence-specific DNA binding by MYC/MAX to low-affinity non-E-box motifs." PLoS One **12**(7): e0180147.

Alt, J. R., A. Bouska, M. R. Fernandez, R. L. Cerny, H. Xiao and C. M. Eischen (2005). "Mdm2 binds to Nbs1 at sites of DNA damage and regulates double strand break repair." J Biol Chem **280**(19): 18771-18781.

Alvarez, S., M. Diaz, J. Flach, S. Rodriguez-Acebes, A. J. Lopez-Contreras, D. Martinez, M. Canamero, et al. (2015). "Replication stress caused by low MCM expression limits fetal erythropoiesis and hematopoietic stem cell functionality." Nat Commun **6**: 8548.

Amati, B., K. Alevizopoulos and J. Vlach (1998). "Myc and the cell cycle." Front Biosci **3**: d250-268.

Amati, B., M. W. Brooks, N. Levy, T. D. Littlewood, G. I. Evan and H. Land (1993). "Oncogenic activity of the c-Myc protein requires dimerization with Max." Cell **72**(2): 233-245.

Amati, B., T. D. Littlewood, G. I. Evan and H. Land (1993). "The c-Myc protein induces cell cycle progression and apoptosis through dimerization with Max." EMBO J **12**(13): 5083-5087.

American Cancer Society (2017). "Survival rates and factors that affect prognosis (outlook) for non-hodgkin lymphoma." Retrieved April 28, 2018, from https://www.cancer.org/cancer/non-hodgkin-lymphoma/detection-diagnosis-staging/factors-prognosis.html.

An, J., E. Gonzalez-Avalos, A. Chawla, M. Jeong, I. F. Lopez-Moyado, W. Li, M. A. Goodell, et al. (2015). "Acute loss of TET function results in aggressive myeloid cancer in mice." Nat Commun **6**: 10071.

Arabi, A., S. Wu, K. Ridderstrale, H. Bierhoff, C. Shiue, K. Fatyol, S. Fahlen, et al. (2005). "c-Myc associates with ribosomal DNA and activates RNA polymerase I transcription." Nat Cell Biol **7**(3): 303-310.

Arai, F. and T. Suda (2007). "Maintenance of quiescent hematopoietic stem cells in the osteoblastic niche." Ann N Y Acad Sci **1106**: 41-53.

Armitage, J. O., R. D. Gascoyne, M. A. Lunning and F. Cavalli (2017). "Non-Hodgkin lymphoma." Lancet **390**(10091): 298-310.

Askew, D. S., R. A. Ashmun, B. C. Simmons and J. L. Cleveland (1991). "Constitutive c-myc expression in an IL-3-dependent myeloid cell line suppresses cell cycle arrest and accelerates apoptosis." Oncogene **6**(10): 1915-1922.

Attwooll, C., E. Lazzerini Denchi and K. Helin (2004). "The E2F family: specific functions and overlapping interests." EMBO J **23**(24): 4709-4716.

Badu-Nkansah, A., A. C. Mason, B. F. Eichman and D. Cortez (2016). "Identification of a Substrate Recognition Domain in the Replication Stress Response Protein Zinc Finger Ran-binding Domain-containing Protein 3 (ZRANB3)." J Biol Chem **291**(15): 8251-8257.

Baldridge, M. T., K. Y. King, N. C. Boles, D. C. Weksberg and M. A. Goodell (2010). "Quiescent haematopoietic stem cells are activated by IFN-gamma in response to chronic infection." Nature **465**(7299): 793-797.

Ball, H. L., M. R. Ehrhardt, D. A. Mordes, G. G. Glick, W. J. Chazin and D. Cortez (2007). "Function of a conserved checkpoint recruitment domain in ATRIP proteins." Mol Cell Biol **27**(9): 3367-3377.

Bansbach, C. E., R. Betous, C. A. Lovejoy, G. G. Glick and D. Cortez (2009). "The annealing helicase SMARCAL1 maintains genome integrity at stalled replication forks." Genes Dev **23**(20): 2405-2414.

Bansbach, C. E., C. F. Boerkoel and D. Cortez (2010). "SMARCAL1 and replication stress: an explanation for SIOD?" Nucleus **1**(3): 245-248.

Baradaran-Heravi, A., K. S. Cho, B. Tolhuis, M. Sanyal, O. Morozova, M. Morimoto, L. I. Elizondo, et al. (2012). "Penetrance of biallelic SMARCAL1 mutations is associated with environmental and genetic disturbances of gene expression." Hum Mol Genet **21**(11): 2572-2587.

Baradaran-Heravi, A., A. Raams, J. Lubieniecka, K. S. Cho, K. A. DeHaai, M. Basiratnia, P. O. Mari, et al. (2012). "SMARCAL1 deficiency predisposes to non-Hodgkin lymphoma and hypersensitivity to genotoxic agents in vivo." Am J Med Genet A **158A**(9): 2204-2213.

Barnes, D. E. and T. Lindahl (2004). "Repair and genetic consequences of endogenous DNA base damage in mammalian cells." Annu Rev Genet **38**: 445-476.

Barrett, J., M. J. Birrer, G. J. Kato, H. Dosaka-Akita and C. V. Dang (1992). "Activation domains of L-Myc and c-Myc determine their transforming potencies in rat embryo cells." Mol Cell Biol **12**(7): 3130-3137.

Bartkova, J., N. Rezaei, M. Liontos, P. Karakaidos, D. Kletsas, N. Issaeva, L. V. Vassiliou, et al. (2006). "Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints." Nature **444**(7119): 633-637.

Bass, T. E., J. W. Luzwick, G. Kavanaugh, C. Carroll, H. Dungrawala, G. G. Glick, M. D. Feldkamp, et al. (2016). "ETAA1 acts at stalled replication forks to maintain genome integrity." Nat Cell Biol **18**(11): 1185-1195.

Bell, S. P. and B. Stillman (1992). "ATP-dependent recognition of eukaryotic origins of DNA replication by a multiprotein complex." Nature **357**(6374): 128-134.

Benveniste, P., C. Frelin, S. Janmohamed, M. Barbara, R. Herrington, D. Hyam and N. N. Iscove (2010). "Intermediate-term hematopoietic stem cells with extended but time-limited reconstitution potential." Cell Stem Cell **6**(1): 48-58.

Bermejo, R., T. Capra, R. Jossen, A. Colosio, C. Frattini, W. Carotenuto, A. Cocito, et al. (2011). "The replication checkpoint protects fork stability by releasing transcribed genes from nuclear pores." Cell **146**(2): 233-246.

Bermudez, V. P., L. A. Lindsey-Boltz, A. J. Cesare, Y. Maniwa, J. D. Griffith, J. Hurwitz and A. Sancar (2003). "Loading of the human 9-1-1 checkpoint complex onto DNA by the checkpoint clamp loader hRad17-replication factor C complex in vitro." Proc Natl Acad Sci U S A **100**(4): 1633-1638.

Bernitz, J. M., H. S. Kim, B. MacArthur, H. Sieburg and K. Moore (2016). "Hematopoietic Stem Cells Count and Remember Self-Renewal Divisions." Cell **167**(5): 1296-1309 e1210.

Beroukhim, R., C. H. Mermel, D. Porter, G. Wei, S. Raychaudhuri, J. Donovan, J. Barretina, et al. (2010). "The landscape of somatic copy-number alteration across human cancers." Nature **463**(7283): 899-905.

Bester, A. C., M. Roniger, Y. S. Oren, M. M. Im, D. Sarni, M. Chaoat, A. Bensimon, et al. (2011). "Nucleotide deficiency promotes genomic instability in early stages of cancer development." Cell **145**(3): 435-446.

Betous, R., F. B. Couch, A. C. Mason, B. F. Eichman, M. Manosas and D. Cortez (2013). "Substrate-selective repair and restart of replication forks by DNA translocases." Cell Rep **3**(6): 1958-1969.

Betous, R., A. C. Mason, R. P. Rambo, C. E. Bansbach, A. Badu-Nkansah, B. M. Sirbu, B. F. Eichman, et al. (2012). "SMARCAL1 catalyzes fork regression and Holliday junction migration to maintain genome stability during DNA replication." Genes Dev **26**(2): 151-162.

Bhat, K. P., R. Betous and D. Cortez (2015). "High-affinity DNA-binding domains of replication protein A (RPA) direct SMARCAL1-dependent replication fork remodeling." J Biol Chem **290**(7): 4110-4117.

Bhatia, K., K. Huppi, G. Spangler, D. Siwarski, R. Iyer and I. Magrath (1993). "Point mutations in the c-Myc transactivation domain are common in Burkitt's lymphoma and mouse plasmacytomas." Nat Genet **5**(1): 56-61.

Bhatia, K., G. Spangler, G. Gaidano, N. Hamdy, R. Dalla-Favera and I. Magrath (1994). "Mutations in the coding region of c-myc occur frequently in acquired immunodeficiency syndrome-associated lymphomas." Blood **84**(3): 883-888.

Bhatia, V., S. I. Barroso, M. L. Garcia-Rubio, E. Tumini, E. Herrera-Moyano and A. Aguilera (2014). "BRCA2 prevents R-loop accumulation and associates with TREX-2 mRNA export factor PCID2." Nature **511**(7509): 362-365.

Bianchi, J., S. G. Rudd, S. K. Jozwiakowski, L. J. Bailey, V. Soura, E. Taylor, I. Stevanovic, et al. (2013). "PrimPol bypasses UV photoproducts during eukaryotic chromosomal DNA replication." Mol Cell **52**(4): 566-573.

Bignell, G. R., C. D. Greenman, H. Davies, A. P. Butler, S. Edkins, J. M. Andrews, G. Buck, et al. (2010). "Signatures of mutation and selection in the cancer genome." Nature **463**(7283): 893-898.

Blackford, A. N. and S. P. Jackson (2017). "ATM, ATR, and DNA-PK: The Trinity at the Heart of the DNA Damage Response." Mol Cell **66**(6): 801-817.

Blackwell, T. K., L. Kretzner, E. M. Blackwood, R. N. Eisenman and H. Weintraub (1990). "Sequence-specific DNA binding by the c-Myc protein." Science **250**(4984): 1149-1151.

Blackwood, E. M. and R. N. Eisenman (1991). "Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc." Science **251**(4998): 1211-1217.

Blastyak, A., I. Hajdu, I. Unk and L. Haracska (2010). "Role of double-stranded DNA translocase activity of human HLTF in replication of damaged DNA." Mol Cell Biol **30**(3): 684-693.

Blow, J. J. and A. Dutta (2005). "Preventing re-replication of chromosomal DNA." Nat Rev Mol Cell Biol **6**(6): 476-486.

Boerkoel, C. F., H. Takashima, J. John, J. Yan, P. Stankiewicz, L. Rosenbarker, J. L. Andre, et al. (2002). "Mutant chromatin remodeling protein SMARCAL1 causes Schimke immuno-osseous dysplasia." Nat Genet **30**(2): 215-220.

Bouchard, C., K. Thieke, A. Maier, R. Saffrich, J. Hanley-Hyde, W. Ansorge, S. Reed, et al. (1999). "Direct induction of cyclin D2 by Myc contributes to cell cycle progression and sequestration of p27." EMBO J **18**(19): 5321-5333.

Bouska, A., T. Lushnikova, S. Plaza and C. M. Eischen (2008). "Mdm2 promotes genetic instability and transformation independent of p53." Mol Cell Biol **28**(15): 4862-4874.

Brown, E. J. and D. Baltimore (2000). "ATR disruption leads to chromosomal fragmentation and early embryonic lethality." Genes Dev **14**(4): 397-402.

Brush, G. S., D. M. Morrow, P. Hieter and T. J. Kelly (1996). "The ATM homologue MEC1 is required for phosphorylation of replication protein A in yeast." Proc Natl Acad Sci U S A **93**(26): 15075-15080.

Bryder, D., D. J. Rossi and I. L. Weissman (2006). "Hematopoietic stem cells: the paradigmatic tissue-specific stem cell." Am J Pathol **169**(2): 338-346.

Burnham, D. R., B. Nijholt, I. De Vlaminck, J. Quan, T. Yusufzai and C. Dekker (2017). "Annealing helicase HARP closes RPA-stabilized DNA bubbles non-processively." Nucleic Acids Res **45**(8): 4687-4695.

Burrow, A. A., L. E. Williams, L. C. Pierce and Y. H. Wang (2009). "Over half of breakpoints in gene pairs involved in cancer-specific recurrent translocations are mapped to human chromosomal fragile sites." BMC Genomics **10**: 59.

Campaner, S. and B. Amati (2012). "Two sides of the Myc-induced DNA damage response: from tumor suppression to tumor maintenance." Cell Div **7**(1): 6.

Carroll, C., A. Badu-Nkansah, T. Hunley, A. Baradaran-Heravi, D. Cortez and H. Frangoul (2013). "Schimke Immunoosseous Dysplasia associated with undifferentiated carcinoma and a novel SMARCAL1 mutation in a child." Pediatr Blood Cancer **60**(9): E88-90.

Carroll, C., C. E. Bansbach, R. Zhao, S. Y. Jung, J. Qin and D. Cortez (2014). "Phosphorylation of a C-terminal auto-inhibitory domain increases SMARCAL1 activity." Nucleic Acids Res **42**(2): 918-925.

Cayrou, C., P. Coulombe, A. Vigneron, S. Stanojcic, O. Ganier, I. Peiffer, E. Rivals, et al. (2011). "Genome-scale analysis of metazoan replication origins reveals their organization in specific but flexible sites defined by conserved features." Genome Res **21**(9): 1438-1449.

Ceccaldi, R., P. Sarangi and A. D. D'Andrea (2016). "The Fanconi anaemia pathway: new players and new functions." Nat Rev Mol Cell Biol **17**(6): 337-349.

Center for Disease Control and Prevention (2016). "United States Health Report 2016." Retrieved April 26, 2018, from https://www.cdc.gov/nchs/data/hus/hus16.pdf#019.

Challen, G. A., N. Boles, K. K. Lin and M. A. Goodell (2009). "Mouse hematopoietic stem cell identification and analysis." Cytometry A **75**(1): 14-24.

Cheng, T., N. Rodrigues, H. Shen, Y. Yang, D. Dombkowski, M. Sykes and D. T. Scadden (2000). "Hematopoietic stem cell quiescence maintained by p21cip1/waf1." Science **287**(5459): 1804-1808.

Cher, M. L., G. S. Bova, D. H. Moore, E. J. Small, P. R. Carroll, S. S. Pin, J. I. Epstein, et al. (1996). "Genetic alterations in untreated metastases and androgen-independent prostate cancer detected by comparative genomic hybridization and allelotyping." Cancer Res **56**(13): 3091-3102.

Cheshier, S. H., S. S. Prohaska and I. L. Weissman (2007). "The effect of bleeding on hematopoietic stem cell cycling and self-renewal." Stem Cells Dev **16**(5): 707-717.

Ciccia, A., A. L. Bredemeyer, M. E. Sowa, M. E. Terret, P. V. Jallepalli, J. W. Harper and S. J. Elledge (2009). "The SIOD disorder protein SMARCAL1 is an RPA-interacting protein involved in replication fork restart." Genes Dev **23**(20): 2415-2425.

Ciccia, A. and S. J. Elledge (2010). "The DNA damage response: making it safe to play with knives." Mol Cell **40**(2): 179-204.

Ciccia, A., A. V. Nimonkar, Y. Hu, I. Hajdu, Y. J. Achar, L. Izhar, S. A. Petit, et al. (2012). "Polyubiquitinated PCNA recruits the ZRANB3 translocase to maintain genomic integrity after replication stress." Mol Cell **47**(3): 396-409.

Cimprich, K. A. and D. Cortez (2008). "ATR: an essential regulator of genome integrity." Nat Rev Mol Cell Biol **9**(8): 616-627.

Claassen, G. F. and S. R. Hann (2000). "A role for transcriptional repression of p21CIP1 by c-Myc in overcoming transforming growth factor beta -induced cell-cycle arrest." Proc Natl Acad Sci U S A **97**(17): 9498-9503.

Clark, H. M., T. Yano, T. Otsuki, E. S. Jaffe, D. Shibata and M. Raffeld (1994). "Mutations in the coding region of c-MYC in AIDS-associated and other aggressive lymphomas." Cancer Res **54**(13): 3383-3386.

Clewing, J. M., B. C. Antalfy, T. Lucke, B. Najafian, K. M. Marwedel, A. Hori, R. M. Powel, et al. (2007). "Schimke immuno-osseous dysplasia: a clinicopathological correlation." J Med Genet **44**(2): 122-130.

Clewing, J. M., H. Fryssira, D. Goodman, S. F. Smithson, E. A. Sloan, S. Lou, Y. Huang, et al. (2007). "Schimke immunoosseous dysplasia: suggestions of genetic diversity." Hum Mutat **28**(3): 273-283.

Cobb, J. A., L. Bjergbaek, K. Shimada, C. Frei and S. M. Gasser (2003). "DNA polymerase stabilization at stalled replication forks requires Mec1 and the RecQ helicase Sgs1." EMBO J **22**(16): 4325-4336.

Cole, K. A., J. Huggins, M. Laquaglia, C. E. Hulderman, M. R. Russell, K. Bosse, S. J. Diskin, et al. (2011). "RNAi screen of the protein kinome identifies checkpoint kinase 1 (CHK1) as a therapeutic target in neuroblastoma." Proc Natl Acad Sci U S A **108**(8): 3336-3341.

Coleman, M. A., J. A. Eisen and H. W. Mohrenweiser (2000). "Cloning and characterization of HARP/SMARCAL1: a prokaryotic HepA-related SNF2 helicase protein from human and mouse." Genomics **65**(3): 274-282.

Cortes-Ledesma, F. and A. Aguilera (2006). "Double-strand breaks arising by replication through a nick are repaired by cohesin-dependent sister-chromatid exchange." EMBO Rep **7**(9): 919-926.

Cortez, D. (2015). "Preventing replication fork collapse to maintain genome integrity." DNA Repair (Amst) **32**: 149-157.

Cortez, D., S. Guntuku, J. Qin and S. J. Elledge (2001). "ATR and ATRIP: partners in checkpoint signaling." Science **294**(5547): 1713-1716.

Costa, A., I. Ilves, N. Tamberg, T. Petojevic, E. Nogales, M. R. Botchan and J. M. Berger (2011). "The structural basis for MCM2-7 helicase activation by GINS and Cdc45." Nat Struct Mol Biol **18**(4): 471-477.

Costes, A. and S. A. Lambert (2012). "Homologous recombination as a replication fork escort: fork-protection and recovery." Biomolecules **3**(1): 39-71.

Cotta-Ramusino, C., D. Fachinetti, C. Lucca, Y. Doksani, M. Lopes, J. Sogo and M. Foiani (2005). "Exo1 processes stalled replication forks and counteracts fork reversal in checkpoint-defective cells." Mol Cell **17**(1): 153-159.

Cottini, F., T. Hideshima, R. Suzuki, Y. T. Tai, G. Bianchini, P. G. Richardson, K. C. Anderson, et al. (2015). "Synthetic Lethal Approaches Exploiting DNA Damage in Aggressive Myeloma." Cancer Discov **5**(9): 972-987.

Couch, F. B., C. E. Bansbach, R. Driscoll, J. W. Luzwick, G. G. Glick, R. Betous, C. M. Carroll, et al. (2013). "ATR phosphorylates SMARCAL1 to prevent replication fork collapse." Genes Dev **27**(14): 1610-1623.

Couzin-Frankel, J. (2013). "Breakthrough of the year 2013. Cancer immunotherapy." Science **342**(6165): 1432-1433.

Cox, K. E., A. Marechal and R. L. Flynn (2016). "SMARCAL1 Resolves Replication Stress at ALT Telomeres." Cell Rep **14**(5): 1032-1040.

Dalla-Favera, R., M. Bregni, J. Erikson, D. Patterson, R. C. Gallo and C. M. Croce (1982). "Human c-myc onc gene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells." Proc Natl Acad Sci U S A **79**(24): 7824-7827.

Dang, C. V. (2012). "MYC on the path to cancer." Cell **149**(1): 22-35.

Dang, C. V. (2013). "MYC, metabolism, cell growth, and tumorigenesis." Cold Spring Harb Perspect Med **3**(8).

Dang, C. V. (2014). "Gene regulation: fine-tuned amplification in cells." Nature **511**(7510): 417-418.

Dang, C. V., K. A. O'Donnell, K. I. Zeller, T. Nguyen, R. C. Osthus and F. Li (2006). "The c-Myc target gene network." Semin Cancer Biol **16**(4): 253-264.

Dave, S. S., K. Fu, G. W. Wright, L. T. Lam, P. Kluin, E. J. Boerma, T. C. Greiner, et al. (2006). "Molecular diagnosis of Burkitt's lymphoma." N Engl J Med **354**(23): 2431-2442.

Davis, A. C., M. Wims, G. D. Spotts, S. R. Hann and A. Bradley (1993). "A null c-myc mutation causes lethality before 10.5 days of gestation in homozygotes and reduced fertility in heterozygous female mice." Genes Dev **7**(4): 671-682.

de Haan, G. and S. S. Lazare (2018). "Aging of hematopoietic stem cells." Blood **131**(5): 479-487.

de Klein, A., M. Muijtjens, R. van Os, Y. Verhoeven, B. Smit, A. M. Carr, A. R. Lehmann, et al. (2000). "Targeted disruption of the cell-cycle checkpoint gene ATR leads to early embryonic lethality in mice." Curr Biol **10**(8): 479-482.

De Piccoli, G., Y. Katou, T. Itoh, R. Nakato, K. Shirahige and K. Labib (2012). "Replisome stability at defective DNA replication forks is independent of S phase checkpoint kinases." Mol Cell **45**(5): 696-704.

Delacroix, S., J. M. Wagner, M. Kobayashi, K. Yamamoto and L. M. Karnitz (2007). "The Rad9-Hus1-Rad1 (9-1-1) clamp activates checkpoint signaling via TopBP1." Genes Dev **21**(12): 1472-1477.

DePamphilis, M. L., J. J. Blow, S. Ghosh, T. Saha, K. Noguchi and A. Vassilev (2006). "Regulating the licensing of DNA replication origins in metazoa." Curr Opin Cell Biol **18**(3): 231-239.

Di Micco, R., M. Fumagalli, A. Cicalese, S. Piccinin, P. Gasparini, C. Luise, C. Schurra, et al. (2006). "Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication." Nature **444**(7119): 638-642.

Dominguez-Sola, D., C. Y. Ying, C. Grandori, L. Ruggiero, B. Chen, M. Li, D. A. Galloway, et al. (2007). "Non-transcriptional control of DNA replication by c-Myc." Nature **448**(7152): 445-451.

Duesberg, P. H., K. Bister and P. K. Vogt (1977). "The RNA of avian acute leukemia virus MC29." Proc Natl Acad Sci U S A **74**(10): 4320-4324.

Dungrawala, H., K. L. Rose, K. P. Bhat, K. N. Mohni, G. G. Glick, F. B. Couch and D. Cortez (2015). "The Replication Checkpoint Prevents Two Types of Fork Collapse without Regulating Replisome Stability." Mol Cell **59**(6): 998-1010.

Duzdevich, D., M. D. Warner, S. Ticau, N. A. Ivica, S. P. Bell and E. C. Greene (2015). "The dynamics of eukaryotic replication initiation: origin specificity, licensing, and firing at the single-molecule level." Mol Cell **58**(3): 483-494.

Dykstra, B., S. Olthof, J. Schreuder, M. Ritsema and G. de Haan (2011). "Clonal analysis reveals multiple functional defects of aged murine hematopoietic stem cells." J Exp Med **208**(13): 2691-2703.

Eberhardy, S. R. and P. J. Farnham (2001). "c-Myc mediates activation of the cad promoter via a post-RNA polymerase II recruitment mechanism." J Biol Chem **276**(51): 48562-48571.

Eberhardy, S. R. and P. J. Farnham (2002). "Myc recruits P-TEFb to mediate the final step in the transcriptional activation of the cad promoter." J Biol Chem **277**(42): 40156-40162.

Egle, A., A. W. Harris, P. Bouillet and S. Cory (2004). "Bim is a suppressor of Myc-induced mouse B cell leukemia." Proc Natl Acad Sci U S A **101**(16): 6164-6169.

Egler, R. A., E. Fernandes, K. Rothermund, S. Sereika, N. de Souza-Pinto, P. Jaruga, M. Dizdaroglu, et al. (2005). "Regulation of reactive oxygen species, DNA damage, and c-Myc function by peroxiredoxin 1." Oncogene **24**(54): 8038-8050.

Eilers, M., D. Picard, K. R. Yamamoto and J. M. Bishop (1989). "Chimaeras of myc oncoprotein and steroid receptors cause hormone-dependent transformation of cells." Nature **340**(6228): 66-68.

Eilers, M., S. Schirm and J. M. Bishop (1991). "The MYC protein activates transcription of the alpha-prothymosin gene." EMBO J **10**(1): 133-141.

Eischen, C. M., G. Packham, J. Nip, B. E. Fee, S. W. Hiebert, G. P. Zambetti and J. L. Cleveland (2001). "Bcl-2 is an apoptotic target suppressed by both c-Myc and E2F-1." Oncogene **20**(48): 6983-6993.

Eischen, C. M., J. D. Weber, M. F. Roussel, C. J. Sherr and J. L. Cleveland (1999). "Disruption of the ARF-Mdm2-p53 tumor suppressor pathway in Myc-induced lymphomagenesis." Genes Dev **13**(20): 2658-2669.

Eischen, C. M., D. Woo, M. F. Roussel and J. L. Cleveland (2001). "Apoptosis triggered by Myc-induced suppression of Bcl-X(L) or Bcl-2 is bypassed during lymphomagenesis." Mol Cell Biol **21**(15): 5063-5070.

Escot, C., C. Theillet, R. Lidereau, F. Spyratos, M. H. Champeme, J. Gest and R. Callahan (1986). "Genetic alteration of the c-myc protooncogene (MYC) in human primary breast carcinomas." Proc Natl Acad Sci U S A **83**(13): 4834-4838.

Essers, M. A., S. Offner, W. E. Blanco-Bose, Z. Waibler, U. Kalinke, M. A. Duchosal and A. Trumpp (2009). "IFNalpha activates dormant haematopoietic stem cells in vivo." Nature **458**(7240): 904-908.

Evan, G. I., A. H. Wyllie, C. S. Gilbert, T. D. Littlewood, H. Land, M. Brooks, C. M. Waters, et al. (1992). "Induction of apoptosis in fibroblasts by c-myc protein." Cell **69**(1): 119-128.

Evrin, C., P. Clarke, J. Zech, R. Lurz, J. Sun, S. Uhle, H. Li, et al. (2009). "A double-hexameric MCM2-7 complex is loaded onto origin DNA during licensing of eukaryotic DNA replication." Proc Natl Acad Sci U S A **106**(48): 20240-20245.

Feijoo, C., C. Hall-Jackson, R. Wu, D. Jenkins, J. Leitch, D. M. Gilbert and C. Smythe (2001). "Activation of mammalian Chk1 during DNA replication arrest: a role for Chk1 in the intra-S phase checkpoint monitoring replication origin firing." J Cell Biol **154**(5): 913-923.

Felsher, D. W. and J. M. Bishop (1999). "Transient excess of MYC activity can elicit genomic instability and tumorigenesis." Proc Natl Acad Sci U S A **96**(7): 3940-3944.

Felsher, D. W., A. Zetterberg, J. Zhu, T. Tlsty and J. M. Bishop (2000). "Overexpression of MYC causes p53-dependent G2 arrest of normal fibroblasts." Proc Natl Acad Sci U S A **97**(19): 10544-10548.

Fernandez, P. C., S. R. Frank, L. Wang, M. Schroeder, S. Liu, J. Greene, A. Cocito, et al. (2003). "Genomic targets of the human c-Myc protein." Genes Dev **17**(9): 1115-1129.

Ferrao, P. T., E. P. Bukczynska, R. W. Johnstone and G. A. McArthur (2012). "Efficacy of CHK inhibitors as single agents in MYC-driven lymphoma cells." Oncogene **31**(13): 1661-1672.

Flach, J., S. T. Bakker, M. Mohrin, P. C. Conroy, E. M. Pietras, D. Reynaud, S. Alvarez, et al. (2014). "Replication stress is a potent driver of functional decline in ageing haematopoietic stem cells." Nature **512**(7513): 198-202.

Flach, J. and M. Milyavsky (2018). "Replication stress in hematopoietic stem cells in mouse and man." Mutat Res **808**: 74-82.

Follonier, C., J. Oehler, R. Herrador and M. Lopes (2013). "Friedreich's ataxia-associated GAA repeats induce replication-fork reversal and unusual molecular junctions." Nat Struct Mol Biol **20**(4): 486-494.

Fragkos, M., O. Ganier, P. Coulombe and M. Mechali (2015). "DNA replication origin activation in space and time." Nat Rev Mol Cell Biol **16**(6): 360-374.

Frank, S. R., T. Parisi, S. Taubert, P. Fernandez, M. Fuchs, H. M. Chan, D. M. Livingston, et al. (2003). "MYC recruits the TIP60 histone acetyltransferase complex to chromatin." EMBO Rep **4**(6): 575-580.

Furnari, B., N. Rhind and P. Russell (1997). "Cdc25 mitotic inducer targeted by chk1 DNA damage checkpoint kinase." Science **277**(5331): 1495-1497.

Gaillard, H., T. Garcia-Muse and A. Aguilera (2015). "Replication stress and cancer." Nat Rev Cancer **15**(5): 276-289.

Gaillard, H., E. Herrera-Moyano and A. Aguilera (2013). "Transcription-associated genome instability." Chem Rev **113**(11): 8638-8661.

Galaktionov, K., X. Chen and D. Beach (1996). "Cdc25 cell-cycle phosphatase as a target of c-myc." Nature **382**(6591): 511-517.

Gan, W., Z. Guan, J. Liu, T. Gui, K. Shen, J. L. Manley and X. Li (2011). "R-loop-mediated genomic instability is caused by impairment of replication fork progression." Genes Dev **25**(19): 2041-2056.

Gao, P., I. Tchernyshyov, T. C. Chang, Y. S. Lee, K. Kita, T. Ochi, K. I. Zeller, et al. (2009). "c-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism." Nature **458**(7239): 762-765.

Ge, X. Q., D. A. Jackson and J. J. Blow (2007). "Dormant origins licensed by excess Mcm2-7 are required for human cells to survive replicative stress." Genes Dev **21**(24): 3331-3341.

Gerstein, M. B., A. Kundaje, M. Hariharan, S. G. Landt, K. K. Yan, C. Cheng, X. J. Mu, et al. (2012). "Architecture of the human regulatory network derived from ENCODE data." Nature **489**(7414): 91-100.

Ghosal, G., J. Yuan and J. Chen (2011). "The HARP domain dictates the annealing helicase activity of HARP/SMARCAL1." EMBO Rep **12**(6): 574-580.

Gomez-Roman, N., C. Grandori, R. N. Eisenman and R. J. White (2003). "Direct activation of RNA polymerase III transcription by c-Myc." Nature **421**(6920): 290-294.

Gorrini, C., M. Squatrito, C. Luise, N. Syed, D. Perna, L. Wark, F. Martinato, et al. (2007). "Tip60 is a haplo-insufficient tumour suppressor required for an oncogene-induced DNA damage response." Nature **448**(7157): 1063-1067.

Gottipati, P., T. N. Cassel, L. Savolainen and T. Helleday (2008). "Transcription-associated recombination is dependent on replication in Mammalian cells." Mol Cell Biol **28**(1): 154-164.

Grandori, C., N. Gomez-Roman, Z. A. Felton-Edkins, C. Ngouenet, D. A. Galloway, R. N. Eisenman and R. J. White (2005). "c-Myc binds to human ribosomal DNA and stimulates transcription of rRNA genes by RNA polymerase I." Nat Cell Biol **7**(3): 311-318.

Grandori, C., K. J. Wu, P. Fernandez, C. Ngouenet, J. Grim, B. E. Clurman, M. J. Moser, et al. (2003). "Werner syndrome protein limits MYC-induced cellular senescence." Genes Dev **17**(13): 1569-1574.

Gregory, M. A. and S. R. Hann (2000). "c-Myc proteolysis by the ubiquitin-proteasome pathway: stabilization of c-Myc in Burkitt's lymphoma cells." Mol Cell Biol **20**(7): 2423-2435.

Guccione, E., F. Martinato, G. Finocchiaro, L. Luzi, L. Tizzoni, V. Dall' Olio, G. Zardo, et al. (2006). "Myc-binding-site recognition in the human genome is determined by chromatin context." Nat Cell Biol **8**(7): 764-770.

Guryanova, O. A., K. Shank, B. Spitzer, L. Luciani, R. P. Koche, F. E. Garrett-Bakelman, C. Ganzel, et al. (2016). "DNMT3A mutations promote anthracycline resistance in acute myeloid leukemia via impaired nucleosome remodeling." Nat Med **22**(12): 1488-1495.

Haahr, P., S. Hoffmann, M. A. Tollenaere, T. Ho, L. I. Toledo, M. Mann, S. Bekker-Jensen, et al. (2016). "Activation of the ATR kinase by the RPA-binding protein ETAA1." Nat Cell Biol **18**(11): 1196-1207.

Halazonetis, T. D., V. G. Gorgoulis and J. Bartek (2008). "An oncogene-induced DNA damage model for cancer development." Science **319**(5868): 1352-1355.

Hanada, K., M. Budzowska, S. L. Davies, E. van Drunen, H. Onizawa, H. B. Beverloo, A. Maas, et al. (2007). "The structure-specific endonuclease Mus81 contributes to replication restart by generating double-strand DNA breaks." Nat Struct Mol Biol **14**(11): 1096-1104.

Hanahan, D. and R. A. Weinberg (2000). "The hallmarks of cancer." Cell **100**(1): 57-70.

Hanahan, D. and R. A. Weinberg (2011). "Hallmarks of cancer: the next generation." Cell **144**(5): 646-674.

Harbour, J. W. and D. C. Dean (2000). "The Rb/E2F pathway: expanding roles and emerging paradigms." Genes Dev **14**(19): 2393-2409.

Harbour, J. W., R. X. Luo, A. Dei Santi, A. A. Postigo and D. C. Dean (1999). "Cdk phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G1." Cell **98**(6): 859-869.

Harris, A. W., C. A. Pinkert, M. Crawford, W. Y. Langdon, R. L. Brinster and J. M. Adams (1988). "The E mu-myc transgenic mouse. A model for high-incidence spontaneous lymphoma and leukemia of early B cells." J Exp Med **167**(2): 353-371.

Hashimoto, Y., F. Puddu and V. Costanzo (2011). "RAD51- and MRE11-dependent reassembly of uncoupled CMG helicase complex at collapsed replication forks." Nat Struct Mol Biol **19**(1): 17-24.

Hashimoto, Y., A. Ray Chaudhuri, M. Lopes and V. Costanzo (2010). "Rad51 protects nascent DNA from Mre11-dependent degradation and promotes continuous DNA synthesis." Nat Struct Mol Biol **17**(11): 1305-1311.

Hayward, W. S., B. G. Neel and S. M. Astrin (1981). "Activation of a cellular onc gene by promoter insertion in ALV-induced lymphoid leukosis." Nature **290**(5806): 475-480.

Heffernan, T. P., D. A. Simpson, A. R. Frank, A. N. Heinloth, R. S. Paules, M. Cordeiro-Stone and W. K. Kaufmann (2002). "An ATR- and Chk1-dependent S checkpoint inhibits replicon initiation following UVC-induced DNA damage." Mol Cell Biol **22**(24): 8552-8561.

Helmrich, A., M. Ballarino and L. Tora (2011). "Collisions between replication and transcription complexes cause common fragile site instability at the longest human genes." Mol Cell **44**(6): 966-977.

Hemann, M. T., A. Bric, J. Teruya-Feldstein, A. Herbst, J. A. Nilsson, C. Cordon-Cardo, J. L. Cleveland, et al. (2005). "Evasion of the p53 tumour surveillance network by tumour-derived MYC mutants." Nature **436**(7052): 807-811.

Herbst, A., M. T. Hemann, K. A. Tworkowski, S. E. Salghetti, S. W. Lowe and W. P. Tansey (2005). "A conserved element in Myc that negatively regulates its proapoptotic activity." EMBO Rep **6**(2): 177-183.

Herkert, B. and M. Eilers (2010). "Transcriptional repression: the dark side of myc." Genes Cancer **1**(6): 580-586.

Hermeking, H. and D. Eick (1994). "Mediation of c-Myc-induced apoptosis by p53." Science **265**(5181): 2091-2093.

Hermeking, H., C. Rago, M. Schuhmacher, Q. Li, J. F. Barrett, A. J. Obaya, B. C. O'Connell, et al. (2000). "Identification of CDK4 as a target of c-MYC." Proc Natl Acad Sci U S A **97**(5): 2229-2234.

Hockensmith, J. W., A. F. Wahl, S. Kowalski and R. A. Bambara (1986). "Purification of a calf thymus DNA-dependent adenosinetriphosphatase that prefers a primer-template junction effector." Biochemistry **25**(24): 7812-7821.

Hoglund, A., L. M. Nilsson, S. V. Muralidharan, L. A. Hasvold, P. Merta, M. Rudelius, V. Nikolova, et al. (2011). "Therapeutic implications for the induced levels of Chk1 in Myc-expressing cancer cells." Clin Cancer Res **17**(22): 7067-7079.

Hu, S., A. Balakrishnan, R. A. Bok, B. Anderton, P. E. Larson, S. J. Nelson, J. Kurhanewicz, et al. (2011). "13C-pyruvate imaging reveals alterations in glycolysis that precede c-Myc-induced tumor formation and regression." Cell Metab **14**(1): 131-142.

Huang, J., S. Liu, M. A. Bellani, A. K. Thazhathveetil, C. Ling, J. P. de Winter, Y. Wang, et al. (2013). "The DNA translocase FANCM/MHF promotes replication traverse of DNA interstrand crosslinks." Mol Cell **52**(3): 434-446.

Humblet, C., M. P. Defresne, R. Greimers, A. M. Rongy and J. Boniver (1989). "Further studies on the mechanism of radiation induced thymic lymphoma prevention by bone marrow transplantation in C57BL mice." Leukemia **3**(11): 813-818.

Ibarra, A., E. Schwob and J. Mendez (2008). "Excess MCM proteins protect human cells from replicative stress by licensing backup origins of replication." Proc Natl Acad Sci U S A **105**(26): 8956-8961.

Im, J. S., S. H. Ki, A. Farina, D. S. Jung, J. Hurwitz and J. K. Lee (2009). "Assembly of the Cdc45-Mcm2-7-GINS complex in human cells requires the Ctf4/And-1, RecQL4, and Mcm10 proteins." Proc Natl Acad Sci U S A **106**(37): 15628-15632.

Iritani, B. M. and R. N. Eisenman (1999). "c-Myc enhances protein synthesis and cell size during B lymphocyte development." Proc Natl Acad Sci U S A **96**(23): 13180-13185.

Itkin, T., A. Ludin, B. Gradus, S. Gur-Cohen, A. Kalinkovich, A. Schajnovitz, Y. Ovadya, et al. (2012). "FGF-2 expands murine hematopoietic stem and progenitor cells via proliferation of stromal cells, c-Kit activation, and CXCL12 down-regulation." Blood **120**(9): 1843-1855.

Javier, R. T. and J. S. Butel (2008). "The history of tumor virology." Cancer Res **68**(19): 7693-7706.

Jiang, G., A. Espeseth, D. J. Hazuda and D. M. Margolis (2007). "c-Myc and Sp1 contribute to proviral latency by recruiting histone deacetylase 1 to the human immunodeficiency virus type 1 promoter." J Virol **81**(20): 10914-10923.

Jiricny, J. (2006). "The multifaceted mismatch-repair system." Nat Rev Mol Cell Biol **7**(5): 335-346.

Johnston, J. M. and W. L. Carroll (1992). "c-myc hypermutation in Burkitt's lymphoma." Leuk Lymphoma **8**(6): 431-439.

Jones, R. M., O. Mortusewicz, I. Afzal, M. Lorvellec, P. Garcia, T. Helleday and E. Petermann (2013). "Increased replication initiation and conflicts with transcription underlie Cyclin E-induced replication stress." Oncogene **32**(32): 3744-3753.

Jones, S. (2004). "An overview of the basic helix-loop-helix proteins." Genome Biol **5**(6): 226.

Kanao, R. and C. Masutani (2017). "Regulation of DNA damage tolerance in mammalian cells by post-translational modifications of PCNA." Mutat Res **803-805**: 82-88.

Kaplan, H. S. and M. B. Brown (1952). "A quantitative dose-response study of lymphoid-tumor development in irradiated C 57 black mice." J Natl Cancer Inst **13**(1): 185-208.

Kaplan, H. S., W. H. Carnes, M. B. Brown and B. B. Hirsch (1956). "Indirect induction of lymphomas in irradiated mice. I. Tumor incidence and morphology in mice bearing nonirradiated thymic grafts." Cancer Res **16**(5): 422-425.

Karn, J., J. V. Watson, A. D. Lowe, S. M. Green and W. Vedeckis (1989). "Regulation of cell cycle duration by c-myc levels." Oncogene **4**(6): 773-787.

Kato, J., H. Matsushime, S. W. Hiebert, M. E. Ewen and C. J. Sherr (1993). "Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4." Genes Dev **7**(3): 331-342.

Kawabata, T., S. W. Luebben, S. Yamaguchi, I. Ilves, I. Matise, T. Buske, M. R. Botchan, et al. (2011). "Stalled fork rescue via dormant replication origins in unchallenged S phase promotes proper chromosome segregation and tumor suppression." Mol Cell **41**(5): 543-553.

Keka, I. S., Mohiuddin, Y. Maede, M. M. Rahman, T. Sakuma, M. Honma, T. Yamamoto, et al. (2015). "Smarcal1 promotes double-strand-break repair by nonhomologous end-joining." Nucleic Acids Res **43**(13): 6359-6372.

Kelly, K., B. H. Cochran, C. D. Stiles and P. Leder (1983). "Cell-specific regulation of the c-myc gene by lymphocyte mitogens and platelet-derived growth factor." Cell **35**(3 Pt 2): 603-610.

Kiel, M. J., O. H. Yilmaz, T. Iwashita, O. H. Yilmaz, C. Terhorst and S. J. Morrison (2005). "SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells." Cell **121**(7): 1109-1121.

Kim, J. C. and S. M. Mirkin (2013). "The balancing act of DNA repeat expansions." Curr Opin Genet Dev **23**(3): 280-288.

Kim, W. Y. and N. E. Sharpless (2006). "The regulation of INK4/ARF in cancer and aging." Cell **127**(2): 265-275.

Kolinjivadi, A. M., V. Sannino, A. De Antoni, K. Zadorozhny, M. Kilkenny, H. Techer, G. Baldi, et al. (2017). "Smarcal1-Mediated Fork Reversal Triggers Mre11-Dependent Degradation of Nascent DNA in the Absence of Brca2 and Stable Rad51 Nucleofilaments." Mol Cell **67**(5): 867-881 e867.

Kominami, R. and O. Niwa (2006). "Radiation carcinogenesis in mouse thymic lymphomas." Cancer Sci **97**(7): 575-581.

Koster, D. A., A. Crut, S. Shuman, M. A. Bjornsti and N. H. Dekker (2010). "Cellular strategies for regulating DNA supercoiling: a single-molecule perspective." Cell **142**(4): 519-530.

Kotsantis, P., R. M. Jones, M. R. Higgs and E. Petermann (2015). "Cancer therapy and replication stress: forks on the road to perdition." Adv Clin Chem **69**: 91-138.

Kotsantis, P., E. Petermann and S. J. Boulton (2018). "Mechanisms of Oncogene-Induced Replication Stress: Jigsaw Falling into Place." Cancer Discov **8**(5): 537-555.

Kunkel, T. A. (2009). "Evolving views of DNA replication (in)fidelity." Cold Spring Harb Symp Quant Biol **74**: 91-101.

Kurland, J. F. and W. P. Tansey (2008). "Myc-mediated transcriptional repression by recruitment of histone deacetylase." Cancer Res **68**(10): 3624-3629.

Kuzminov, A. (2001). "Single-strand interruptions in replicating chromosomes cause double-strand breaks." Proc Natl Acad Sci U S A **98**(15): 8241-8246.

Labi, V., M. Erlacher, G. Krumschnabel, C. Manzl, A. Tzankov, J. Pinon, A. Egle, et al. (2010). "Apoptosis of leukocytes triggered by acute DNA damage promotes lymphoma formation." Genes Dev **24**(15): 1602-1607.

Labib, K. (2010). "How do Cdc7 and cyclin-dependent kinases trigger the initiation of chromosome replication in eukaryotic cells?" Genes Dev **24**(12): 1208-1219.

Land, H., L. F. Parada and R. A. Weinberg (1983). "Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes." Nature **304**(5927): 596-602.

Lawrence, M. S., P. Stojanov, C. H. Mermel, J. T. Robinson, L. A. Garraway, T. R. Golub, M. Meyerson, et al. (2014). "Discovery and saturation analysis of cancer genes across 21 tumour types." Nature **505**(7484): 495-501.

Lee, C. M. and E. P. Reddy (1999). "The v-myc oncogene." Oncogene **18**(19): 2997-3003.

Lee, J., A. Kumagai and W. G. Dunphy (2007). "The Rad9-Hus1-Rad1 checkpoint clamp regulates interaction of TopBP1 with ATR." J Biol Chem **282**(38): 28036-28044.

Legouy, E., R. DePinho, K. Zimmerman, R. Collum, G. Yancopoulos, L. Mitsock, R. Kriz, et al. (1987). "Structure and expression of the murine L-myc gene." EMBO J **6**(11): 3359-3366.

Lehmann, A. R., A. Niimi, T. Ogi, S. Brown, S. Sabbioneda, J. F. Wing, P. L. Kannouche, et al. (2007). "Translesion synthesis: Y-family polymerases and the polymerase switch." DNA Repair (Amst) **6**(7): 891-899.

Lemacon, D., J. Jackson, A. Quinet, J. R. Brickner, S. Li, S. Yazinski, Z. You, et al. (2017). "MRE11 and EXO1 nucleases degrade reversed forks and elicit MUS81-dependent fork rescue in BRCA2-deficient cells." Nat Commun **8**(1): 860.

Leone, G., R. Sears, E. Huang, R. Rempel, F. Nuckolls, C. H. Park, P. Giangrande, et al. (2001). "Myc requires distinct E2F activities to induce S phase and apoptosis." Mol Cell **8**(1): 105-113.

Letessier, A., G. A. Millot, S. Koundrioukoff, A. M. Lachages, N. Vogt, R. S. Hansen, B. Malfoy, et al. (2011). "Cell-type-specific replication initiation programs set fragility of the FRA3B fragile site." Nature **470**(7332): 120-123.

Leukemia and Lymphoma Society (2018). "Facts and Statistics." Retrieved April 28, 2018, from https://www.lls.org/facts-and-statistics/facts-and-statistics-overview.

Levens, D. (2002). "Disentangling the MYC web." Proc Natl Acad Sci U S A **99**(9): 5757-5759.

Lewis, J. S., S. Jergic and N. E. Dixon (2016). Chapter Two - The E. coli DNA Replication Fork. The Enzymes. L. S. Kaguni and M. T. Oliveira, Academic Press. **39:** 31-88.

Li, L. H., C. Nerlov, G. Prendergast, D. MacGregor and E. B. Ziff (1994). "c-Myc represses transcription in vivo by a novel mechanism dependent on the initiator element and Myc box II." EMBO J **13**(17): 4070-4079.

Li, Q. and C. V. Dang (1999). "c-Myc overexpression uncouples DNA replication from mitosis." Mol Cell Biol **19**(8): 5339-5351.

Lin, C. Y., J. Loven, P. B. Rahl, R. M. Paranal, C. B. Burge, J. E. Bradner, T. I. Lee, et al. (2012). "Transcriptional amplification in tumor cells with elevated c-Myc." Cell **151**(1): 56-67.

Liu, Q., S. Guntuku, X. S. Cui, S. Matsuoka, D. Cortez, K. Tamai, G. Luo, et al. (2000). "Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint." Genes Dev **14**(12): 1448-1459.

Lopez-Girona, A., K. Tanaka, X. B. Chen, B. A. Baber, C. H. McGowan and P. Russell (2001). "Serine-345 is required for Rad3-dependent phosphorylation and function of checkpoint kinase Chk1 in fission yeast." Proc Natl Acad Sci U S A **98**(20): 11289-11294.

Lucca, C., F. Vanoli, C. Cotta-Ramusino, A. Pellicioli, G. Liberi, J. Haber and M. Foiani (2004). "Checkpoint-mediated control of replisome-fork association and signalling in response to replication pausing." Oncogene **23**(6): 1206-1213.

Lundberg, A. S. and R. A. Weinberg (1998). "Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-cdk complexes." Mol Cell Biol **18**(2): 753-761.

Lutterbach, B. and S. R. Hann (1994). "Hierarchical phosphorylation at N-terminal transformation-sensitive sites in c-Myc protein is regulated by mitogens and in mitosis." Mol Cell Biol **14**(8): 5510-5522.

Macheret, M. and T. D. Halazonetis (2015). "DNA replication stress as a hallmark of cancer." Annu Rev Pathol **10**: 425-448.

Macheret, M. and T. D. Halazonetis (2018). "Intragenic origins due to short G1 phases underlie oncogene-induced DNA replication stress." Nature **555**(7694): 112-116.

Machwe, A., L. Xiao, J. Groden and D. K. Orren (2006). "The Werner and Bloom syndrome proteins catalyze regression of a model replication fork." Biochemistry **45**(47): 13939-13946.

Machwe, A., L. Xiao, R. G. Lloyd, E. Bolt and D. K. Orren (2007). "Replication fork regression in vitro by the Werner syndrome protein (WRN): holliday junction formation,

the effect of leading arm structure and a potential role for WRN exonuclease activity." Nucleic Acids Res **35**(17): 5729-5747.

Maclean, K. H., U. B. Keller, C. Rodriguez-Galindo, J. A. Nilsson and J. L. Cleveland (2003). "c-Myc augments gamma irradiation-induced apoptosis by suppressing Bcl-XL." Mol Cell Biol **23**(20): 7256-7270.

Mai, S., J. Hanley-Hyde and M. Fluri (1996). "c-Myc overexpression associated DHFR gene amplification in hamster, rat, mouse and human cell lines." Oncogene **12**(2): 277-288.

Mailand, N., I. Gibbs-Seymour and S. Bekker-Jensen (2013). "Regulation of PCNA-protein interactions for genome stability." Nat Rev Mol Cell Biol **14**(5): 269-282.

Majka, J., A. Niedziela-Majka and P. M. Burgers (2006). "The checkpoint clamp activates Mec1 kinase during initiation of the DNA damage checkpoint." Mol Cell **24**(6): 891-901.

Malumbres, M. and M. Barbacid (2009). "Cell cycle, CDKs and cancer: a changing paradigm." Nat Rev Cancer **9**(3): 153-166.

Marteijn, J. A., H. Lans, W. Vermeulen and J. H. Hoeijmakers (2014). "Understanding nucleotide excision repair and its roles in cancer and ageing." Nat Rev Mol Cell Biol **15**(7): 465-481.

Masai, H., C. Taniyama, K. Ogino, E. Matsui, N. Kakusho, S. Matsumoto, J. M. Kim, et al. (2006). "Phosphorylation of MCM4 by Cdc7 kinase facilitates its interaction with Cdc45 on the chromatin." J Biol Chem **281**(51): 39249-39261.

Mason, A. C., R. P. Rambo, B. Greer, M. Pritchett, J. A. Tainer, D. Cortez and B. F. Eichman (2014). "A structure-specific nucleic acid-binding domain conserved among DNA repair proteins." Proc Natl Acad Sci U S A **111**(21): 7618-7623.

Mateyak, M. K., A. J. Obaya, S. Adachi and J. M. Sedivy (1997). "Phenotypes of c-Myc-deficient rat fibroblasts isolated by targeted homologous recombination." Cell Growth Differ **8**(10): 1039-1048.

Matos, J., M. G. Blanco, S. Maslen, J. M. Skehel and S. C. West (2011). "Regulatory control of the resolution of DNA recombination intermediates during meiosis and mitosis." Cell **147**(1): 158-172.

Matsushime, H., M. E. Ewen, D. K. Strom, J. Y. Kato, S. K. Hanks, M. F. Roussel and C. J. Sherr (1992). "Identification and properties of an atypical catalytic subunit (p34PSK-J3/cdk4) for mammalian D type G1 cyclins." Cell **71**(2): 323-334.

Matsuzaki, K., V. Borel, C. A. Adelman, D. Schindler and S. J. Boulton (2015). "FANCJ suppresses microsatellite instability and lymphomagenesis independent of the Fanconi anemia pathway." Genes Dev **29**(24): 2532-2546.

Maya-Mendoza, A., J. Ostrakova, M. Kosar, A. Hall, P. Duskova, M. Mistrik, J. M. Merchut-Maya, et al. (2015). "Myc and Ras oncogenes engage different energy metabolism programs and evoke distinct patterns of oxidative and DNA replication stress." Mol Oncol **9**(3): 601-616.

Maya-Mendoza, A., E. Petermann, D. A. Gillespie, K. W. Caldecott and D. A. Jackson (2007). "Chk1 regulates the density of active replication origins during the vertebrate S phase." EMBO J **26**(11): 2719-2731.

McMahon, S. B., H. A. Van Buskirk, K. A. Dugan, T. D. Copeland and M. D. Cole (1998). "The novel ATM-related protein TRRAP is an essential cofactor for the c-Myc and E2F oncoproteins." Cell **94**(3): 363-374.

McMahon, S. B., M. A. Wood and M. D. Cole (2000). "The essential cofactor TRRAP recruits the histone acetyltransferase hGCN5 to c-Myc." Mol Cell Biol **20**(2): 556-562.

McMurray, C. T. (2010). "Mechanisms of trinucleotide repeat instability during human development." Nat Rev Genet **11**(11): 786-799.

Mechali, M. (2010). "Eukaryotic DNA replication origins: many choices for appropriate answers." Nat Rev Mol Cell Biol **11**(10): 728-738.

Mellon, P., A. Pawson, K. Bister, G. S. Martin and P. H. Duesberg (1978). "Specific RNA sequences and gene products of MC29 avian acute leukemia virus." Proc Natl Acad Sci U S A **75**(12): 5874-5878.

Menssen, A. and H. Hermeking (2002). "Characterization of the c-MYC-regulated transcriptome by SAGE: identification and analysis of c-MYC target genes." Proc Natl Acad Sci U S A **99**(9): 6274-6279.

Meyer, N. and L. Z. Penn (2008). "Reflecting on 25 years with MYC." Nat Rev Cancer **8**(12): 976-990.

Meyerson, M. and E. Harlow (1994). "Identification of G1 kinase activity for cdk6, a novel cyclin D partner." Mol Cell Biol **14**(3): 2077-2086.

Michalak, E. M., C. J. Vandenberg, A. R. Delbridge, L. Wu, C. L. Scott, J. M. Adams and A. Strasser (2010). "Apoptosis-promoted tumorigenesis: gamma-irradiation-induced thymic lymphomagenesis requires Puma-driven leukocyte death." Genes Dev **24**(15): 1608-1613.

Mizushima, T., N. Takahashi and B. Stillman (2000). "Cdc6p modulates the structure and DNA binding activity of the origin recognition complex in vitro." Genes Dev **14**(13): 1631-1641.

Montagnoli, A., B. Valsasina, D. Brotherton, S. Troiani, S. Rainoldi, P. Tenca, A. Molinari, et al. (2006). "Identification of Mcm2 phosphorylation sites by S-phase-regulating kinases." J Biol Chem **281**(15): 10281-10290.

Morita, Y., H. Ema and H. Nakauchi (2010). "Heterogeneity and hierarchy within the most primitive hematopoietic stem cell compartment." J Exp Med **207**(6): 1173-1182.

Moser, R., M. Toyoshima, K. Robinson, K. E. Gurley, H. L. Howie, J. Davison, M. Morgan, et al. (2012). "MYC-driven tumorigenesis is inhibited by WRN syndrome gene deficiency." Mol Cancer Res **10**(4): 535-545.

Munoz, I. M., K. Hain, A. C. Declais, M. Gardiner, G. W. Toh, L. Sanchez-Pulido, J. M. Heuckmann, et al. (2009). "Coordination of structure-specific nucleases by human SLX4/BTBD12 is required for DNA repair." Mol Cell **35**(1): 116-127.

Murga, M., S. Campaner, A. J. Lopez-Contreras, L. I. Toledo, R. Soria, M. F. Montana, L. Artista, et al. (2011). "Exploiting oncogene-induced replicative stress for the selective killing of Myc-driven tumors." Nat Struct Mol Biol **18**(12): 1331-1335.

Musgrove, E. A., C. E. Caldon, J. Barraclough, A. Stone and R. L. Sutherland (2011). "Cyclin D as a therapeutic target in cancer." Nat Rev Cancer **11**(8): 558-572.

Muthuswami, R., P. A. Truman, L. D. Mesner and J. W. Hockensmith (2000). "A eukaryotic SWI2/SNF2 domain, an exquisite detector of double-stranded to single-stranded DNA transition elements." J Biol Chem **275**(11): 7648-7655.

Nag, S., J. Qin, K. S. Srivenugopal, M. Wang and R. Zhang (2013). "The MDM2-p53 pathway revisited." J Biomed Res **27**(4): 254-271.

Nam, E. A. and D. Cortez (2011). "ATR signalling: more than meeting at the fork." Biochem J **436**(3): 527-536.

National Cancer Institute (2017). "Cancer Statistics." Retrieved April 26, 2018, from https://www.cancer.gov/about-cancer/understanding/statistics.

Neel, B. G., S. C. Jhanwar, R. S. Chaganti and W. S. Hayward (1982). "Two human c-onc genes are located on the long arm of chromosome 8." Proc Natl Acad Sci U S A **79**(24): 7842-7846.

Neelsen, K. J. and M. Lopes (2015). "Replication fork reversal in eukaryotes: from dead end to dynamic response." Nat Rev Mol Cell Biol **16**(4): 207-220.

Neelsen, K. J., I. M. Zanini, R. Herrador and M. Lopes (2013). "Oncogenes induce genotoxic stress by mitotic processing of unusual replication intermediates." J Cell Biol **200**(6): 699-708.

Nelson, D. M., X. Ye, C. Hall, H. Santos, T. Ma, G. D. Kao, T. J. Yen, et al. (2002). "Coupling of DNA synthesis and histone synthesis in S phase independent of cyclin/cdk2 activity." Mol Cell Biol **22**(21): 7459-7472.

Nesbit, C. E., L. E. Grove, X. Yin and E. V. Prochownik (1998). "Differential apoptotic behaviors of c-myc, N-myc, and L-myc oncoproteins." Cell Growth Differ **9**(9): 731-741.

Nesbit, C. E., J. M. Tersak and E. V. Prochownik (1999). "MYC oncogenes and human neoplastic disease." Oncogene **18**(19): 3004-3016.

Nie, Z., G. Hu, G. Wei, K. Cui, A. Yamane, W. Resch, R. Wang, et al. (2012). "c-Myc is a universal amplifier of expressed genes in lymphocytes and embryonic stem cells." Cell **151**(1): 68-79.

Nombela-Arrieta, C., J. Ritz and L. E. Silberstein (2011). "The elusive nature and function of mesenchymal stem cells." Nat Rev Mol Cell Biol **12**(2): 126-131.

O'Driscoll, M., V. L. Ruiz-Perez, C. G. Woods, P. A. Jeggo and J. A. Goodship (2003). "A splicing mutation affecting expression of ataxia-telangiectasia and Rad3-related protein (ATR) results in Seckel syndrome." Nat Genet **33**(4): 497-501.

Oguro, H., L. Ding and S. J. Morrison (2013). "SLAM family markers resolve functionally distinct subpopulations of hematopoietic stem cells and multipotent progenitors." Cell Stem Cell **13**(1): 102-116.

Orford, K. W. and D. T. Scadden (2008). "Deconstructing stem cell self-renewal: genetic insights into cell-cycle regulation." Nat Rev Genet **9**(2): 115-128.

Otto, T. and P. Sicinski (2017). "Cell cycle proteins as promising targets in cancer therapy." Nat Rev Cancer **17**(2): 93-115.

Ozenne, P., B. Eymin, E. Brambilla and S. Gazzeri (2010). "The ARF tumor suppressor: structure, functions and status in cancer." Int J Cancer **127**(10): 2239-2247.

Ozeri-Galai, E., R. Lebofsky, A. Rahat, A. C. Bester, A. Bensimon and B. Kerem (2011). "Failure of origin activation in response to fork stalling leads to chromosomal instability at fragile sites." Mol Cell **43**(1): 122-131.

Parker, M. W., M. R. Botchan and J. M. Berger (2017). "Mechanisms and regulation of DNA replication initiation in eukaryotes." Crit Rev Biochem Mol Biol **52**(2): 107-144.

Payne, G. S., J. M. Bishop and H. E. Varmus (1982). "Multiple arrangements of viral DNA and an activated host oncogene in bursal lymphomas." Nature **295**(5846): 209-214.

Peng, C. Y., P. R. Graves, R. S. Thoma, Z. Wu, A. S. Shaw and H. Piwnica-Worms (1997). "Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216." Science **277**(5331): 1501-1505.

Petermann, E., M. L. Orta, N. Issaeva, N. Schultz and T. Helleday (2010). "Hydroxyurea-stalled replication forks become progressively inactivated and require two different RAD51-mediated pathways for restart and repair." Mol Cell **37**(4): 492-502.

Peukert, K., P. Staller, A. Schneider, G. Carmichael, F. Hanel and M. Eilers (1997). "An alternative pathway for gene regulation by Myc." EMBO J **16**(18): 5672-5686.

Poole, L. A. and D. Cortez (2017). "Functions of SMARCAL1, ZRANB3, and HLTF in maintaining genome stability." Crit Rev Biochem Mol Biol **52**(6): 696-714.

Poole, L. A., R. Zhao, G. G. Glick, C. A. Lovejoy, C. M. Eischen and D. Cortez (2015). "SMARCAL1 maintains telomere integrity during DNA replication." Proc Natl Acad Sci U S A **112**(48): 14864-14869.

Postow, L., E. M. Woo, B. T. Chait and H. Funabiki (2009). "Identification of SMARCAL1 as a component of the DNA damage response." J Biol Chem **284**(51): 35951-35961.

Prakash, S., R. E. Johnson and L. Prakash (2005). "Eukaryotic translesion synthesis DNA polymerases: specificity of structure and function." Annu Rev Biochem **74**: 317-353.

Puccetti, M. V., M. A. Fischer, M. P. Arrate, K. L. Boyd, R. J. Duszynski, R. Betous, D. Cortez, et al. (2017). "Defective replication stress response inhibits lymphomagenesis and impairs lymphocyte reconstitution." Oncogene **36**(18): 2553-2564.

Pusapati, R. V., R. J. Rounbehler, S. Hong, J. T. Powers, M. Yan, K. Kiguchi, M. J. McArthur, et al. (2006). "ATM promotes apoptosis and suppresses tumorigenesis in response to Myc." Proc Natl Acad Sci U S A **103**(5): 1446-1451.

Quelle, D. E., F. Zindy, R. A. Ashmun and C. J. Sherr (1995). "Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest." Cell **83**(6): 993-1000.

Quinet, A., A. T. Vessoni, C. R. Rocha, V. Gottifredi, D. Biard, A. Sarasin, C. F. Menck, et al. (2014). "Gap-filling and bypass at the replication fork are both active mechanisms for tolerance of low-dose ultraviolet-induced DNA damage in the human genome." DNA Repair (Amst) **14**: 27-38.

Ragland, R. L., S. Patel, R. S. Rivard, K. Smith, A. A. Peters, A. K. Bielinsky and E. J. Brown (2013). "RNF4 and PLK1 are required for replication fork collapse in ATR-deficient cells." Genes Dev **27**(20): 2259-2273.

Ralf, C., I. D. Hickson and L. Wu (2006). "The Bloom's syndrome helicase can promote the regression of a model replication fork." J Biol Chem **281**(32): 22839-22846.

Rao, P. H., V. V. Murty, D. C. Louie and R. S. Chaganti (1998). "Nonsyntenic amplification of MYC with CDK4 and MDM2 in a malignant mixed tumor of salivary gland." Cancer Genet Cytogenet **105**(2): 160-163.

Ray Chaudhuri, A., A. K. Ahuja, R. Herrador and M. Lopes (2015). "Poly(ADP-ribosyl) glycohydrolase prevents the accumulation of unusual replication structures during unperturbed S phase." Mol Cell Biol **35**(5): 856-865.

Ray Chaudhuri, A., Y. Hashimoto, R. Herrador, K. J. Neelsen, D. Fachinetti, R. Bermejo, A. Cocito, et al. (2012). "Topoisomerase I poisoning results in PARP-mediated replication fork reversal." Nat Struct Mol Biol **19**(4): 417-423.

Reimann, M., C. Loddenkemper, C. Rudolph, I. Schildhauer, B. Teichmann, H. Stein, B. Schlegelberger, et al. (2007). "The Myc-evoked DNA damage response accounts for treatment resistance in primary lymphomas in vivo." Blood **110**(8): 2996-3004.

Remus, D., F. Beuron, G. Tolun, J. D. Griffith, E. P. Morris and J. F. Diffley (2009). "Concerted loading of Mcm2-7 double hexamers around DNA during DNA replication origin licensing." Cell **139**(4): 719-730.

Remus, D. and J. F. Diffley (2009). "Eukaryotic DNA replication control: lock and load, then fire." Curr Opin Cell Biol **21**(6): 771-777.

Robbins, S. L., R. S. Cotran, V. Kumar, A. K. Abbas and J. C. Aster (2015). Pathologic basis of disease. Philadelphia, PA, Saunders Elsevier.

Robinson, K., N. Asawachaicharn, D. A. Galloway and C. Grandori (2009). "c-Myc accelerates S-phase and requires WRN to avoid replication stress." PLoS One **4**(6): e5951.

Rohban, S. and S. Campaner (2015). "Myc induced replicative stress response: How to cope with it and exploit it." Biochim Biophys Acta **1849**(5): 517-524.

Rosenwald, I. B. (1996). "Upregulated expression of the genes encoding translation initiation factors eIF-4E and eIF-2alpha in transformed cells." Cancer Lett **102**(1-2): 113-123.

Roussel, M., S. Saule, C. Lagrou, C. Rommens, H. Beug, T. Graf and D. Stehelin (1979). "Three new types of viral oncogene of cellular origin specific for haematopoietic cell transformation." Nature **281**(5731): 452-455.

Sabnis, H. S., R. R. Somasagara and K. D. Bunting (2017). "Targeting MYC Dependence by Metabolic Inhibitors in Cancer." Genes (Basel) **8**(4).

Sabo, A., T. R. Kress, M. Pelizzola, S. de Pretis, M. M. Gorski, A. Tesi, M. J. Morelli, et al. (2014). "Selective transcriptional regulation by Myc in cellular growth control and lymphomagenesis." Nature **511**(7510): 488-492.

Sado, T., H. Kamisaku and E. Kubo (1991). "Bone marrow-thymus interactions during thymic lymphomagenesis induced by fractionated radiation exposure in B10 mice: analysis using bone marrow transplantation between Thy 1 congenic mice." J Radiat Res **32 Suppl 2**: 168-180.

Saintigny, Y., F. Delacote, G. Vares, F. Petitot, S. Lambert, D. Averbeck and B. S. Lopez (2001). "Characterization of homologous recombination induced by replication inhibition in mammalian cells." EMBO J **20**(14): 3861-3870.

Sakofsky, C. J. and A. Malkova (2017). "Break induced replication in eukaryotes: mechanisms, functions, and consequences." Crit Rev Biochem Mol Biol **52**(4): 395-413.

Saldivar, J. C., D. Cortez and K. A. Cimprich (2017). "The essential kinase ATR: ensuring faithful duplication of a challenging genome." Nat Rev Mol Cell Biol **18**(10): 622-636.

Salghetti, S. E., A. A. Caudy, J. G. Chenoweth and W. P. Tansey (2001). "Regulation of transcriptional activation domain function by ubiquitin." Science **293**(5535): 1651-1653.

Sanchez, Y., C. Wong, R. S. Thoma, R. Richman, Z. Wu, H. Piwnica-Worms and S. J. Elledge (1997). "Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage to Cdk regulation through Cdc25." Science **277**(5331): 1497-1501.

Sanjiv, K., A. Hagenkort, J. M. Calderon-Montano, T. Koolmeister, P. M. Reaper, O. Mortusewicz, S. A. Jacques, et al. (2016). "Cancer-Specific Synthetic Lethality between ATR and CHK1 Kinase Activities." Cell Rep **14**(2): 298-309.

Sankar, N., R. K. Kadeppagari and B. Thimmapaya (2009). "c-Myc-induced aberrant DNA synthesis and activation of DNA damage response in p300 knockdown cells." J Biol Chem **284**(22): 15193-15205.

Sanyal, M., M. Morimoto, A. Baradaran-Heravi, K. Choi, N. Kambham, K. Jensen, S. Dutt, et al. (2015). "Lack of IL7Ralpha expression in T cells is a hallmark of T-cell immunodeficiency in Schimke immuno-osseous dysplasia (SIOD)." Clin Immunol **161**(2): 355-365.

Sarkies, P., P. Murat, L. G. Phillips, K. J. Patel, S. Balasubramanian and J. E. Sale (2012). "FANCJ coordinates two pathways that maintain epigenetic stability at G-quadruplex DNA." Nucleic Acids Res **40**(4): 1485-1498.

Satyanarayana, A. and P. Kaldis (2009). "Mammalian cell-cycle regulation: several Cdks, numerous cyclins and diverse compensatory mechanisms." Oncogene **28**(33): 2925-2939.

Schlacher, K., N. Christ, N. Siaud, A. Egashira, H. Wu and M. Jasin (2011). "Double-strand break repair-independent role for BRCA2 in blocking stalled replication fork degradation by MRE11." Cell **145**(4): 529-542.

Schlacher, K., H. Wu and M. Jasin (2012). "A distinct replication fork protection pathway connects Fanconi anemia tumor suppressors to RAD51-BRCA1/2." Cancer Cell **22**(1): 106-116.

Schoppy, D. W., R. L. Ragland, O. Gilad, N. Shastri, A. A. Peters, M. Murga, O. Fernandez-Capetillo, et al. (2012). "Oncogenic stress sensitizes murine cancers to hypomorphic suppression of ATR." J Clin Invest **122**(1): 241-252.

Sclafani, R. A. and T. M. Holzen (2007). "Cell cycle regulation of DNA replication." Annu Rev Genet **41**: 237-280.

Sebesta, M., C. D. O. Cooper, A. Ariza, C. J. Carnie and D. Ahel (2017). "Structural insights into the function of ZRANB3 in replication stress response." Nat Commun **8**: 15847.

Seoane, J., H. V. Le and J. Massague (2002). "Myc suppression of the p21(Cip1) Cdk inhibitor influences the outcome of the p53 response to DNA damage." Nature **419**(6908): 729-734.

Seoane, J., C. Pouponnot, P. Staller, M. Schader, M. Eilers and J. Massague (2001). "TGFbeta influences Myc, Miz-1 and Smad to control the CDK inhibitor p15INK4b." Nat Cell Biol **3**(4): 400-408.

Sheiness, D. and J. M. Bishop (1979). "DNA and RNA from uninfected vertebrate cells contain nucleotide sequences related to the putative transforming gene of avian myelocytomatosis virus." J Virol **31**(2): 514-521.

Sheiness, D., L. Fanshier and J. M. Bishop (1978). "Identification of nucleotide sequences which may encode the oncogenic capacity of avian retrovirus MC29." J Virol **28**(2): 600-610.

Sherr, C. J. and J. M. Roberts (2004). "Living with or without cyclins and cyclin-dependent kinases." Genes Dev **18**(22): 2699-2711.

Shi, Y., J. M. Glynn, L. J. Guilbert, T. G. Cotter, R. P. Bissonnette and D. R. Green (1992). "Role for c-myc in activation-induced apoptotic cell death in T cell hybridomas." Science **257**(5067): 212-214.

Shibuya, H., M. Yoneyama, J. Ninomiya-Tsuji, K. Matsumoto and T. Taniguchi (1992). "IL-2 and EGF receptors stimulate the hematopoietic cell cycle via different signaling pathways: demonstration of a novel role for c-myc." Cell **70**(1): 57-67.

Shim, H., C. Dolde, B. C. Lewis, C. S. Wu, G. Dang, R. A. Jungmann, R. Dalla-Favera, et al. (1997). "c-Myc transactivation of LDH-A: implications for tumor metabolism and growth." Proc Natl Acad Sci U S A **94**(13): 6658-6663.

Shima, N. and K. D. Pederson (2017). "Dormant origins as a built-in safeguard in eukaryotic DNA replication against genome instability and disease development." DNA Repair (Amst) **56**: 166-173.

Singhi, A. D., A. Cimino-Mathews, R. B. Jenkins, F. Lan, S. R. Fink, H. Nassar, R. Vang, et al. (2012). "MYC gene amplification is often acquired in lethal distant breast cancer metastases of unamplified primary tumors." Mod Pathol **25**(3): 378-387.

Smits, V. A., P. M. Reaper and S. P. Jackson (2006). "Rapid PIKK-dependent release of Chk1 from chromatin promotes the DNA-damage checkpoint response." Curr Biol **16**(2): 150-159.

Sogo, J. M., M. Lopes and M. Foiani (2002). "Fork reversal and ssDNA accumulation at stalled replication forks owing to checkpoint defects." Science **297**(5581): 599-602.

Sorensen, C. S. and R. G. Syljuasen (2012). "Safeguarding genome integrity: the checkpoint kinases ATR, CHK1 and WEE1 restrain CDK activity during normal DNA replication." Nucleic Acids Res **40**(2): 477-486.

Speck, C. and B. Stillman (2007). "Cdc6 ATPase activity regulates ORC x Cdc6 stability and the selection of specific DNA sequences as origins of DNA replication." J Biol Chem **282**(16): 11705-11714.

Spranger, J., G. K. Hinkel, H. Stoss, W. Thoenes, D. Wargowski and F. Zepp (1991). "Schimke immuno-osseous dysplasia: a newly recognized multisystem disease." J Pediatr **119**(1 Pt 1): 64-72.

Srinivasan, S. V., D. Dominguez-Sola, L. C. Wang, O. Hyrien and J. Gautier (2013). "Cdc45 is a critical effector of myc-dependent DNA replication stress." Cell Rep **3**(5): 1629-1639.

Staller, P., K. Peukert, A. Kiermaier, J. Seoane, J. Lukas, H. Karsunky, T. Moroy, et al. (2001). "Repression of p15INK4b expression by Myc through association with Miz-1." Nat Cell Biol **3**(4): 392-399.

Stasik, C. J., H. Nitta, W. Zhang, C. H. Mosher, J. R. Cook, R. R. Tubbs, J. M. Unger, et al. (2010). "Increased MYC gene copy number correlates with increased mRNA levels in diffuse large B-cell lymphoma." Haematologica **95**(4): 597-603.

Steckel, M., M. Molina-Arcas, B. Weigelt, M. Marani, P. H. Warne, H. Kuznetsov, G. Kelly, et al. (2012). "Determination of synthetic lethal interactions in KRAS oncogene-

dependent cancer cells reveals novel therapeutic targeting strategies." Cell Res **22**(8): 1227-1245.

Steffen, D. (1984). "Proviruses are adjacent to c-myc in some murine leukemia virus-induced lymphomas." Proc Natl Acad Sci U S A **81**(7): 2097-2101.

Stone, J., T. de Lange, G. Ramsay, E. Jakobovits, J. M. Bishop, H. Varmus and W. Lee (1987). "Definition of regions in human c-myc that are involved in transformation and nuclear localization." Mol Cell Biol **7**(5): 1697-1709.

Strieder, V. and W. Lutz (2002). "Regulation of N-myc expression in development and disease." Cancer Lett **180**(2): 107-119.

Sun, Y., P. Y. Liu, C. J. Scarlett, A. Malyukova, B. Liu, G. M. Marshall, K. L. MacKenzie, et al. (2014). "Histone deacetylase 5 blocks neuroblastoma cell differentiation by interacting with N-Myc." Oncogene **33**(23): 2987-2994.

Taglialatela, A., S. Alvarez, G. Leuzzi, V. Sannino, L. Ranjha, J. W. Huang, C. Madubata, et al. (2017). "Restoration of Replication Fork Stability in BRCA1- and BRCA2-Deficient Cells by Inactivation of SNF2-Family Fork Remodelers." Mol Cell **68**(2): 414-430 e418.

Takizawa, H., R. R. Regoes, C. S. Boddupalli, S. Bonhoeffer and M. G. Manz (2011). "Dynamic variation in cycling of hematopoietic stem cells in steady state and inflammation." J Exp Med **208**(2): 273-284.

Tansey, W. P. (2014). "Mammalian Myc proteins and cancer." New Journal of Science **2014**.

Taub, R., I. Kirsch, C. Morton, G. Lenoir, D. Swan, S. Tronick, S. Aaronson, et al. (1982). "Translocation of the c-myc gene into the immunoglobulin heavy chain locus in human Burkitt lymphoma and murine plasmacytoma cells." Proc Natl Acad Sci U S A **79**(24): 7837-7841.

Toledo, L. I., M. Altmeyer, M. B. Rask, C. Lukas, D. H. Larsen, L. K. Povlsen, S. Bekker-Jensen, et al. (2013). "ATR prohibits replication catastrophe by preventing global exhaustion of RPA." Cell **155**(5): 1088-1103.

Tsantoulis, P. K., A. Kotsinas, P. P. Sfikakis, K. Evangelou, M. Sideridou, B. Levy, L. Mo, et al. (2008). "Oncogene-induced replication stress preferentially targets common fragile sites in preneoplastic lesions. A genome-wide study." Oncogene **27**(23): 3256-3264.

Uribesalgo, I., M. Buschbeck, A. Gutierrez, S. Teichmann, S. Demajo, B. Kuebler, J. F. Nomdedeu, et al. (2011). "E-box-independent regulation of transcription and differentiation by MYC." Nat Cell Biol **13**(12): 1443-1449.

Vafa, O., M. Wade, S. Kern, M. Beeche, T. K. Pandita, G. M. Hampton and G. M. Wahl (2002). "c-Myc can induce DNA damage, increase reactive oxygen species, and mitigate p53 function: a mechanism for oncogene-induced genetic instability." Mol Cell **9**(5): 1031-1044.

Vennstrom, B., D. Sheiness, J. Zabielski and J. M. Bishop (1982). "Isolation and characterization of c-myc, a cellular homolog of the oncogene (v-myc) of avian myelocytomatosis virus strain 29." J Virol **42**(3): 773-779.

Vousden, K. H. and C. Prives (2009). "Blinded by the Light: The Growing Complexity of p53." Cell **137**(3): 413-431.

Vujanovic, M., J. Krietsch, M. C. Raso, N. Terraneo, R. Zellweger, J. A. Schmid, A. Taglialatela, et al. (2017). "Replication Fork Slowing and Reversal upon DNA Damage Require PCNA Polyubiquitination and ZRANB3 DNA Translocase Activity." Mol Cell **67**(5): 882-890 e885.

Wagner, A. J., J. M. Kokontis and N. Hay (1994). "Myc-mediated apoptosis requires wild-type p53 in a manner independent of cell cycle arrest and the ability of p53 to induce p21waf1/cip1." Genes Dev **8**(23): 2817-2830.

Wallace, S. S. (2014). "Base excision repair: a critical player in many games." DNA Repair (Amst) **19**: 14-26.

Walter, D., A. Lier, A. Geiselhart, F. B. Thalheimer, S. Huntscha, M. C. Sobotta, B. Moehrle, et al. (2015). "Exit from dormancy provokes DNA-damage-induced attrition in haematopoietic stem cells." Nature **520**(7548): 549-552.

Walworth, N. C. and R. Bernards (1996). "rad-dependent response of the chk1-encoded protein kinase at the DNA damage checkpoint." Science **271**(5247): 353-356.

Wang, H., J. Guan, H. Wang, A. R. Perrault, Y. Wang and G. Iliakis (2001). "Replication protein A2 phosphorylation after DNA damage by the coordinated action of ataxia telangiectasia-mutated and DNA-dependent protein kinase." Cancer Res **61**(23): 8554-8563.

Wang, J. C. (2002). "Cellular roles of DNA topoisomerases: a molecular perspective." Nat Rev Mol Cell Biol **3**(6): 430-440.

Ward, I. M. and J. Chen (2001). "Histone H2AX is phosphorylated in an ATR-dependent manner in response to replicational stress." J Biol Chem **276**(51): 47759-47762.

Weinberg, R. A. (2014). The biology of cancer.

Welcker, M., A. Orian, J. E. Grim, R. N. Eisenman and B. E. Clurman (2004). "A nucleolar isoform of the Fbw7 ubiquitin ligase regulates c-Myc and cell size." Curr Biol **14**(20): 1852-1857.

Welcker, M., A. Orian, J. Jin, J. E. Grim, J. W. Harper, R. N. Eisenman and B. E. Clurman (2004). "The Fbw7 tumor suppressor regulates glycogen synthase kinase 3 phosphorylation-dependent c-Myc protein degradation." Proc Natl Acad Sci U S A **101**(24): 9085-9090.

Weston, R., H. Peeters and D. Ahel (2012). "ZRANB3 is a structure-specific ATP-dependent endonuclease involved in replication stress response." Genes Dev **26**(14): 1558-1572.

Wilson, N. K., D. G. Kent, F. Buettner, M. Shehata, I. C. Macaulay, F. J. Calero-Nieto, M. Sanchez Castillo, et al. (2015). "Combined Single-Cell Functional and Gene Expression Analysis Resolves Heterogeneity within Stem Cell Populations." Cell Stem Cell **16**(6): 712-724.

Wise, D. R., R. J. DeBerardinis, A. Mancuso, N. Sayed, X. Y. Zhang, H. K. Pfeiffer, I. Nissim, et al. (2008). "Myc regulates a transcriptional program that stimulates mitochondrial glutaminolysis and leads to glutamine addiction." Proc Natl Acad Sci U S A **105**(48): 18782-18787.

Wold, M. S. and T. Kelly (1988). "Purification and characterization of replication protein A, a cellular protein required for in vitro replication of simian virus 40 DNA." Proc Natl Acad Sci U S A **85**(8): 2523-2527.

Wu, Y., K. Shin-ya and R. M. Brosh, Jr. (2008). "FANCJ helicase defective in Fanconia anemia and breast cancer unwinds G-quadruplex DNA to defend genomic stability." Mol Cell Biol **28**(12): 4116-4128.

Xie, M., Y. Yen, T. K. Owonikoko, S. S. Ramalingam, F. R. Khuri, W. J. Curran, P. W. Doetsch, et al. (2014). "Bcl2 induces DNA replication stress by inhibiting ribonucleotide reductase." Cancer Res **74**(1): 212-223.

Yamamoto, R., Y. Morita, J. Ooehara, S. Hamanaka, M. Onodera, K. L. Rudolph, H. Ema, et al. (2013). "Clonal analysis unveils self-renewing lineage-restricted progenitors generated directly from hematopoietic stem cells." Cell **154**(5): 1112-1126.

Yang, L., D. Bryder, J. Adolfsson, J. Nygren, R. Mansson, M. Sigvardsson and S. E. Jacobsen (2005). "Identification of Lin(-)Sca1(+)kit(+)CD34(+)Flt3- short-term hematopoietic stem cells capable of rapidly reconstituting and rescuing myeloablated transplant recipients." Blood **105**(7): 2717-2723.

Yilmaz, O. H., M. J. Kiel and S. J. Morrison (2006). "SLAM family markers are conserved among hematopoietic stem cells from old and reconstituted mice and markedly increase their purity." Blood **107**(3): 924-930.

Yin, X. Y., L. Grove, N. S. Datta, M. W. Long and E. V. Prochownik (1999). "C-myc overexpression and p53 loss cooperate to promote genomic instability." Oncogene **18**(5): 1177-1184.

Youle, R. J. and A. Strasser (2008). "The BCL-2 protein family: opposing activities that mediate cell death." Nat Rev Mol Cell Biol **9**(1): 47-59.

Yu, C., H. Gan, J. Han, Z. X. Zhou, S. Jia, A. Chabes, G. Farrugia, et al. (2014). "Strand-specific analysis shows protein binding at replication forks and PCNA unloading from lagging strands when forks stall." Mol Cell **56**(4): 551-563.

Yuan, J., G. Ghosal and J. Chen (2009). "The annealing helicase HARP protects stalled replication forks." Genes Dev **23**(20): 2394-2399.

Yuan, J., G. Ghosal and J. Chen (2012). "The HARP-like domain-containing protein AH2/ZRANB3 binds to PCNA and participates in cellular response to replication stress." Mol Cell **47**(3): 410-421.

Yuan, Z., A. Riera, L. Bai, J. Sun, S. Nandi, C. Spanos, Z. A. Chen, et al. (2017). "Structural basis of Mcm2-7 replicative helicase loading by ORC-Cdc6 and Cdt1." Nat Struct Mol Biol **24**(3): 316-324.

Yunis, J. J. and A. L. Soreng (1984). "Constitutive fragile sites and cancer." Science **226**(4679): 1199-1204.

Yusufzai, T. and J. T. Kadonaga (2008). "HARP is an ATP-driven annealing helicase." Science **322**(5902): 748-750.

Yusufzai, T. and J. T. Kadonaga (2010). "Annealing helicase 2 (AH2), a DNA-rewinding motor with an HNH motif." Proc Natl Acad Sci U S A **107**(49): 20970-20973.

Yusufzai, T., X. Kong, K. Yokomori and J. T. Kadonaga (2009). "The annealing helicase HARP is recruited to DNA repair sites via an interaction with RPA." Genes Dev **23**(20): 2400-2404.

Zeller, K. I., X. Zhao, C. W. Lee, K. P. Chiu, F. Yao, J. T. Yustein, H. S. Ooi, et al. (2006). "Global mapping of c-Myc binding sites and target gene networks in human B cells." Proc Natl Acad Sci U S A **103**(47): 17834-17839.

Zellweger, R., D. Dalcher, K. Mutreja, M. Berti, J. A. Schmid, R. Herrador, A. Vindigni, et al. (2015). "Rad51-mediated replication fork reversal is a global response to genotoxic treatments in human cells." J Cell Biol **208**(5): 563-579.

Zeman, M. K. and K. A. Cimprich (2014). "Causes and consequences of replication stress." Nat Cell Biol **16**(1): 2-9.

Zhang, H., P. Gao, R. Fukuda, G. Kumar, B. Krishnamachary, K. I. Zeller, C. V. Dang, et al. (2007). "HIF-1 inhibits mitochondrial biogenesis and cellular respiration in VHL-deficient renal cell carcinoma by repression of C-MYC activity." Cancer Cell **11**(5): 407-420.

Zhang, X., X. Zhao, W. Fiskus, J. Lin, T. Lwin, R. Rao, Y. Zhang, et al. (2012). "Coordinated silencing of MYC-mediated miR-29 by HDAC3 and EZH2 as a therapeutic target of histone modification in aggressive B-Cell lymphomas." Cancer Cell **22**(4): 506-523.

Zhang, X. Y., L. M. DeSalle and S. B. McMahon (2006). "Identification of novel targets of MYC whose transcription requires the essential MbII domain." Cell Cycle **5**(3): 238-241.

Zimmerman, K. M., R. M. Jones, E. Petermann and P. A. Jeggo (2013). "Diminished origin-licensing capacity specifically sensitizes tumor cells to replication stress." Mol Cancer Res **11**(4): 370-380.

Zindy, F., C. M. Eischen, D. H. Randle, T. Kamijo, J. L. Cleveland, C. J. Sherr and M. F. Roussel (1998). "Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization." Genes Dev **12**(15): 2424-2433.

Zou, L. and S. J. Elledge (2003). "Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes." Science **300**(5625): 1542-1548.

Zou, L., D. Liu and S. J. Elledge (2003). "Replication protein A-mediated recruitment and activation of Rad17 complexes." Proc Natl Acad Sci U S A **100**(24): 13827-13832.

Zou, Y., Y. Liu, X. Wu and S. M. Shell (2006). "Functions of human replication protein A (RPA): from DNA replication to DNA damage and stress responses." J Cell Physiol **208**(2): 267-273.