Chromatin structuring factor HMGA2 controls subtelomere stability and cancer chemosensitivity

Syed Moiz Ahmed

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**CHROMATIN STRUCTURING FACTOR HMGA2
CONTROLS SUBTELOMERE STABILITY AND CANCER
CHEMOSENSITIVITY**

SYED MOIZ AHMED

SCHOOL OF BIOLOGICAL SCIENCES

2020

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SUBTELOMERE STABILITY AND CANCER
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SYED MOIZ AHMED

SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to the Nanyang Technological
University in partial fulfilment of the requirement for the
degree of Doctor of Philosophy

2020

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research done by me except where otherwise stated in this thesis. The thesis work has not been submitted for a degree or professional qualification to any other university or institution. I declare that this thesis is written by myself and is free of plagiarism and of sufficient grammatical clarity to be examined. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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[Prof. Peter Dröge]

Authorship Attribution Statement

This thesis contains material from two papers published in the following peer-reviewed journals in which I am listed as the first author.

Chapter **3.1 and 3.2** are published as **Ahmed, S. M.**, P. D. Ramani, S. Q. R. Wong, X. Zhao, R. Ivanyi-Nagy, T. C. Leong, C. Chua, Z. Li, H. Hentze, I. B. Tan, J. Yan, R. DasGupta and P. Droge. The chromatin structuring protein HMGA2 influences human subtelomere stability and cancer chemosensitivity. PLoS One 14(5): e0215696 (2019) DOI: 10.1371/journal.pone.0215696

The contributions of the co-authors are as follows:

- I, Dr. Priya Ramani (NTU) and Dr. Peter Dröge (NTU) conceptualized and designed the study.
- Manuscript was drafted, edited and revised by me, Dr. Priya Ramani (NTU) and Dr. Peter Dröge (NTU).
- Pulsed field gel electrophoresis was conducted by me and Dr. Priya Ramani (NTU). Data analysis and quantification of all the PFGE experiments was done by me.
- Cell cytotoxicity assays, southern blot experiments, protein expression analysis, dot blot / gel retardation assays were performed and evaluated by me.
- Dr. Zhizhong Li (Novartis, California) provided the H1299 HMGA2 knockout cell line.
- *In vivo* tumor xenograft studies were conducted by Mr. Stephen Wong (GIS, A*STAR). Western blot analysis was done by me and Mr. Stephen Wong (GIS, A*STAR).
- Dr. Tang Choong Leong (Singapore General Hospital) provided colorectal tumor samples for *in vivo* studies

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- The single molecule analysis that was done by Dr. Xiaodan Zhao (NUS) and Prof. Jie Yan (NUS) has not been included in the thesis.
- PFGE experiments, southern blot experiments, protein expression analysis and cell cytotoxicity assays done by Dr. Priya Ramani (NTU) has not been included in the thesis.
- The sequencing analysis that was done by Dr. Priya Ramani (NTU) and Dr. Roland Ivanyi-Nagy (NTU) has not been included in the thesis.

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- I performed all the experiments and analyzed the data.
- Manuscript was drafted, edited and revised by me and Prof. Peter Dröge (NTU).

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Abbreviations

2,3 M	2,3 hook mutant
AFM	atomic force microscopy
AML	acute myeloid leukemia
ATCC	american type culture collection
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia and Rad3 related
BRCA1/2	breast cancer type 1/2 gene
CAS9	CRISPR associated protein 9
CDC2/6	cell division cycle 2/6
CFS	common fragile site
CHEF	clamped homogeneous electric field
CHK1	checkpoint kinase 1
CPT	camptothecin
CRISPR	clustered regularly interspaced short palindromic repeats
CSC	cancer stem-like cells
CSM3	chromosome segregation in meiosis 3
DIG	digoxigenin
DMSO	dimethyl sulfoxide
DOX	doxycycline
DSB	double strand break
ERCC1	excision repair 1, endonuclease non-catalytic subunit
ESCs	embryonic stem cells
ETOP	etoposide
EXO1	exonuclease 1
FANCD2	fanconi anemia group D2
FEN1	flap structure-specific endonuclease
HMGA2	high mobility group AT hook 2
HRP	horseradish peroxidase

hTERT	human telomerase reverse transcriptase
HU	hydroxyurea
ICE	<i>in vivo</i> complex of enzyme assay
IL-2	interleukin-2
KO	knockout
LET-7	lethal-7
MCM2-7	minichromosome maintenance 2-7
MBD4	methyl-CpG binding domain 4
MERB	merbarone
MRC1	mediator of the replication checkpoint
MRE11	meiotic recombination 11 homolog
ORC	origin recognition complex
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PFGE	pulsed field gel electrophoresis
POL	polymerase
PVDF	polyvinylidene fluoride
RIF1	Rap1-interacting factor 1
RFC	replication factor C
RIPA	radioimmunoprecipitation assay
RLU	relative light units
RPA	replication protein A
RPC	replisome progression complex
RPMI	roswell park memorial institute
scDNA	supercoiled deoxyribonucleic acid
SDS	sodium dodecyl sulphate
shRNA	short hairpin ribonucleic acid
TBS	tris-buffered saline
TGF- β	transforming growth factor- β
TOF1	topoisomerase interacting factor 1

TOP1/2	topoisomerase type 1 and 2
TOP1cc	TOP1-DNA cleavage complexes
TOP2cc	TOP2-DNA cleavage complexes
WT	wildtype
XRCC1	x-ray repair cross-complementing protein-1
XPA	xeroderma pigmentosum, complementation group A

Abstract

The maintenance of eukaryotic genomes in a complete and undamaged state is critical for any tightly regulated DNA process such as replication and transcription to function efficiently. With DNA damage response and repair systems predominantly slow, cells must be equipped with factors that work at the root of the problem, tackling genome-wide challenges that are thrown at them, for example, during chemotherapy.

The oncofetal architectural factor HMGA2, by utilizing its three DNA binding domains has been shown to bind stalled replication forks and prevent their collapse into genotoxic DNA strand breaks in fast replicating cancer cells, hESCs as well as in other heterologous yeast and bacterial cell systems. However, the exact mechanism behind its fork protection function remained elusive.

A recent report found HMGA2 to bind supercoiled DNA, one of the many transient structures generated at stalled replication forks, with high affinity. In addition, studies in yeast found HMGA2 to prevent the formation of a potentially topologically driven, pathological fork structure, thereby preventing fork collapse and hinting at a possible mechanism of fork protection. To dwell deeper into HMGA2's proposed chaperone function, I utilized four different cancer cell line model systems and induced replication/topological stress by targeting DNA polymerase or topoisomerases.

I identified that the expression levels of HMGA2 are critical in determining sensitivity to SN38, a clinically active TOP1 inhibitor and found that cancer cells that express high levels of HMGA2 are resistant to SN38 whereas, surprisingly, low to moderate HMGA2 expression sensitized the cells to drug induced DNA damage. I show that this differential chemosensitivity to SN38 correlated with HMGA2-mediated TOP1-DNA cleavage complexes (TOP1cc) formation, highlighting HMGA2 can either interfere with drug binding to TOP1 generating low levels of the toxic TOP1cc or increase SN38-TOP1-DNA interactions, leading to higher TOP1cc levels. Further analysis also revealed that specific subtelomeric regions (30-100 kb) are extremely sensitive to the topological stress induced by DNA synthesis or TOP1 inhibitors. Furthermore, I corroborated our *ex vivo* observations in an *in vivo* setting, using patient-derived tumor xenografts in mice and found HMGA2 expression to determine the effectiveness of a clinically relevant TOP1 poison.

Next, I showed here for the first time that HMGA2 expression confers resistance against both a TOP2 poison (etoposide) and catalytic inhibitor (merbarone), that cause DNA damage by stabilizing the intermediate TOP2-DNA cleavage complexes (TOP2cc) and by inducing topological stress, respectively. I attribute this wide-spread protection across various cell lines tested in this study to the constraint of sc DNA and/or catalytic activation of TOP2A mediated by HMGA2, which, remarkably, could provide a mechanistic explanation for the recent observation of poor clinical outcomes in bone-marrow derived mononuclear cells of acute myeloid leukemia patients that express HMGA2.

Taken together, our study reveals a fascinating aspect of HMGA2 dependent fork protection mechanism that counteracts replication induced DNA topological stress and marks a potential clinical impact that could aid chemotherapy related decision making, in particular for leukemic and colorectal cancer patients.

Graphical Abstract

1. Introduction

1.1 Eukaryotic DNA replication

Ever since the seminal discovery of DNA structure over 60 years ago (Watson and Crick 1953), the fundamental mechanism of DNA replication has been successfully elucidated across different cell systems (Chagin, Stear et al. 2010). DNA is precisely and efficiently duplicated during each cell cycle through a series of tightly regulated processes encompassing replication initiation, elongation and termination. With eukaryotic genomes substantially larger compared to their prokaryotic counterparts, replication is initiated at several thousand specialized sites called 'origin of replication' to allow rapid DNA duplication (Mendez and Stillman 2003) (Wu, Liu et al. 2014).

Figure 1- Replication initiation. A tightly regulated process that involves loading of the double hexamer Mcm2-7 helicase onto origins followed by subsequent recruitment of protein factors that activate the loaded helicase. Reproduced from (Marks, Smith et al. 2016) with permission.

First, a six-subunit origin recognition complex (ORC), an inactive heterohexameric Mcm2-7 helicase, and various other initiator proteins assemble at origins during the early G1 phase, forming 'pre-replication' structures in a process called origin licensing.

Following the activation of cyclin-dependent kinases during S phase, additional initiator proteins that include Mcm10, Cdc45, GINS, RPA and Pol ϵ are recruited at the origin forming a 'pre-initiation' complex (Labib 2010) (Costa, Hood et al. 2013). This process, called origin firing, (**Fig. 1**) leads to the activation of the multi-subunit CMG (Cdc45, Mcm2-7, GINS) helicase complex (Boos, Frigola et al. 2012), that will, in turn, initiate unwinding at the origin and track along single stranded parental DNA (Yardimci, Loveland et al. 2010) (Bleichert, Botchan et al. 2017) (O'Donnell and Li 2018) (Reuswig and Pfander 2019).

Such tight regulation ensures that the selected origin is firing only once throughout the entire cell cycle, preventing complications such as re-replication (Arias and Walter 2007). The activation of replicative helicase also triggers the assembly of a functional replisome, a complex conglomerate of protein factors that is essential for DNA elongation (Bell and Labib 2016) (Kelly and Callegari 2019).

Figure 2- DNA elongation. Following the activation of replicative helicase (CMG) and recruitment of key factors, a pair of replisomes are formed, generating forks that move in opposite direction (bidirectional). Reproduced from (Wei and Zhao 2017) with permission.

The functional replisome progression complex (RPC) associates with DNA polymerases to initiate replication elongation (**Fig. 2**) (Bell and Labib 2016) (Gambus,

Jones et al. 2006). DNA Pol α -primase, a specialized enzyme complex that initiates DNA synthesis on both the leading and lagging strands, first adds a short RNA primer (~10 nucleotides) template that is then briefly extended by DNA Pol α . The specific directionality of DNA synthesis allows for the continuous extension of the leading strand by Pol ϵ (Pursell, Isoz et al. 2007) whereas the lagging strand is extended discontinuously by Pol δ (Nick McElhinny, Gordenin et al. 2008), recruited at the fork by processivity factors PCNA and RFC (Jeruzalmi, O'Donnell et al. 2002) (Stillman 2008) (Hedglin, Kumar et al. 2013) (Georgescu, Schauer et al. 2015). These discontinuous, short stretches of DNA called Okazaki fragments, are then processed by FEN1 endonuclease and DNA ligase to generate a continuous DNA strand (Garg, Stith et al. 2004). As a barrage of proteins work in sync to elongate DNA strands, the CMG helicase continues to translocate away from the origin, bypassing replication roadblocks until the forks converge about midway of two origins (Hawkins, Retkute et al. 2013) (McGuffee, Smith et al. 2013), marking the beginning of replication termination.

Although still not completely understood, replication termination occurs either at specialized termination sites or when replication forks converge (Fachinetti, Bermejo et al. 2010). During this process, the replicative CMG helicase encounters replicated dsDNA, leading to the polyubiquitylation of its MCM7 protein subunit which is then recognized by protein factors that help unload CMG helicase from the chromatin and dismantle the complex into its individual components (Maric, Maculins et al. 2014) (Dewar, Budzowska et al. 2015) (Gambus 2017). This triggers the replisome factors which are either directly or indirectly in contact with the replicative helicase to dissociate from the chromatin (Dewar and Walter 2017). Finally, the intertwined daughter strands are resolved, resulting in fully replicated daughter DNA.

1.2 Replication stress

The integrity and smooth progression of replication forks, typically recognized as 'Y'-shaped DNA structures, is crucial for the cell to duplicate its genome (Branzei and Foiani 2010). However, these structures frequently encounter obstacles and barriers that include DNA lesions (damaged bases/abasic sites), template strand breaks, DNA secondary structures, covalent DNA-protein complexes, the transcription machinery, inverted repeats, fragile sites (e.g. telomeres) and unresolved DNA entanglements,

leading to fork stalling (Mirkin and Mirkin 2007) (Branzei and Foiani 2010) (Minca and Kowalski 2011) (Hashimoto, Puddu et al. 2011) (Aguilera and Garcia-Muse 2013) (Gelot, Magdalou et al. 2015) (Lu, Wang et al. 2015). In addition, the lack of key replication factors such as dNTPs and RPA proteins, that are required for replication elongation (Poli, Tsaponina et al. 2012) and single stranded DNA (ssDNA) protection (Toledo, Altmeyer et al. 2013), respectively, also induce replication stress (**Fig. 3**).

Figure 3- Barriers to replication fork progression. Depiction of cell intrinsic as well as extrinsic factors that hinder fork stability. Modified from (Higa, Fujita et al. 2017)

When left unresolved, prolonged fork stalling could generate large stretches of unprotected ssDNA, as a result of helicase uncoupling from the replisome (Byun, Pacek et al. 2005), that are prone to nucleolytic attack (Stahl, Droge et al. 1986). In addition, the cleavage of abasic sites or other DNA lesions by endonucleases as well as collision with protein-DNA adducts on the parental DNA generate double stranded DNA breaks causing unscheduled rearrangements, mutations and ultimately cell death (Alexander and Orr-Weaver 2016) (Cortez 2019).

As a consequence, several cellular mechanisms are in place to overcome fork obstacles or barriers, that include but are not limited to lesion skipping (Yeeles and Mariani 2011) (Mariani 2018), template switching (Branzei and Foiani 2007), firing of dormant origins (Blow and Ge 2008) (Errico and Costanzo 2012), activation of cell cycle checkpoints (Iyer and Rhind 2013) and remodelling of replication forks (Sidorova 2017). However, most of these mechanisms activate error prone polymerases and utilize repair and recombination pathways that could contribute to mutagenesis and genetic rearrangements, ultimately proving deleterious (Bi 2015) (Kai and Wang 2003).

(Branzei and Szakal 2017). Alternatively, recent reports suggest the existence of protein factors that physically associate at the replication fork and prevent fork stalling, providing somewhat safer and more reliable alternatives of fork protection and restart compared to the ones described above (Rickman and Smogorzewska 2019). In this context, DNA repair factors BRCA and FANCD2 were found to stabilize and protect nascent DNA strands on stalled forks from degradation as well as prevent the formation of pathological fork structures that can lead to genome instability (Schlacher, Christ et al. 2011) (Schlacher, Wu et al. 2012). In addition, replication factors Mrc1 and Tof1-Csm3 were found to prevent the uncoupling of replisome from helicase, thereby, preventing the accumulation and subsequent degradation of ssDNA (Gellon, Kaushal et al. 2019). Another DNA repair factor RIF1 was also found to promote genome stability by binding and stabilizing replication stress-induced four-way junctions that are prone to nuclease digestion (Mukherjee, Tripathi et al. 2019).

Adding to the growing repertoire of protein factors that promote replication fork protection, our laboratory discovered a novel function for HMGA2, an oncofetal chromatin structuring protein, that appears to chaperone stalled forks and prevent their endonucleolytic collapse (Yu, Lim et al. 2014). As the protein influences a range of nuclear processes and function, I now focus first on understanding its diverse role in modulating cellular mechanisms.

1.3 High mobility group (HMG) proteins- General overview

Eukaryotic chromosomes are packed by the highly conserved and dynamic histone proteins, namely H1, H2A, H2B, H3 and H4 that form the fundamental building blocks of chromatin called nucleosomes. These multiprotein complexes have been extensively studied as they regulate chromatin structure and control DNA dependent processes such as replication, transcription and repair (Strahl and Allis 2000) (Morales, Giamarchi et al. 2001). The profound alterations in chromatin structure is also brought about by non-canonical histone proteins (such as the variants of H2A and H3) that have evolved to play diverse roles in chromosomal processes (Henikoff and Smith 2015). In addition, several non-histone chromatin factors have been described to regulate chromatin mechanics and were found to be indispensable to eukaryotic chromosome structure and function (Bustin and Reeves 1996) (Einck and Bustin 1985). Among these, the major and best characterized proteins are the high mobility

group (HMG) proteins, designated so as a result of their high electrophoretic mobility in acidic polyacrylamide gels and defined by their highly charged amino acid content (Bustin, Lehn et al. 1990).

HMG proteins are classified into three superfamilies based on their DNA binding motifs, namely HMGA (high mobility group AT-hook), previously called HMGI/Y (AT Hook Motif), HMGB or HMG1/2 (HMG Box Motif) and HMGN or HMG14/17 (Nucleosomal binding domain) (Bustin 2001), and have been shown to interact with specific DNA structures rather than precise DNA sequences (Bustin and Reeves 1996). These DNA structures include synthetic four-way junction structures, non-B-form structures (bent, kinked and unwound), base-unpaired regions of AT-rich DNA and distorted regions of DNA found on isolated nucleosome core particles (Reeves 2001) (Cleynen and Van de Ven 2008) (Catez and Hock 2010). High mobility group proteins are also called architectural transcription factors because of their ability to modulate transcription of certain genes by altering chromatin structure both locally and globally at promotor and enhancer regions (Bustin 1999). Among the three HMG superfamily proteins, HMGA (AT Hook motifs) proteins preferentially bind to the minor groove of AT-rich regions on B-DNA both *in vivo* and *in vitro* (Reeves and Nissen 1990) (Colombo, Burger et al. 2017) making them more exclusive in terms of their cellular function compared to the other HMG families which have little/no nucleotide sequence preference (Reeves and Adair 2005).

1.3.1 HMGA proteins and their structural characteristics

The human high mobility group AT-hook (HMGA) gene family comprises only two members, namely HMGA1 and HMGA2, located on chromosome 6 (6p21) (Friedmann, Holth et al. 1993) and chromosome 12 (12q14-15) (Chau, Patel et al. 1995), respectively. The HMGA1 gene transcript has three splice variants encoding HMGA1a (107aa), HMGA1b (96aa) and HMGA1c (179aa), whereas the HMGA2 gene encodes a single protein product HMGA2 (109aa) (**Fig. 4**). However, recent studies have found splice variants for HMGA2, that are key for the molecular identity of human hematopoietic stem cells (Cesana, Guo et al. 2018).

Figure 4- The HMGA superfamily. HMGA proteins containing the distinctive DNA binding domains (DB) with the characteristic AT-hooks (blue) and acidic C-terminal domains (pink). Note that the amino acid length described is devoid of the initiator methionine. The amino acid sequence of HMGA1c is different (green) as compared to HMGA1a or HMGA1b. Reproduced from (Ozturk, Singh et al. 2014).

HMGA are low molecular weight proteins, characterized by their three AT-Hooks (DNA binding domains), except for HMGA1c (two AT-Hooks) (**Fig. 4**) and their acidic C-terminal domain, which is thought to be important for protein-protein interactions and possibly for the recruitment of various factors to enhanceosomes (Pfannkuche, Summer et al. 2009) (Ashar, Chouinard et al. 2010). The three independent AT-hooks and the C-terminal domain arise from distinct exons and contribute to about 50% of the amino acid sequence identity between HMGA1 and HMGA2 (Cleyne and Van de Ven 2008).

```

HMGA1a 1 MSESSESSKSSQPLASKQEKDGTGTE---KRGRGRPRKQPPVSPGTALVGSQKEPS-EVPTPKRERG59
HMGA1b 1 MSESSESSKSSQPLASKQEKDGTGTE---KRGRGRPRKQPP*****KEPS-EVPTPKRERG48
HMGA1c 1 MSESSESSKSSQPLASKQEKDGTGTE---KRGRGRPRKQPPVSPGTALVGSQKEPS-EVPTPKRERG59
HMGA2 1 MSARGEGAGQPSTSAQGQPAAPAPQKRGRGRPRKQQQ-----EPTGE-PSPKRERG50

HMGA1a 60 RPKGSKNKGAAG---TRKTTTTPG-RKGRGRPRKLEK-----EEEEGIS-QESSEEEQ107
HMGA1b 49 RPKGSKNKGAAG---TRKTTTTPG-RKGRGRPRKLEK-----EEEEGIS-QESSEEEQ96
HMGA1c 60 RPKGSKKNWRRRKRRASRRSPRRSSDHCVPAPHRSSFLLGLDSFAPLPPPPPLPOAHHH121
HMGA2 51 RPKGSKNKSPSKAAQ-KKAEAT-GEKRRGRGRPRKWPQQVVQKKPAQEETEETSSQESAED-109

HMGA1c 122 HRLWPPPPSSTCALTTTLHSTPAAAGLPWAEWGAFFPWPQFPAPPAPRIHTCPPGQG179

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Figure 5- Amino acid alignment of human HMGA proteins. Residues in the AT-hooks and in the C-terminal tail are conserved. Reproduced from (Cleyne and Van de Ven 2008) with permission.

The AT-hooks containing 8 to 9 amino acids have a highly conserved sequence consisting of Arg-Gly-Arg-Pro flanked by lysine and arginine as shown in **Fig. 5**. The

arrangement of the AT-hooks in the primary amino acid sequence creates a unique binding module, which leads to the protein binding to about 15 nucleotides of AT-rich DNA (1-1.5 helical turn) of the minor groove (Huth, Bewley et al. 1997) (Cui, Wei et al. 2005) (Pfannkuche, Summer et al. 2009) (Kohl, Zhong et al. 2019) in a co-operative manner (Yie, Liang et al. 1997).

Figure 6- HMGA2 secondary structure. Secondary structure prediction of HMGA2 indicating extensive (more than 70%) random coils with few helical conformations (in red). PSIPRED 4.0 software was used to illustrate predicted secondary structure (Jones 1999)

Structural analysis using circular dichroism (CD), gel filtration and nuclear magnetic resonance (NMR) spectroscopy revealed that both HMGA1 (Evans, Zajicek et al. 1995) (Reeves and Beckerbauer 2001) (Kohl, Zhong et al. 2019) and HMGA2 (Frost, Baez et al. 2015) (Krahn, Meier et al. 2017) lack secondary or tertiary structure formation in solution (**Fig. 6**) and are grouped as intrinsically disordered or unstructured proteins (Dunker, Cortese et al. 2005) (Uversky, Oldfield et al. 2005). However, upon binding to DNA, HMGA proteins undergo a conformational change forming a more planar, crescent shaped structure that ultimately bends specific AT-rich DNA (Reeves 2001) (Slama-Schwok, Zakrzewska et al. 2000) (Chen, Young et al. 2010) (Colombo, Burger et al. 2017). Furthermore, HMGA proteins can recognize and bind atypical DNA substrates such as supercoiled DNA (Nissen and Reeves 1995) (Zhao, Peter et al. 2017), single stranded DNA (Zhao, Peter et al. 2017) and three- and four-way DNA junctions (Hill and Reeves 1997) (Hill, Pedulla et al. 1999) (Yu, Lim et al. 2014). Depending on the type, composition, length or organization of its substrate, the multivalent DNA binding molecule can cause localized changes without the requirement of energy in form of ATP hydrolysis. Such conformational changes are thought to be a result of several processes that work in sync and include mechanisms such as the binding of AT-hooks to DNA either in cis or trans configuration, non-covalent cross linking of different dsDNA segments mediated by HMGA proteins, binding to the same DNA molecule by multiple HMGA proteins and

neutralization of negative charge on the DNA backbone (Reeves and Beckerbauer 2001) (Reeves 2001) (Pfannkuche, Summer et al. 2009).

1.3.2 Biological relevance of *HMGA*

Despite sharing extensive sequence homology with more than 70% identity within the three AT hook domains, the expression pattern of *HMGA1* and *HMGA2* is different, suggesting that they might carry out distinct roles, in particular during organismal development (Cleynen and Van de Ven 2008) (Colombo, Burger et al. 2017). Both proteins are highly expressed in embryonic stem (ES) cells. In normal human somatic cells, *HMGA1* is expressed at low levels whereas *HMGA2* is absent (Yanagisawa and Resar 2014) (Chiappetta, Avantaggiato et al. 1996) (Gattas, Quade et al. 1999).

Figure 7- Growth defects in *Hmga* knockout mice. (A) Comparison of *Hmga2* knockout (KO) mice (left) having a ‘pygmy’ phenotype (40-60% reduction in size) as compared to wild-type (WT) mice (right). Reproduced from (Li, Gilbert et al. 2012) with permission (B) Comparison of *Hmga1/2* double knockout (A1/A2-KO) mice (left) having a ‘super-pygmy’ phenotype (75% reduction in size) as compared to wild-type (WT) mice (right). Reproduced from (Federico, Forzati et al. 2014).

Hmga1 knockout mice develop a phenotype resembling human type 2 diabetes (Foti, Chiefari et al. 2005) and cardiac hypertrophy (Fedele, Fidanza et al. 2006), whereas the homozygous deletion of *Hmga2* in mice leads to a ‘pygmy’ or ‘small body’ phenotype with severe reduction in fat tissue (Zhou, Benson et al. 1995). Interestingly, *Hmga1/Hmga2* double knockout in mice leads to a ‘superpygmy’ phenotype with severely reduced life span, pointing to their crucial role in development (Federico, Forzati et al. 2014). *HMGA2* has also been implicated in the regulation of growth, as it influences beak size in finches (Lamichhaney, Han et al. 2016) and body size in rabbits and other mammals (Carneiro, Hu et al. 2017). Haploinsufficiency for *HMGA2* as a result of microdeletions of the *HMGA2* gene on chromosome 12q14 has been

associated with short stature and growth restriction (Mari, Hermanns et al. 2009) (Alyaqoub, Pyatt et al. 2012). Furthermore, single nucleotide polymorphisms as well as truncations in human *HMGA2* leading to protein overexpression have been associated with increased height and overgrowth respectively (Ligon, Moore et al. 2005) (Weedon, Lettre et al. 2007) (Fusco, Babu et al. 2016).

1.3.3 Expression pattern of *HMGA2*

The expression of *HMGA2* is tightly regulated, and its dysregulation in adult somatic cells is heavily linked to tumorigenesis as it plays a causal role in the development of mesenchymal neoplasms (Wood, Maher et al. 2000) (Fedele, Battista et al. 2002) (Zaidi, Okada et al. 2006). In addition, gene rearrangements in *HMGA2* have been reported to lead to overexpression of *HMGA2* in numerous benign mesenchymal tumors (Ashar, Fejzo et al. 1995) (Schoenmakers, Wanschura et al. 1995) (Ligon, Moore et al. 2005). Apart from transforming growth factor- β (TGF- β)/Smad signalling (Thuault, Valcourt et al. 2006) and β -catenin (Wend, Runke et al. 2013) that have been reported to directly influence and promote expression of *HMGA2* by regulating its promoter activity, *HMGA2* is also downregulated by several miRNAs (Kim, Song et al. 2015) (Ye and Gui 2018) as well as repression complexes (ZBRK1/BRCA1/CtIP) that recognizes and represses the *HMGA2* promoter (Ahmed, Tsai et al. 2010). One of the earliest reports identified let-7, a founding member of the miRNA family, to downregulate *HMGA2* in somatic cells by interacting with its 3' untranslated region (Lee and Dutta 2007) (**Fig. 8**), thereby, implicating *HMGA2* to be a critical factor for stemness (Yu, Yao et al. 2007) (Lee, Han et al. 2016).

Figure 8- Regulation of chromatin factor *HMGA2* by let-7 miRNA. Expression of *HMGA2* inversely correlates with let-7 miRNA levels in embryonic stem cells and somatic cells. Reproduced from (Droge and Davey 2008) with permission.

The chromosomal translocations in the HMGA2 locus could generate shortened or chimeric fusion products, that lack C-terminal and 3' untranslated regions which contain multiple sites for let-7 miRNA binding, leading to increased HMGA2 mRNA expression (Mayr, Hemann et al. 2007). Such altered gene products are still functional as they carry the three DNA-binding domains and are implicated in cellular transformation (Schoenmakers, Wanschura et al. 1995) (Fedele, Berlingieri et al. 1998). Furthermore, higher expression of HMGA2 in cancer cells was also found to be regulated by LIN28, a stem cell-specific RNA binding protein that negatively regulates let-7 biogenesis, ultimately causing the degradation of let-7 precursors and hence derepressing HMGA2 expression (Piskounova, Polytarchou et al. 2011).

1.3.4 HMGA and cancer progression

HMGA2 is re-activated in a wide range of solid tumors (Pallante, Sepe et al. 2015), including lung (Meyer, Loeschke et al. 2007), colorectal (Wang, Liu et al. 2011), breast (Wu, Zhan et al. 2016), pancreatic (Piscuoglio, Zlobec et al. 2012), ovarian (Mahajan, Liu et al. 2010) and testicular (Franco, Esposito et al. 2008) carcinomas, as well as in hematological malignancies (Meyer, Krispeneit et al. 2007) (Tan, Wei et al. 2016) with high expression correlating to severe tumor grade and poor patient survival.

The protein is heavily implicated in cancer development as it promotes epithelial-to-mesenchymal transition (EMT) by directly regulating E-cadherin expression and several other EMT-related genes (Thuault, Valcourt et al. 2006) (Wu, Liu et al. 2011) (Wu and Wei 2013) (Ding, Wang et al. 2014), leading to invasion and metastasis (Sun, Song et al. 2013) (Morishita, Zaidi et al. 2013). It also facilitates cancer progression by regulating the expression of pro-inflammatory cytokines such as IL-2/IL-15 through the NF- κ B pathway (Baldassarre, Fedele et al. 2001) (Huang, Li et al. 2017), and controls cell proliferation by enhancing the activity of E2F1, a transcription factor required to transcribe several replication-related (CDC6, thymidine kinase) and cell-cycle control (cyclin E, cyclin A, CDC2) genes (Helin 1998) (Fedele, Visone et al. 2006) (**Fig. 9**).

Additionally, HMGA2 was found to regulate self-renewal of stem cells, in particular cancer stem-like cells (CSCs) by either abolishing differentiation or regulating transcription of key pluripotency genes (Yu, Yao et al. 2007) (Nishino, Kim et al. 2008) (Copley, Babovic et al. 2013) (Chien, Wang et al. 2015) (Giancotti, Bergamin et al. 2018), highlighting its role in metastasis and recurrence, leading to poor prognosis

(Sun, Sun et al. 2017). Remarkably, HMGA2 was also shown to increase hTERT (human telomerase reverse transcriptase) expression levels, by interfering with hTERT repressor protein function and increasing hTERT promoter activity, shedding new light on its role in promoting tumorigenesis (Li, Lin et al. 2011), although, this was later challenged in mouse cells (Morshedi, Ren et al. 2013).

Figure 9- HMGA2 and cancer progression. Schematic overview of the regulation of HMGA2 expression and key mechanisms regulated by HMGA proteins leading to cancer cell progression.

1.3.5 HMGA2 and genome stability

One of the most significant effects in promoting tumorigenesis, at least during the early stages of cancer progression, comes from the ability of HMGA family proteins to interfere with DNA repair (Reeves and Adair 2005) (Reeves 2015). HMGA2 was reported to interfere with the transcription of ERCC1, a key factor that is involved in nucleotide excision repair (Borrmann, Schwanbeck et al. 2003). It also interferes with DNA-dependent protein kinase function that is required for double strand break repair (Li, Boo et al. 2009). Furthermore, the homologous HMGA1 is also implicated, as it downregulates the expression of base excision repair factors (XRCC1 and MBD4), nucleotide excision repair factor (XPA) (Adair, Kwon et al. 2005) (Adair, Maloney et al. 2007) as well as double strand break repair factor (BRCA1) (Baldassarre, Battista et al. 2003). Taken together, these studies implicate the oncogenic potential of HMGA2,

as it impairs DNA damage response, leading to the accumulation of genetic mutations, further causing genomic instability (**Fig. 9**).

On the contrary, HMGA proteins have also been implicated in promoting DNA repair, protecting tumor cells from genotoxic stress induced by several chemotherapeutic agents leading to chemoresistance. HMGA proteins were found to regulate ATM promoter expression as part of a positive feedback loop in response to DNA damage (Palmieri, Valentino et al. 2011) and were also found to sustain ATR-CHK1 hyperphosphorylation that leads to prolonged cell cycle arrest, allowing repair of DNA damage (Natarajan, Hombach-Klonisch et al. 2013). Furthermore, Summer, Li et al. (2009) identified HMGA2 to possess lyase activities that recognise and cleave apyrimidinic/apurinic sites, thereby promoting base excision repair function. In addition, HMGA proteins interfere with the induction of apoptosis, allowing cancer cells to escape the genotoxic insults thrown at them (Pierantoni, Rinaldo et al. 2006) (Frasca, Rustighi et al. 2006) (Esposito, Tornincasa et al. 2010) (Esmailzadeh, Mansoori et al. 2017). Consistent with these studies, our laboratory described a novel function for HMGA2, preventing DNA fragmentation in the presence of hydroxyurea (Yu, Lim et al. 2014), a chemotherapeutic drug that targets ribonucleotide diphosphate reductase depleting dNTP pools, leading to fork stalling and uncoupling of helicase from DNA polymerase (Madaan, Kaushik et al. 2012) (Berti and Vindigni 2016).

Prolonged fork stalling can be lethal, as it leads to fork collapse either through endonucleolytic attack (Hanada, Budzowska et al. 2007) or replication run-off events (Ray Chaudhuri, Hashimoto et al. 2012), contributing to the formation of double strand breaks and ultimately cell death. Thus, several cellular mechanisms are in place to help stalled forks resume DNA synthesis (Berti and Vindigni 2016). One particularly interesting stalled fork processing mechanism, called fork regression or reversal, has been a topic of intense debate recently. Initially reported to promote fork restart (Higgins, Kato et al. 1976), replication fork reversal which involves the formation of a four-way assembly resembling a Holliday junction (also called chicken foot-like structure), due to the displacement and base pairing of nascent daughter strands and rewinding of parental strands (**Fig. 10**) (Atkinson and McGlynn 2009), is now considered to be deleterious. The transient pausing mediated by fork reversal, serves as a physiological intermediate in the reactivation and repair of stalled forks, that would otherwise collapse generating DSBs (Ray Chaudhuri, Hashimoto et al. 2012)

(Neelsen, Zanini et al. 2013) (Zeman and Cimprich 2014) (Quinet, Lemacon et al. 2017). However, recent reports suggest that regressed forks are subject to cleavage by nucleases such as MRE11 or EXO1 and thus constitute pathological structures (Branzei and Foiani 2010) (Neelsen, Zanini et al. 2013) (Jossen and Bermejo 2013) (Neelsen and Lopes 2015) (Mijic, Zellweger et al. 2017) (Lemacon, Jackson et al. 2017), generating double strand breaks. Additionally, fork regression possibly involves replisome removal which could have harmful consequences (Meng and Zhao 2017), and the strand exchange that occurs during this process, especially on repeat sequences, could lead to misalignments, thereby inducing chromosomal rearrangements and genome instability (McMurray 2010) (Follonier, Oehler et al. 2013).

Figure 10- Fork reversal. Formation and processing of 'chicken foot' regressed forks during replication fork stalling mediated by hydroxyurea (HU). Adapted from (Przetocka, Porro et al. 2018) with permission.

HMGA2 was reported to prevent fork regression in yeast; a mechanism that could potentially explain at least partially, its proposed fork chaperone function (Yu, Lim et al. 2014). However, the underlying molecular mechanisms behind its fork protection remain to be identified. Interestingly, fork regression is a DNA topology-driven phenomenon that relieves topological stress, generated when positive supercoiling ahead of the replication fork is not resolved fast enough (Postow, Ullsperger et al. 2001) (Schvartzman and Stasiak 2004) (Jossen and Bermejo 2013) (Keszthelyi, Minchell et al. 2016). In this context, it is worthwhile to better understand the DNA topological events at the replication fork.

1.4 DNA topological stress

One of the biggest hurdles that replication forks encounter during their journey to duplicate eukaryotic genome is the generation of topological stress. Despite the extensive knowledge of the protein factors that maintain topological homeostasis

(Pommier, Sun et al. 2016), accumulated since the first description of DNA supercoiling more than 50 years ago (Vinograd, Lebowitz et al. 1965) (Germond, Hirt et al. 1975), the molecular mechanisms that co-ordinate their function still remains poorly understood (Szafran, Gongerowska et al. 2016) (Guo, Haakonsen et al. 2018) (Goffart, Hangan et al. 2019).

The double helical nature of DNA poses a challenge during complementary DNA strand separation, leading to entanglements (Bednar, Furrer et al. 1994) (Yu and Droge 2014). On open-ended linear DNA, these entanglements can diffuse away due to the axial rotation of the double helix. However, for complex eukaryotic chromatin that is large and often anchored physically to the nuclear lamina or chromosome scaffolds, this free rotation is impeded. In this context, the rotational drag generated by large protein-DNA complexes that are often free to rotate about the DNA helix, causes compensatory changes elsewhere in the DNA molecule such as supercoiling, generating topological stress along the chromosome. Depending on the winding of DNA segments either in the same or opposite direction of the DNA helix, positive and negative supercoils are generated respectively (Cozzarelli and Wang 1990) (Droge 1994) (Koster, Crut et al. 2010) (Gilbert and Allan 2014) (Keszthelyi, Minchell et al. 2016).

The torsional stress might have different functional consequences, as it can influence the formation of distinct DNA structures, such as Z-DNA (Lee, Kim et al. 2010), P-DNA (Allemand, Bensimon et al. 1998), cruciforms (Mizuuchi, Mizuuchi et al. 1982), R-loops (Drolet, Broccoli et al. 2003) and G-quadruplexes (Kouzine and Levens 2007) (Xia, Zheng et al. 2018), negatively impacting DNA transactions such as replication or transcription (Liu and Wang 1987) (Koster, Crut et al. 2010) (Baranello, Levens et al. 2012). Thus, cells must ensure topological changes are controlled and fine-tuned to allow smooth progression of these processes.

The replication machinery, while translocating along the DNA helix, generates positive supercoiling (topological stress) in the un-replicated segment, ahead of the replication fork, facilitated by the intrinsic mechanical properties of eukaryotic chromatin (Kouzine and Levens 2019). These torsionally soft, positive supercoils promote nucleosome instability impacting replication progression (Sheinin, Li et al. 2013) (Le, Gao et al. 2019). Behind the fork, in the replicated region, pre-catenanes are generated as a

result of the intertwining of the two sister strands (**Fig. 11**). Such torsional stress hampers strand unwinding, as the replisome is unable to translocate further, leading to fork stalling (Espeli and Marians 2004) (Deweese, Osheroff et al. 2008). Furthermore, when two replication forks come in close proximity to each other (convergence), the topological stress generated ahead of the fork can only be compensated through pre-catenane formation, slowing down DNA synthesis (Gambus 2017) (Dewar and Walter 2017).

Figure 11- Consequences of progressing molecular motors on DNA. Both the transcription (left panel) and the replication machineries (right panel) are potent generators of torsional stress. If not resolved quickly, this topological stress can lead to genome instability. Modified from (Jeppsson, Carlborg et al. 2014).

In addition, the RNA polymerase when anchored during transcription, generates positive supercoiling ahead and negative supercoiling behind the enzyme (**Fig. 11**), as proposed by the ‘twin-supercoiled domain model’ (Liu and Wang 1987) (Wu, Shyy et al. 1988). Negative supercoiling affects DNA melting probabilities at promoters allowing strand separation, required for transcription initiation, and modulates gene expression by promoting the binding of regulatory proteins and transcription factors such as TFIID to DNA (Koster, Crut et al. 2010) (Baranello, Levens et al. 2012) (Tabuchi, Handa et al. 1993) (Teves and Henikoff 2014) (Seol and Neuman 2016). On the other hand, positive supercoiling can block transcription initiation and mRNA synthesis (Revyakin, Ebright et al. 2004) (Gartenberg and Wang 1992).

1.5 Topoisomerases

The human genome encodes six topoisomerases that act to prevent the topological stress generated when DNA transactions occur. These enzymes are classified into two types, TOP1 and TOP2, based on their ability to cleave the phosphodiester backbone of DNA on either a single strand or on both strands, respectively. TOP1-

type enzymes are further divided into TOP1A and TOP1B, based on their ability to form 5' or 3' DNA-tyrosyl intermediates, respectively. TOP2 are divided into two isozymes, TOP2A and TOP2B, with the former playing important roles in chromosome segregation and replication, and the latter heavily involved in transcriptional regulation (Pommier, Sun et al. 2016) (Wang 2002) (Pommier, Leo et al. 2010).

In general, torsional stress is mitigated by both TOP1/2 that directly relax positively supercoiled DNA ahead of the fork, or through a fork rotation mechanism that counteracts the mechanical strain ahead of the fork by generating intertwined daughter duplexes (pre-catenanes) behind the fork (Postow, Crisona et al. 2001) (Bermejo, Doksani et al. 2007). These pre-catenanes are then resolved by TOP2 allowing daughter chromosome segregation (Lucas, Germe et al. 2001).

1.5.1 TOP1- Mechanism of action

Human TOP1 relaxes supercoiled DNA by first forming a clamp around the duplex DNA and nicking only a single strand by a transesterification reaction, where the catalytic tyrosine becomes covalently attached to the 3' DNA end forming a DNA-3' phosphotyrosyl enzyme intermediate cleavage complex (also called TOP1cc). This is followed by controlled rotation of the 5' nicked DNA strand around the remaining intact complementary strand permitting supercoiled DNA relaxation and ultimately religation of the nicked DNA strand as shown in **Fig. 12** (Pommier 2006). Normally, steady state concentration of the transiently formed TOP1cc is low, as religation is kinetically favoured over the cleavage reaction (Champoux 2001).

Figure 12- TOP1B-mediated relaxation of supercoiled DNA. Depiction of the three critical steps involved in DNA relaxation that include nicking of the DNA strand, controlled rotation and religation. Reproduced from (Pommier 2006) with permission.

1.5.2 TOP1 Poisons

Most genotoxic agents currently used to treat cancers inhibit enzymes that are directly or indirectly involved in DNA replication, and ultimately induce fork stalling/collapse, triggering single/double strand break formation and apoptosis (Kaina 2003) (Zellweger, Dalcher et al. 2015). These genotoxic agents can generate different types of DNA lesions as a result of base depurination, base oxidation, base deamination, base methylation as well as DNA intra-/interstrand and DNA-protein crosslinks (Swift and Golsteyn 2014).

TOP1 is targeted by chemotherapeutic drugs as they take advantage of the inherent ability of the enzyme to generate single strand nicks in the parental DNA. The most widely used drugs are the water soluble camptothecin (CPT) derivatives topotecan and irinotecan (CPT-11) that have been approved for treatment of ovarian (Bai, Li et al. 2016), lung (Stewart 2004) and colorectal cancers (Douillard 2005). CPT is a structural inhibitor of TOP1, derived from the bark of the Chinese tree *Camptotheca acuminata* and acts by inhibiting the religation step, leading to the stabilization of TOP1cc that comprises the nicked DNA strand (single strand break). Hence, these anticancer agents are termed 'interfacial TOP1 poisons', as they bind at the interface

of the TOP1-DNA complex and intercalate between the base pairs flanking the cleavage site as shown in **Fig. 13** (Pommier 2006) (Pommier 2009). Although topotecan can intercalate directly at the cleavage complex (Staker, Hjerrild et al. 2002), irinotecan is a prodrug that must first be converted to a more potent active metabolite, called SN-38 (Ohtsuka, Inoue et al. 2003).

Figure 13- Mechanism of action of TOP1 poison. Stabilization of DNA-TOP1 covalent complex (TOP1cc) by the interfacial poison SN38. Reproduced from (Pommier 2009) with permission.

Two distinct but mutually not excluding mechanisms have been proposed by different groups to account for the induction of DNA damage by TOP1 inhibitors. The first highlights the fact that TOP1 poisons stabilize the intermediate complex (TOP1cc), leading to widespread accumulation of trapped TOP1 with nicked single-stranded DNA, which can later be converted to double strand breaks upon collision with replisomes/helicases and RNA polymerases (Strumberg, Pilon et al. 2000) (Kuzminov 2001) (Pommier, Redon et al. 2003). Hence, the reversible ternary DNA-CPT-TOP1 complexes are converted into cytotoxic lesions leading to apoptosis (**Fig. 14**) (Li and Liu 2001).

Figure 14- CPT-induced cell cytotoxicity. Two possible scenarios depicting the consequences of CPT-mediated inhibition of TOP1 activity. Modified from (Koster, Palle et al. 2007) with permission.

The second, more recently proposed mechanism for TOP1 poison-induced DNA damage involves topological stress that is generated when TOP1 activity is inhibited. This generates unresolved positive supercoils (Koster, Palle et al. 2007) (Puddu, Salguero et al. 2017) ahead of the fork, as depicted in **Fig. 14**, leading to fork reversal (Ray Chaudhuri, Hashimoto et al. 2012) (Postow, Ullsperger et al. 2001) and ultimately fork collapse.

1.5.3- TOP2- Mechanism of action

Although TOP2A and TOP2B are both homodimeric proteins that share extensive homology and comparable catalytic activities, TOP2A is predominantly implicated to function at the replication fork (Drake, Hofmann et al. 1989) (Chikamori, Grozav et al. 2010) (Pommier, Sun et al. 2016), while TOP2B is found at transcription sites (Pommier, Sun et al. 2016) (Calderwood 2016) (Nelson, Haffner et al. 2018). These isozymes catalyze DNA relaxation by first binding two different DNA segments, which juxtapose each other in scDNA, and cleaving the first double stranded DNA ('G-segment'), forming the transient TOP2A-DNA cleavage complex (TOP2cc), which allows the second double stranded DNA ('T-segment') to pass through the DNA gate. This is then followed by religation and release of the 'G-segment', restoring the catalytic cycle. The enzyme's activity and in particular the double stranded DNA passage reaction, is dependent on ATP binding and hydrolysis (McClendon and Osheroff 2007) (Pommier, Leo et al. 2010), as shown in **Fig. 15**, and each reaction cycle relaxes two supercoils from the substrate DNA.

Figure 15- TOP2-mediated DNA relaxation. Illustration of key steps of DNA relaxation by TOP2 highlighting 'G-segment' (green) binding, followed by DNA cleavage (TOP2cc) and religation of the double strand break. The intact 'T-segment' (pink) is passed through the cleavage segment leading to a change in linking number. Reproduced from (Vos, Tretter et al. 2011) with permission.

The steady state levels of TOP2cc need to be tightly regulated, as both overly high or low levels can be detrimental to the cell. A rise in TOP2cc levels can generate permanent double strand breaks and induce apoptosis as a result of fork collisions with these lesions (Wilstermann and Osheroff 2003) (Fortune and Osheroff 2000), whereas low TOP2cc levels can lead to decreased growth rates and mitotic failure (McClendon and Osheroff 2007), presumably by the built-up of supercoiling in chromatin.

1.5.4- TOP2 Poisons

TOP2 also serves as a target for anti-cancer drugs, proving invaluable in the treatment of several solid tumors as well as in hematological malignancies, either alone or in

combination therapy (Pommier, Leo et al. 2010) (Pommier 2013) (Hevener, Verstak et al. 2018).

Based on their mode of action, TOP2 targeting drugs can be classified into two main categories. TOP2 poisons are clinically relevant compounds that stabilize the toxic TOP2cc and induce DNA damage or apoptosis. These are further classified as intercalating (anthracyclines such as doxorubicin or daunorubicin and mitoxantrone) and non-intercalating agents (etoposide and teniposide) that stabilize TOP2cc, although via alternate mechanisms. The intercalating drugs bind DNA directly and stabilize TOP2cc at drug-bound DNA sites (Capranico, Kohn et al. 1990) whereas the non-intercalating agents have negligible affinity for DNA and involve drug-TOP2 interactions to stabilize TOP2cc (Froelich-Ammon and Osheroff 1995).

Figure 16- Inhibition of TOP2 by etoposide. Schematic highlighting TOP2 poison (etoposide) interfering with a key step in the TOP2 enzymatic cycle. Modified from (Vos, Tretter et al. 2011) with permission.

Etoposide derived from the rhizome of *Podophyllum peltatum* was discovered more than 35 years ago, and is now widely used in the treatment of small cell lung cancer and testicular cancers (Rezonja, Knez et al. 2013) (Hevener, Verstak et al. 2018). It mediates the inhibition of TOP2 by reversibly binding at the interface between the enzyme and cleaved DNA, with two drug binding sites in the TOP2 dimer (Bromberg, Burgin et al. 2003), thereby preventing the religation of the transient double strand break in the 'G-segment' (see **Fig. 16**). This stabilizes the toxic TOP2cc to accumulate to dangerous levels, which can generate permanent double strand breaks following collisions with the incoming replication machinery (see **Fig. 17**) (Tammaro, Barr et al. 2013) (Baranello, Kouzine et al. 2014) (Yan, Tammaro et al. 2016).

Figure 17- Consequences of TOP2 poisoning. TOP2 poison (etoposide) induce DNA damage through the stabilization of deleterious TOP2cc. Adapted from (Berti, Ray Chaudhuri et al. 2013) with permission.

1.5.5- TOP2 catalytic inhibitors

A second class of heterogeneous compounds that interfere with the catalytic cycle of TOP2 are catalytic inhibitors. They are utilized for their activity as cardio-protectors and efficacy modulators for other drugs and are currently under clinical evaluation for anti-tumor activity (Larsen, Escargueil et al. 2003) (Nitiss 2009). They are further divided into ATP hydrolysis inhibitors (Iwai, Hara et al. 1997), DNA cleavage inhibitors (Fortune and Osheroff 1998), TOP2-DNA binding inhibitors (Classen, Olland et al. 2003) and ATP binding site inhibitors (Chene, Rudloff et al. 2009) based on the inhibition of different steps in the enzymatic cycle.

Figure 18- Catalytic inhibition of TOP2 by merbarone. Schematic highlighting catalytic inhibitor (merbarone) interfering with the cleavage reaction of the TOP2 enzymatic cycle. Modified from (Vos, Tretter et al. 2011) with permission.

The TOP2 catalytic inhibitor merbarone inhibits the TOP2-mediated DNA cleavage reaction, thereby preventing the trapping of TOP2 in a covalent complex with DNA (see **Fig. 18**) (Fortune and Osheroff 1998). Although merbarone induces DNA damage and is currently being clinically tested as an anti-tumor agent, the mechanism behind its genotoxicity remains to be elucidated (Drake, Hofmann et al. 1989) (Larsen,

Escargueil et al. 2003). With TOP2 enzymatic activity blocked, the possibility that the alterations in supercoiling homeostasis can stall replication forks, thereby inducing fork collapse, is reasonable but yet to be explored (Wang and Eastmond 2002) (Wang, Roy et al. 2007).

1.5.6 Targeting topoisomerases- Current perspective

Although targeting topoisomerases has had a significant clinical impact on cancer patient survival over the years, their use is sometimes limited due to adverse side-effects. In particular, TOP2 poisons, such as etoposide, are implicated in chromosomal rearrangements that can lead to therapy related acute leukemias (Felix 2001) (Pendleton, Lindsey et al. 2014). Anthracyclins, another class of TOP2 poisons, induce cardiotoxicity leading to severe therapy-related complications (Menna, Salvatorelli et al. 2008). TOP1 poisons such as irinotecan and topotecan have also been reported to induce neutropenia and severe diarrhoea (Kweekel, Guchelaar et al. 2008) (Garst 2007), with a potential to induce secondary malignancies (Attia, Aleisa et al. 2009). In addition, the emergence of mutant topoisomerases (Pommier, Letaurtre et al. 1994) (Kubo, Yoshikawa et al. 1996) (Chikamori, Grabowski et al. 2003), activation of multiple drug resistance (Hofmann and Mattern 1993) (Sohn, Lee et al. 2006) (Wijdeven, Pang et al. 2015) or DNA repair pathways (Mehta, Awah et al. 2018), changes in topoisomerase gene expression (Takano, Kohno et al. 1992) (Froelich-Ammon and Osheroff 1995) or localization (Feldhoff, Mirski et al. 1994), could all reduce treatment efficacy, ultimately rendering currently used topoisomerase poisons ineffective and resulting in poor patient survival.

Hence, there is a growing need to identify and understand the molecular mechanisms that regulate the efficacy of these highly promising TOP inhibitors, which are also currently being tested for their role as anti-inflammatory and anti-viral agents (Cuya, Bjornsti et al. 2017). In addition, the search for novel bioactive molecules and delivery approaches that have higher efficacy to target topoisomerases, as well as the development of alternative regimens that prevent therapy-related secondary translocations are currently under way (Reichardt, Tabone et al. 2018) (Hevener, Verstak et al. 2018) (Liang, Wu et al. 2019) (You and Gao 2019). The influence of these novel approaches could potentially help negate side-effects and improve clinical outcomes.

1.6 Objectives of the study

A novel role in replication fork chaperoning mediated by the chromatin structuring factor HMGA2 has been previously described in our laboratory, although, the underlying mechanism behind this fork protection was not clear. *In vitro* studies from our laboratory have hinted at the possibility that the chaperoning function involves control/regulation of local changes in DNA topology. I hypothesized that HMGA2 could be a modulator of dynamic chromosomal DNA supercoil changes, which could contribute to fork stability during replication stress. The objective of the present work was to address the precise role of human HMGA2 protein during perturbed replication and to provide a more accurate and complete appreciation of how the protein functions in replication fork fidelity.

Furthermore, I will examine the functional connection between HMGA2 and topoisomerases at replication forks *in vivo*, using cancer cell models that allow the alteration of HMGA2 expression as well as by blocking topoisomerase function. Considering that the protein was found to bind and constrain both positive and negative supercoiled DNA with high affinity *in vitro*, and implicated in a myriad of functions *in vivo*, the possibility of more than one mechanism of fork protection exists. Such mechanism(s) could confer unprecedented advantage to cancer cells under induced replication stress which I aim to uncover using cell-based functional assays and rescue studies.

2. Materials and methods

2.1 Chemicals / Reagents

Names	Source	Identifier
doxycycline hyclate	Sigma, St. Louis, Missouri, USA	#D9891
hydroxyurea	Sigma, St. Louis, Missouri, USA	#H8627
etoposide	Sigma, St. Louis, Missouri, USA	#E1383
merbarone	Santa Cruz, Dallas, TX, USA	#500526
SN38	Abcam, Cambridge, UK	#ab141108
Irinotecan	Active Biochem, Kowloon, Hong Kong	#136572-09-3
TMPyP4	Santa Cruz, Dallas, TX, USA	#204346
protease inhibitor	Roche, Basel, Switzerland	#04693159001
sodium deoxycholate	Sigma, St. Louis, Missouri, USA	#D6750
sodium lauroyl sarcosine	Sigma, St. Louis, Missouri, USA	#L9150
proteinase K	Thermo Scientific, MA, USA	#EO0491
lipofectamine 2000	Invitrogen, Carlsbad, CA, USA	#11668-019

Table 1- List of chemicals / reagents

2.2 Instruments

Names	Supplier
luminescence imager	LAS4000; Fujifilm, Pittsburgh, PA, USA
UV imaging system	Quantum Vilber, Marne Valley, France
dot blot	Cleaver Scientific, Warwickshire, UK
CHEF DRII	Bio-Rad, Hercules, CA, USA
UVP HL-2000 Hybrilinker	Fisher Scientific, New Hampshire, USA
ProBlot hybridization oven	Labnet, New Jersey, USA
TECAN Infinite M200 Pro	Tecan Trading AG, Mannedorf, Switzerland

Table 2- List of instruments

2.3 Cell lines

H1299 cell line (human non-small cell lung carcinoma; #CRL-5803) was obtained from ATCC and its CRISPR-Cas9 mediated HMGA2 knockout (KO) cell line were generated at Novartis, California (a kind gift of Dr. Zhizhong Li). HMGA2 KO cells were generated using CRISPR-Cas9 by lentiviral infection of HMGA2 sgRNA (5'-ggaggcaggatgagcgcacg-3') designed to target the immediate downstream region of the start codon in six alleles, thereby creating frame shifts at the N-terminus. Frameshifts were verified by PCR/sequencing (Ahmed, Ramani et al. 2019). HeLa cells (cervical epithelial adenocarcinoma; #CCL-2) were obtained from ATCC and its three stable HMGA2 expressing clones P2, P8 and P19 were previously generated in Prof. Dröge's laboratory (Sabrina Peter) (Summer, Li et al. 2009). Similarly, A549 cells (human lung epithelial sarcoma; #CCL-185) were obtained from ATCC and its three stable HMGA2 expressing clones 1.3, 1.5 and 1.6 were previously generated in Prof. Dröge's laboratory (Sabrina Peter) (Summer, Li et al. 2009).

Cell lines / Model systems	HMGA2 expression	p53 status
H1299 cells (knockout model)		null
H1299 Parental / H1299	+	
HMGA2 KO	-	
HeLa cells (overexpression model)		wild-type
HeLa Parental / HeLa	-	
P2/P8/P19	+	
A549 cells (overexpression model)		wild-type
A549 Parental / A549	-	
1.3/1.5/1.6	+	
HT1080 cells (knockdown model)		wild-type
HT1080 C1 or HT1080 C2 (Dox-)	+	
HT1080 C1 or HT1080 C2 (Dox+)	-	

Table 3- HMGA2 expression and p53 status in different cancer cell models

Recombinant HT1080 C1 and C2 cells with doxycycline inducible shHMGA2 were previously generated at Prof. Dröge's laboratory (Yu, Lim et al. 2014). HMGA2 knockdown was achieved by 2 µg/ml doxycycline hyclate treatment once every day for four days (Yu, Lim et al. 2014). See **Table 3** for HMGA2 expression profiles and nomenclature for cell lines used in the study. Human embryonic stem cells (hESC) (Genea 047) were obtained from Genea Biocells (Australia). All cell lines were confirmed free of mycoplasma contamination and have been authenticated by short tandem repeat (STR) genotyping (1st BASE, Singapore). HMGA2 expression was routinely analyzed by Western blotting for cell line validation.

2.4 Cell culture and drug treatment

H1299 cells were grown as monolayers at 37°C and 5% CO₂ in RPMI 1640 (Gibco, MA, USA) containing 10% FBS (Gibco) and 1% penicillin-streptomycin (Gibco). HeLa, A549 and HT1080 C1/C2 cell lines were cultured in DMEM (Gibco) containing 10% FBS, 1% L-Glutamine (Gibco) and 1% penicillin-streptomycin under standard culture conditions. hESCs were cultured at 37°C in 5% CO₂ / 5% O₂ in 6 well collagen coated culture plates (Corning® BioCoat™) with M2 media (Genea Biocells) containing 0.25% penicillin-streptomycin. Upon reaching confluency, cells were harvested by first gently washing with PBS (Gibco) followed by trypsinization with passaging solution (Genea Biocells). Detached cells were then collected by neutralization solution (Genea Biocells). All cell lines were treated with either hydroxyurea, etoposide, merbarone, SN38 or TMPyP4 at different concentrations for 24/48 h. For 48 h drug treatment, media was changed every 24 h with fresh drug.

2.5 Western blotting

Monolayer cells were harvested and washed with PBS. Protein lysates were prepared by resuspending cell pellets in ice-cold RIPA lysis buffer (0.01 M Tris-HCl pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 0.1% SDS, 1% triton X-100, 0.1% sodium deoxycholate and 0.14 M NaCl) containing protease inhibitor cocktail. Samples were then sonicated for 3 s (8 times) at low amplitude and spun at 15000 rpm for 20 min at 4°C. Supernatant was collected, and protein concentration was estimated using the protein assay dye (Bio-Rad) with BSA (NEB, Ipswich, MA, USA) used as a standard. Lysates were then heated at 95°C for 10 min with HMGA1, HMGA2 and TOP1 analysed on Bolt™ 4-12% Bis-Tris gels (Invitrogen) whereas TOP2A and TOP2B were analysed on 8% SDS-

PAGE. Following separation, proteins were transferred on to 0.2 μ m PVDF membranes (Bio-Rad). Membranes were blocked for 1 h using the superbloc™ T20 blocking buffer (ThermoFisher) followed by overnight incubation of primary antibody at 4°C. Subsequently, membranes were washed with 1X TBST (0.1% Tween 20) three times (10 mins each) followed by incubation with HRP labelled secondary antibody for 1 h at room temperature. Membranes were washed again in 1X TBST (0.1% Tween 20) three times (10 mins each) and blots were developed with chemiluminescent HRP substrate (Millipore) in a luminescence imager. Densitometric analysis was performed using ImageJ software.

Designation	Species/ Clonality	Source	Identifier	Dilutions Used
α - HMGA1	rabbit monoclonal	CST	7777S (D6A4)	1:1000
α - HMGA2	rabbit polyclonal	CST	5269S RRID:AB_10694917	1:1000
α - HMGA2	rabbit polyclonal	Abcam	ab41878 RRID:AB_2279684	1:200
α -TOP1	rabbit monoclonal	Abcam	ab109374 RRID:AB_10861978	1:2000
α - TOP2A	rabbit polyclonal	TopoGEN	TG2011-1 RRID:AB_1934304	1:2000
α - TOP2B	rabbit monoclonal	Abcam	ab109524 RRID:AB_10859793	1:2000
α - beta-actin	mouse monoclonal	Sigma	A2228 RRID:AB_476697	1:5000
α -GAPDH	mouse monoclonal	Invitrogen	A21994 RRID:AB_2535905	1:4000
HRP- conjugated	goat anti-mouse	Dako	P0447 RRID:AB_2617137	1:20000
HRP- conjugated	goat anti-rabbit	Dako	P0448 RRID:AB_2617138	1:20000
α - TOP1cc	mouse monoclonal	EMD Millipore	MABE1084	1:500

Table 4 - Antibody resources

2.6 Pulsed field gel electrophoresis (PFGE)

DNA fragmentation following drug treatment was analysed using PFGE. Briefly, cells were harvested, resuspended in cell suspension buffer and then cast into a disposable plug mould by mixing resuspended cells with equal volumes of 2% low melting point agarose (Sigma) that was then allowed to solidify at 4°C. The solidified agarose plugs were incubated in lysis buffer at 50°C for 24 h and then washed four times (1 h each) with wash buffer. The plugs were then run on a 1% megabase agarose gel (Bio-Rad) in a CHEF DRII equipment (specific run conditions of 120° field angle, 5 to 30 s switch times, 5 V/cm and 14°C for 17 h in 1X TAE).

Cell Suspension Buffer	Lysis Buffer	Wash Buffer
10 mM Tris, pH 7.2 50 mM EDTA 20 mM NaCl	0.2% sodium deoxycholate 1% sodium lauroyl sarcosine 100 mM EDTA, pH 8 1 mg/ml proteinase K	20 mM Tris, pH 8 50 mM EDTA

Table 5- PFGE buffer compositions

Lambda PFG ladder (NEB) ranging between 48.5-1018 kb was used as a molecular size marker. Subsequently, gels were stained with ethidium bromide for 1 h, de-stained with distilled water and DNA fragments were visualized using UV imaging system.

2.7 Southern blotting

Following DNA fragmentation analysis, telomeric DNA was detected by Southern blotting using the TeloTAGGG telomere length assay kit (Roche, 12209136001) as per the manufacturer's instructions. Briefly, PFGE gels were first depurinated for 10 min at room temperature followed by denaturation (twice) and neutralization (twice) for 15 min each at room temperature (see **Table 6** for buffer composition). Subsequently, an overnight capillary transfer was set up with DNA transferred onto a positively charged nylon membrane (Roche) using 20X SSC transfer buffer. DNA was fixed to the membrane by UV crosslinking at 1.2 J/cm² for 30 s in a HL-2000 Hybrilinker, followed by washing twice in 2X SSC.

Name	Composition
Depurination buffer	0.25 M HCl
Denaturation buffer	0.5 M NaOH, 1.5 M NaCl
Neutralization buffer	0.5 M Tris-Cl, 3 M NaCl, pH 7.5
20X SSC Transfer buffer	3 M NaCl, 0.3 M Sodium citrate, pH 7
Stringent buffer I	2X SSC, 0.1% SDS
Stringent buffer II	0.2X SSC, 0.1% SDS
Maleic acid buffer	0.1 M Maleic acid, 0.15 M NaCl
Wash Buffer	0.1 M Maleic acid, 0.15 M NaCl, 0.3% Tween 20
Detection buffer	0.1 M Tris-Cl, 0.1 M NaCl pH 9.5

Table 6- Southern blot buffer compositions

The membranes were washed in prehybridization buffer for 1 h followed by hybridization overnight using the telomere probe (1:5000). Both prehybridization and hybridization were carried out at 42°C in a hybridization oven. Membranes were then washed twice (5 min each) with stringent buffer I at room temperature followed by two more washes (20 min each) with stringent buffer II at 50°C. Following stringent washes, membranes were first incubated in freshly prepared 1X blocking solution for 30 min before incubating in Anti-DIG-AP working solution for another 30 min. Finally, membranes were washed twice for 15 min each in 1X washing buffer, followed by a short incubation in 1X detection buffer. Next, substrate solution was added drop-wise and the membranes were exposed to X-ray film (Carestream, Rochester, New York, USA) which were then developed in X-OMAT 2000 Processor (Kodak, Rochester, New York, USA).

2.8 Caspase activity

Caspase 3/7® assays (G8090, Promega, Wisconsin, USA) were performed as per the manufacturer's instructions. 5000 cells were seeded in triplicates for each condition and allowed to attach overnight in a white-walled 96-well plate (Corning Inc., New York, USA). Cells were treated with different doses of SN38 for either 6 or 24 h. Following treatment, caspase 3/7 activity was measured by adding 100 µl of room temperature equilibrated caspase reagent to each well and incubating at room

temperature for 1 h. DMSO treated cells were used as vehicle control. Following the incubation period, luminescence was measured using a plate reading luminometer (Infinite M200).

2.9 Cell viability assay

Cell viability assays were performed using the CCK-8 kit (ALX-850-039-0100, Enzo Life Sciences, New York, USA) as per the manufacturer's instructions. Briefly, 5000 cells were seeded in triplicates for each condition and allowed to attach overnight in a 96 well black/clear-bottom plate. Cells were treated with the indicated doses of SN38 for 24 h and etoposide or merbarone for 48 h. DMSO treated cells were used as negative control. Following treatment, 10 μ l of CCK-8 solution was directly added to each well of the plate and incubated for 2 h at 37°C. Absorbance was measured at 450 nm using a microplate reader (Infinite M200).

2.10 Plasmids and purified proteins

The mammalian HMGA2 expression constructs pEF1/*Myc-A-hmga2*-FLAG (HMGA2) and pEF1/*Myc-A-hmga2-hook2/3*Mutant-FLAG (2,3M HMGA2) were previously generated in Peter Dröge's laboratory (Yu, Lim et al. 2014) by inserting the corresponding HMGA2 coding regions between the KpnI and EcoRI sites into pEF1/*Myc-A* plasmid (Invitrogen). All the *in vitro* binding, cleavage and relaxation assays were performed with the Renilla reporter plasmid containing the HSV-TK promoter (pRL-TK, 4045 bp, E2241, Promega).

Recombinant human topoisomerase 2 alpha (TOP2A, 78303Z, Affymetrix, CA, USA) was used for supercoil DNA relaxation assays. Purified human recombinant wild-type and hook 2,3 mutant HMGA2 proteins (0.02M HEPES pH 7.5, 0.5M NaCl, 10% (v/v) glycerol and 2mM TCEP) were provided by the protein production platform (PPP, NTU, Singapore) from BL21 (DE3) Rosetta cells. Standard techniques that include his-tag affinity chromatography and size exclusion chromatography were used for protein purification.

2.11 Gel retardation assay

Wild-type and hook 2,3 mutant HMGA2 proteins were incubated with 0.1 μ g of supercoiled Renilla plasmid at 37°C for 30 min in a buffer containing 0.05 M NaCl, 0.05 M KCl, 5 mM MgCl₂, 0.01 M Tris-Cl, pH 7.9, 0.1 mM EDTA, 1 mM ATP, 15 μ g/ml

BSA. Samples were then mixed with a gel loading dye without SDS (NEB) and analyzed on 0.8% agarose gel (Lonza, Basel, Switzerland) in 1X TAE buffer in the absence of ethidium bromide.

2.12 DNA cleavage assay

The efficacy of etoposide in trapping TOP2 cleavage complexes was determined using the DNA cleavage assay. Indicated amounts of etoposide diluted in DMSO were incubated with 0.1 µg of supercoiled Renilla plasmid at 37°C for 30 min in a buffer containing 0.05 M NaCl, 0.05 M KCl, 5 mM MgCl₂, 0.01 M Tris-Cl, pH 7.9, 0.1 mM EDTA, 1 mM ATP, 15 µg/ml BSA. 4.5 units of human TOP2A was added to each sample to initiate relaxation. The reaction was then stopped with 0.3% (w/v) SDS followed by proteinase K (Thermo Scientific) digestion for 20 min at 37°C. Samples were then analyzed on 0.8% agarose gel (Lonza) in 1X TAE buffer in the absence of ethidium bromide.

2.13 HMGA2 mediated DNA relaxation assay

0.1 µg of supercoiled Renilla plasmid was incubated with different amounts of wild-type and hook 2,3 mutant HMGA2 at 37°C for 30 min in a reaction buffer containing 0.05 M NaCl, 0.05 M KCl, 5 mM MgCl₂, 0.01 M Tris-Cl, pH 7.9, 0.1 mM EDTA, 1 mM ATP, 15 µg/ml BSA. 0.12 units of human TOP2A was added to each sample in order to achieve partial DNA relaxation and the reaction was stopped with 0.3% (w/v) SDS followed by proteinase K digestion. Samples were then analyzed on 0.8% agarose gel (Lonza) in 1X TAE buffer overnight and visualized under UV by staining with ethidium bromide after electrophoresis.

2.14 Complementation assay

Approximately 4×10^5 cells were seeded in 6-well tissue culture plates and allowed to attach overnight. Prior to transfection, media was replaced with penicillin-streptomycin free media and cells were transfected with the indicated amounts of either HMGA2 or 2,3M HMGA2 expression vectors with mock (backbone construct) used as control. Lipofectamine 2000 was used as the transfection reagent. Cells were incubated with the lipofectamine-DNA mixture for 6 h, following which fresh media containing penicillin-streptomycin was added. 24 h post transfection, cells were harvested and split into 6-well tissue culture plates for either Western blot analysis or PFGE (see

sections. 2.5 and 2.6 respectively). For caspase activity or cell viability assays, transfected cells were harvested and seeded as triplicates in a 96-well plate and assayed as described in **sections. 2.8 and 2.9** respectively.

2.15 *In vivo* complex of enzyme (ICE) assay

Human topoisomerase ICE assay kit (TG1020-0, Topogen, Colorado, USA) was used to detect endogenous topoisomerase 1-DNA cleavage complexes (TOP1cc) as per the manufacturer's instructions. Briefly, 1.2×10^6 cells were seeded in 60 mm tissue culture plates and allowed to attach overnight. Cells were then exposed to 20 μ M SN38 for 30 min. Following drug treatment, media was removed, and cells were rinsed twice with pre-warmed PBS. The cells were then lysed with 330 μ l of buffer A and the gooey mass was collected using a cell scraper in a sterile tube. Next, 115 μ l of buffer B was added to the lysate and mixed by inverting the tube 4-6 times followed by incubation for one min at 25°C. The DNA clumped precipitate was then spooled out into a fresh tube and mixed with 200 μ l of buffer C and incubated for one minute. Following incubation, the samples were centrifuged at 13,000 rpm for 2 min at 25°C and the supernatant was discarded. This wash step was repeated twice, and the pellet was allowed to dry for one min after the final wash. 100 μ l of buffer D was added and the pellet was mixed several times and incubated at 37°C for 5 min to dissolve the DNA. Finally, 16 μ l of 0.1 M HEPES was added and the samples were sonicated at low setting to shear high molecular weight genomic DNA. Samples were diluted in 25 mM phosphate buffer and the DNA concentration was estimated using Nano-Drop (Thermo Scientific). Equivalent amounts of DNA were loaded onto a nylon membrane using a dot blot apparatus (Cleaver Scientific) and probed with TOP1cc antibody (see **Table 4**). Spotted DNA was stained by ethidium bromide serving as loading control.

2.16 Magnetic tweezers experiments

DNA molecules were tethered to a functionalized glass coverslip by a superparamagnetic bead (Dynabeads MyOne) of 1 μ m in diameter (Ahmed, Ramani et al. 2019). The force was applied to the DNA molecules through the bead under an inhomogeneous external magnetic field generated by a pair of Neodymium magnets and the force was controlled by the height of the magnets manipulated by a translational micromanipulator (MP-285, Sutter Instruments). A rotation stage (DT-50, Physik Instruments) was used to rotate the magnets in order to wind/unwind torsion-

constrained DNA tethers. The change of linking number (Lk) causes DNA twist deformation and results in the accumulation of twist elastic energy. Above a threshold, the accumulated energy is relaxed through chiral bending into plectonemic supercoiling conformation at forces below 0.5 pN, which is indicated by the DNA extension decreasing linearly as a function of ΔLk .

Rotating the magnetic bead 30 turns results in winding/unwinding DNA and thus +/- supercoiled DNA is formed. Adding 5 nM recombinant human topoisomerase 1 (PROSPEC) is capable of removing any onset supercoiled DNA if transiently formed showing that extension remained nearly at the level of relaxed DNA conformation. A mixture of 5 nM TOP1 and 5 μ M SN38 was added into the channel and the SN38 dependent effect was investigated by multiple cycles of relaxation on both (-) scDNA and (+) scDNA. Similar experiments were performed in the presence of 5 nM TOP1 and 500 nM HMGA2 for both (-) scDNA and (+) scDNA. The buffer solution used in the experiments contained 100 mM KCl and 20 mM Tris (pH 7.4). All experiments were conducted at 23 ± 1 °C.

2.17 *In vivo* PDX analysis

The patient-derived xenograft (PDX) analysis was performed by Stephen Wong (PhD Graduate Student, Genome Institute of Singapore, Agency for Science, Technology and Research). Primary tumor tissues were collected from consented colorectal cancer patients (Singapore General Hospital and National Cancer Centre Singapore) and were used to generate tumor xenografts in mice (Genome Institute of Singapore, Agency for Science, Technology and Research). The IRB or Ethics Committee (SingHealth Institutional Review Board) sanctioned the study (approval no. 2015/2165). Animal studies were approved by the Biological Resource Centre IACUC (protocol #171207) and were conducted in Biological Resource Centre, A*STAR following ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines which are intended to improve the reporting of research using animals (Kilkenny, Browne et al. 2010).

Briefly, 5-6-week-old SCID mice were inoculated with 100 mg PDX tumor material. When tumor volumes, measured using Vernier calipers, reached between 150 mm³ to 350 mm³, mice were randomized into control and treatment groups (n= 6 mice/group). Mice from the control groups received only the vehicle (PEG 400, Sigma and Water

for Injection, BBraun; mixed 1:1 v/v), at a dose volume of 0.1 ml per 10 grams of body weight. Animals in the treatment groups were given Irinotecan at a dose of 50 mg/kg. Both the vehicle and the drug were administered intraperitoneally, once weekly for 6 consecutive weeks. Tumor volume was calculated using the formula $(L \times W^2)/2$ (L, longest axis; W, shortest axis). Tumor growth inhibition was determined using the formula $(C_{\text{dayB}} - T_{\text{dayB}})/(C_{\text{dayB}} - C_{\text{dayA}}) \times 100$, where C is the control group, T is the treatment group, dayA is the mean tumor volume on the day of first dose and dayB is the tumor volume at a specified time point.

2.18 Statistical evaluation

DNA fragmentation as well as cell cytotoxicity data are represented as mean $\pm$ standard deviation (SD), unless otherwise stated. Unpaired two tailed t-test was used to calculate statistical significance. ns- not significant, *P < 0.05, **P < 0.01, ***P < 0.001 and **** p < 0.0001. GraphPad Prism was used for data representation.

2.19 Software

Names	Supplier
ImageJ v1.50	ImageJ, NIH, Maryland, USA (RRID:SCR_003070)
GraphPad Prism 8	GraphPad Software, San Diego, CA, USA (RRID:SCR_015807)

Table 7- Software list

3. Results

3.1 HMGA2 guards hydroxyurea-induced stalled replication forks from collapse

Genome instability is a severe consequence of endogenous replication stress that is characterized by a reduction in replication fork speeds, fork stalling and ultimately fork collapse (Irony-Tur Sinai and Kerem 2018). Reduced dNTP levels have been associated with increased fork stalling, generating excess unprotected ssDNA, potentially as a result of helicase uncoupling from polymerase, ultimately interfering with the DNA replication program (Magdalou, Lopez et al. 2014) (Nedelcheva, Roguev et al. 2005).

Hydroxyurea (HU) diminishes cellular dNTP levels by inhibiting the enzyme ribonucleotide reductase (RNR), leading to replication fork stalling and collapse into DSBs, thereby causing widespread DNA damage (Bianchi, Pontis et al. 1986) (Lundin, Erixon et al. 2002) (Techer, Koundrioukoff et al. 2017). In addition, HU was recently reported to induce fork reversal in human cancer cells (Zellweger, Dalcher et al. 2015). The occurrence and function of regressed forks due to prolonged fork stalling has been a matter of debate for years and is considered a 'double edged sword', with two contradicting models proposed: (i) a physiological intermediate that is required to promote fork restart/repair, aiding replication progression (Atkinson and McGlynn 2009) (Betous, Mason et al. 2012) and (ii) a pathological assembly (chicken foot like structure) that is vulnerable to nucleolytic degradation, generating double strand breaks (Liu 2016) (Neelsen and Lopes 2015) (Mijic, Zellweger et al. 2017).

HMGA2, a nuclear protein, was found to be closely associated with HU-induced stalled replication forks, preventing fork collapse and DNA damage (Yu, Lim et al. 2014). The protein's fork chaperone activity was demonstrated in two human cell systems: HT1080 fibrosarcoma and embryonic stem cells as well as in heterologous yeast and bacteria, with the three AT-hooks indispensable for the protection of stalled forks (Yu, Lim et al. 2014). Moreover, heterologous expression of human HMGA2 was found to diminish HU-induced DNA damage in *Saccharomyces cerevisiae*, by interfering with the occurrence of regressed forks/chicken-foot structures (Yu, Lim et al. 2014).

I have extended these studies to three other human cancer cell model systems that allow us to manipulate the expression levels of HMGA2, either via stable overexpression (HeLa and A549 cell lines) or by HMGA2 knockout (H1299 cell line) (see **Table. 3** for details on HMGA2 expression profiles) (**Fig. 19**).

Figure 19- Western blot analysis to confirm HMGA2 expression levels in three different cancer cell model systems. (A) HMGA2 expression in parental HeLa (cervical carcinoma) cells and three stable HMGA2-overexpressing clones (P2/P8/P19), **(B)** HMGA2 expression in parental A549 (human lung carcinoma) cells and three stable HMGA2-overexpressing clones (1.3/1.5/1.6) and **(C)** HMGA2 expression in parental H1299 (human non-small cell lung carcinoma) and H1299 HMGA2 knockout cell line. β-actin was used as loading control. (A) taken from (Ahmed, Ramani et al. 2019) and (B) taken from (Ahmed and Droge 2019).

In order to examine the effect of HMGA2 expression on fork stalling, we utilized pulsed-field gel electrophoresis (PFGE) to carefully monitor double strand break induction, as a direct measure of fork collapse, after exposing cells to hydroxyurea for 24 h. To minimize DNA damage by external factors such as DNA handling, drug treated cells were harvested, set in agarose plugs and lysed, releasing only cellular DNA (**Fig. 20**).

clones after 24 h HU exposure or control treatment (left panel). DNA fragmentation (both for the >1 Mb as well as the 30-100 kb fractions) was quantified using ImageJ (right panel) (n=3 independent experiments). Note the substantial decrease in the 30-100 kb fragments across the three HMGA2-expressing clones (P2/P8/P19) (marked in red) compared to parental HeLa cells lacking HMGA2. **(B)** PFGE analysis of A549 cells (parental) and three HMGA2-expressing clones (1.3/1.5/1.6) after 24 h HU exposure or control treatment (left panel). DNA fragmentation (both for the >1 Mb as well as the 30-100 kb fractions) was quantified using ImageJ (right panel) (n=3 independent experiments). **(C)** PFGE analysis of H1299 cells (parental) and H1299 HMGA2 knockout cells after 24 h HU exposure (left panel). DNA fragmentation (both for the >1 Mb as well as the 30-100 kb fractions) quantified using ImageJ (right) (n=3 independent experiments). See methods for details on statistical assessment. Note that one experiment used for quantification in (B), was provided by Priya Ramani. Modified from (Ahmed, Ramani et al. 2019).

HMGA2 expression clearly inhibited HU-induced DNA break formation, with stark differences observed in the intensity of the 30-100 kb fragment fractions in all three cancer model systems tested (**Fig. 21**). Quantification of the two fragment fractions after normalization to total DNA loaded (combining both DSB fractions + intact DNA) from independent experiments revealed significant differences in the relatively smaller 30-100 kb DSBs. In addition, HMGA2-expressing clones (P2/P8/P19 and 1.3/1.5/1.6) consistently showed reduced DNA fragmentation compared to the parental cell lines (HeLa and A549, respectively) that lack HMGA2 expression. Overall, the data corroborates and extends our previous study (Yu, Lim et al. 2014) and suggest that HMGA2 guards stalled forks from collapse, most likely by preventing stalled forks from undergoing endonucleolytic attack, ultimately resulting in reduced genome fragmentation in different cellular systems.

3.2 Induced replication stress – TOP1 poison

Fork reversal is an evolutionary conserved mechanism and several in vitro and ex vivo studies have shown that it can occur as a result of excess (+) supercoiling ahead of the stalled fork, creating a fourth DNA branch (Postow, Ullsperger et al. 2001) (Olavarrieta, Martinez-Robles et al. 2002) (Bermejo, Lai et al. 2012). Since HMGA2 reduced formation of regressed forks in yeast cells (Yu, Lim et al. 2014), we hypothesized that HMGA2 could potentially regulate topological problems at stressed forks, thereby promoting their stability and preventing fork collapse. A cluster of single-molecule force spectroscopy methods (atomic force microscopy and magnetic

tweezers) revealed that HMGA2 can bind and constrain, with high affinity, both negative and positive plectonemic DNA, suggesting a plausible mechanism for its fork chaperone function (Zhao, Peter et al. 2017). Additionally, in an attempt to identify HMGA2 antagonists using a cell-based reporter system, our laboratory found evidence for a close functional association between topoisomerase 1 (TOP1) and HMGA2 (Peter, Yu et al. 2016). We decided to further investigate the fork stabilising function of HMGA2 *ex vivo* in the context of topological stress and hence utilized topoisomerase poisons that generate unconstrained supercoiling ahead of the replication machinery (Postow, Crisona et al. 2001) (Pommier, Leo et al. 2010). In addition to the three-cancer model system described above in **section 3.1**, we include a fourth previously described (Yu, Lim et al. 2014) cancer model system, recombinant HT1080 fibrosarcoma cells (two clonal cell populations termed C1 and C2), that allow downregulation of HMGA2 expression upon doxycycline induction of shHMGA2 (**Fig. 22**).

Figure 22- Western blot evaluation to confirm downregulation of HMGA2 expression in recombinant HT1080 C1 and C2 cells. Doxycycline treatment was carried out for four days to allow induction of shHMGA2 stably integrated by lentiviral transduction (Yu, Lim et al. 2014). Taken from (Ahmed and Droge 2019).

We first sought to inhibit TOP1, as it can efficiently remove torsional stress generated at replication forks, thus promoting smooth progression of the replication machinery (Bermejo, Lai et al. 2012) (Keszthelyi, Minchell et al. 2016). TOP1 is widely targeted in chemotherapeutic regimens to treat colon (Fujita, Kubota et al. 2015), lung (Sandler 2002) and ovarian cancers (Gershenson 2002), with novel anticancer drugs against TOP1 being constantly developed (Cinelli 2018). We utilized an interfacial, clinically relevant TOP1 poison, SN38, to study the proposed role of HMGA2 in protecting stalled forks. SN38 prevents TOP1-mediated religation of the DNA, thereby promoting stabilization of the intermediate TOP1-DNA cleavage complexes (TOP1cc). These lesions are dangerous to the genome, as collisions with the replication fork machinery could lead to the rapid generation of double strand breaks (Ryan, Squires et al. 1991)

(Vesela, Chroma et al. 2017). In addition, inhibiting TOP1 function could also lead to the accumulation of positive supercoils ahead of the replication fork, leading to fork stalling and collapse (Koster, Palle et al. 2007) (Menin, Ursich et al. 2018).

3.2.1 HMGA2 expression regulates chemosensitivity to SN38

We assessed the induction of DNA double strand breaks (DSBs) using PFGE as described above after exposure to SN38 in the four cancer cell model systems (previously described in **section 2.3**). Similar to our earlier observations following HU exposure, treatment with SN38 for 48 h also generated two large DNA fragment fractions at the 30-100 kb and >1 Mb range, while the bulky intact DNA remained in the well (See **Fig. 23** and **Fig. 24**).

Figure 23- HMGA2 guards against SN38 induced DNA damage in H1299 and A549 cancer cell models (A) PFGE analysis of H1299 (parental) and HMGA2 knockout cells after treatment with 2 μ M SN38 for 48 h (left panel). DNA fragmentation (both for the >1 Mb as well as the 30-100 kb fractions) was quantified using ImageJ (right panel) (n=3 independent experiments) (B) PFGE analysis of A549 (parental) and its three HMGA2 expressing clones (1.3/1.5/1.6) after treatment with 2 μ M SN38 for 48 h (left panel). DNA fragmentation (both for the >1 Mb as well as the 30-100 kb fractions) was quantified using ImageJ (right panel) (n=3 independent experiments). Note the decrease in 30-100 kb fragments in HMGA2-expressing cells (marked in red)

compared to cells that lack HMGA2. See methods for details on statistical assessment. Note that two experiments used for quantification in (B) were provided by Priya Ramani. Modified from (Ahmed, Ramani et al. 2019).

HMGA2 expression protected against SN38-induced fork collapse in two of our cancer cell model systems (H1299 and A549 cells) (**Fig. 23**). Surprisingly, the other two model systems (HeLa and HT1080 C1/C2), were sensitized to SN38-induced fork collapse in the presence of HMGA2, with significant differences observed in the 30-100 kb fragments (**Fig. 24**).

A

B

C

Figure 24- HMGA2 sensitizes to SN38-induced DNA damage in HeLa and HT1080 cancer cell models. PFGE analysis of (A) HeLa (parental) cells and three HMGA2-expressing clones (P2/P8/P19), (B) HT1080 C1 cells (Dox-/Dox+) and (C) HT1080 C2 cells (Dox-/Dox+) after treatment with 2 μ M SN38 for 48 h. Notice the increase in the 30-100 kb fragment fractions in HMGA2-expressing cells compared to cells that lack HMGA2. DNA fragmentation (both for the >1 Mb as well as the 30-100 kb) was quantified using ImageJ (right panel) after normalization to total DNA. See methods for details on statistical assessment. Taken from (Ahmed, Ramani et al. 2019).

Furthermore, we found that the induction of DNA fragments (>1 Mb and 30-100 kb) following SN38 treatment occurs as early as 6 h (**Fig. 25**), confirming that the fork collapse or DNA damage at replication forks is caused directly by the drug action. As an example, we tested the short-term SN38 exposure (6 h) in the HeLa cancer model system and found HMGA2 expressing P2 cells to be sensitized to SN38-induced replication stress (**Fig. 25**).

Figure 25- Observed SN38-induced genotoxicity in HeLa cells by HMGA2 detectable after 6 h SN38 treatment. PFGE analysis of HeLa (parental) cells and one HMGA2 expressing clone (P2), after treatment with 2 μ M SN38 for 6 h. Notice the rise in 30-100 kb fragments in HMGA2-expressing cells as compared to cells that lack HMGA2. DNA fragmentation (both for the >1 Mb as well as the 30-100 kb fractions) was quantified using ImageJ (right panel) (n=3 independent experiments) See methods for details on statistical assessment. (A) taken from (Ahmed, Ramani et al. 2019).

Western blot analyses of the SN38 target, TOP1, revealed similar endogenous expression levels in the four cancer cell model systems (**Fig. 26**), irrespective of HMGA2 expression, hence ruling out the possibility that the differential expression of TOP1 is responsible for the observed differences in sensitivity to SN38 treatment.

Figure 26- HMGA2 expression does not influence endogenous TOP1 levels across the four cancer cell models. Representative western blot analysis of TOP1 in (A) H1299 (parental) and HMGA2 knockout cells, (B) HeLa (parental) cells and three HMGA2-expressing clones (P2/P8/P19), (C) A549 (parental) and three HMGA2-expressing clones (1.3/1.5/1.6) and (D) HT1080 C1/C2 cells (Dox-/Dox+) (E) Comparative Western blot analysis for TOP1 expression across selected cell lines (left panel) (n=3 independent experiments). Quantification of TOP1 levels was done using the ImageJ software. See methods for details on statistical assessment. Note that one experiment used for quantification in (E) was provided by Priya Ramani. Modified from (Ahmed, Ramani et al. 2019)

3.2.2 HMGA2 influences caspase activation / cell viability in SN38 treated cancer cells

First, to rule out the possibility that the contrasting effects of SN38 treatment observed in different cell lines were due to differences in inherent growth rates, we performed cell viability assays that assess the number of viable cells, comparing cell proliferation rates between H1299 (parental) and HMGA2 KO cell lines over a period of four days

and found no significant differences in growth in the presence (H1299) or absence (HMGA2 knockout) of HMGA2 (**Fig. 27**).

Figure 27- HMGA2 expression does not alter proliferation rates in cancer cells. CCK8 assay in H1299 (parental) and HMGA2 knockout cells evaluated for growth rate changes over 96 h (n=2 independent experiments shown here; each time point had 3 technical replicates). Data plotted as mean $\pm$ SD. Statistical assessment was done using two-way ANOVA (Sidak's multiple comparisons). ns not significant. Taken from (Ahmed, Ramani et al. 2019).

Next, to further validate the differential drug sensitivity with respect to HMGA2 expression and to confirm the induction of apoptosis by DNA double strand breaks (Roos and Kaina 2006), we analyzed caspase activity by measuring luminescence following the caspase 3/7-mediated cleavage of a luminogenic substrate, at different concentrations of SN38. We found that the HMGA2 expressing parental H1299 (**Fig. 28A**) and clonal A549 cells (1.3/1.5/1.6) (data not shown; (Ahmed, Ramani et al. 2019)) had significantly lower caspase activity compared to their counterparts lacking HMGA2 after SN38 treatment, thereby validating our PFGE data shown in **Fig. 23A-B**. The opposite effect, a higher caspase 3/7 activity, was clearly observed in the other two cancer models, i.e. the HMGA2 positive P2/P8/P19 clones and HT1080 C2 Dox-, in response to SN38 treatment (**Fig. 28B-C**). In addition, we observed a marked increase in caspase activity in P2 cells that were treated with SN38 for 6 h (**Fig. 28D**), correlating to the PFGE analysis in **Fig. 25**.

Figure 28- Cell line dependent effects of HMGA2 expression on SN38-induced caspase activation. Caspase activity in (A) parental H1299 and HMGA2 knockout cells, (B) parental HeLa cells and three HMGA2-expressing clones (P2/P8/P19) and (C) HT1080 C2 cells (Dox-/Dox+) after SN38 treatment for 24 h. (D) Caspase activity in parental HeLa cells and the HMGA2-expressing clone P2 after SN38 treatment for 6 h. See methods for details on statistical assessment. Taken from (Ahmed, Ramani et al. 2019).

To further confirm these contrasting effects of genotoxicity in HMGA2 expressing cells, we utilized cell survival assays after drug treatment. CCK8 assays in H1299 and HeLa cell systems validated our caspase data (see **Fig. 29A-B**), with HMGA2 expressing H1299 cells resistant to SN38 treatment, highlighting a positive correlation to cell

survival (**Fig. 29A**). In contrast and as expected, the HMGA2-expressing P2 clone was sensitive to SN38 treatment, with significantly reduced cell viability (**Fig. 29B**).

Figure 29- Depending on the cell line, HMGA2 can either protect or sensitize against SN38-induced genotoxic stress. Cell survival assay in **(A)** parental H1299 and HMGA2 knockout cells and **(B)** parental HeLa cells and one HMGA2 expressing clone (P2) after SN38 treatment for 48 h. See methods for details on statistical assessment. Taken from (Ahmed, Ramani et al. 2019).

Taken together, we have uncovered HMGA2's unique ability to either protect or sensitise against SN38-induced replication stress in different cancer cells. However, the underlying mechanism(s) are yet unknown.

3.2.3 HMGA2 overexpression reduces SN38-induced genotoxicity in HMGA2 knockout cells

To further investigate whether fork protection in H1299 cells is due to HMGA2 expression, I decided to complement HMGA2 knockout cells by transiently overexpressing HMGA2. As expected, overexpression of HMGA2 (pEF1-MycA-hmga2-FLAG) recapitulated the protective function observed in **Fig. 23A**, with significantly reduced fragmentation observed in knockout cells expressing HMGA2 compared to mock control (pEF1-MycA-FLAG) (**Fig. 30A**). In addition, this protective effect was abrogated when the 2,3-hook mutant HMGA2, lacking two of the three AT-hooks, was overexpressed in HMGA2 knockout cells, pointing towards the involvement of AT-hooks and HMGA2-DNA interaction in conferring protection (**Fig. 30B**).

Figure 30- HMGA2 overexpression reduces SN38-induced genotoxicity in H1299 HMGA2 KO cells. (A) Representative PFGE analysis (bottom panel) of HMGA2 knockout cells after transient transfection with HMGA2 expression vector followed by 2 μ M SN38 treatment for 48 h. Corresponding Western blot analysis (top panel) of HMGA2 in the transfected HMGA2 knockout cells. Note the decrease in the 30-100 kb fragment fraction in cells complemented with the HMGA2 expression vector. (n=5 independent experiments) (B) PFGE analysis (right panel) of HMGA2 knockout cells after transient transfection of wild-type or 2,3-hook mutant HMGA2 expression vector followed by 2 μ M SN38 treatment for 48 h. Corresponding Western blot analysis (left panel) of HMGA2 in the transfected HMGA2 knockout cells (C) Analysis of apoptosis in HMGA2 knockout cells after transient transfection with HMGA2 expression vector followed by 2 μ M SN38 treatment. Mock transfected cells used as control (n=4 independent experiments) (D) Analysis of apoptosis in HMGA2 knockout cells after transient transfection with wild-type and 2,3-mutant HMGA2 expression vector followed by 2 μ M SN38 treatment. Mock transfected cells used as control (n=1 independent experiment). See methods for details on statistical assessment.

In agreement with these observations, we detected a significant reduction in SN38-induced caspase activation following complementation of H1299 HMGA2 knockout cells with wild-type HMGA2, while expression of the 2,3-hook mutant HMGA2 protein did not affect apoptosis (**Fig. 30C-D**).

3.2.4 HMGA intracellular levels are critical to SN38 induced genotoxic outcomes

An independent recent *in vitro* study from our laboratory had revealed that HMGA2 increases the efficiency of SN38 to trap TOP1 in a covalent complex (TOP1cc), leading to more nicked circular DNA as compared to reactions w/o HMGA2 (Peter, Yu et al. 2016). This effect was dependent on the amount of HMGA2 bound to DNA, with high HMGA2 amounts showing reduced nicking, whereas moderate HMGA2 amounts had more nicked DNA, indicating that the trapping and stabilization of TOP1 in covalent DNA complexes by HMGA2 is not linear. This hinted at the possibility that the variable sensitivity to drug treatment observed in HMGA2 expressing cells (parental H1299 and 1.3/1.5/1.6 are SN38-protected (**Fig. 23A-B**) whereas P2/P8/P19 and HT1080 Dox- are SN38-sensitized (**Fig. 24A-C**)) could be due to differential HMGA2 expression. A comparative Western blot analysis for HMGA2 expression across the tested cell lines revealed that the SN38-protected cells (parental H1299 and 1.3) have high levels of HMGA2 as compared to the SN38-sensitized cells (P2, HT1080 C1 and C2) that have low/moderate HMGA2 expression (**Fig. 31A-B**).

Figure 31- HMGA2 levels vary across tested cell lines. (A) Comparative Western blot analysis for HMGA2 expression across selected cell lines (left panel) (n=3

independent experiments). Quantification of HMGA2 levels was done using the ImageJ software. See methods for details on statistical assessment. Note that one experiment used for the quantification was provided by Priya Ramani. Taken from (Ahmed, Ramani et al. 2019) **(B)** Table depicting the cellular responses to SN38 treatment and its correlation to HMGA2 expression levels in the tested cell lines.

In addition, to exclude the possibility that the observed effect is due to differences in HMGA1 levels, a homologous chromatin factor that was also reported to guard replication forks in *E. coli* (Yu, Lim et al. 2014), we decided to determine HMGA1 expression levels across the tested cell lines. We found that HMGA1 expression is comparable within each cell line model system (**Fig. 32A-D**), as well as across different cell lines (**Fig. 32E**). The comparative Western blot revealed that the SN38-resistant cell lines (H1299 and 1.3) express slightly higher HMGA1 levels compared to their counterparts, although the difference was not statistically significant (**Fig. 32E**). Remarkably, the addition of both HMGA2 and HMGA1 (HMGA) expression values, that is quantified earlier in **Fig. 31A** and **Fig. 32E** respectively, revealed a similar trend of resistant cell lines expressing high HMGA levels as compared to SN38-sensitive cell lines (**Fig. 32F**), with HMGA2 being the critical contributor to this difference, implying that HMGA levels especially HMGA2 is crucial for the cellular outcome following exposure to TOP1 poison.

Figure 32- HMGA1 expression levels are comparable across the four cancer cell models. Western blot analysis of HMGA1 in **(A)** H1299 (parental) and HMGA2 knockout cells, **(B)** HeLa (parental) cells and three HMGA2 expressing clones

(P2/P8/P19), **(C)** A549 (parental) and three HMGA2-expressing clones (1.3/1.5/1.6) and **(D)** HT1080 C1/C2 cells (Dox-/Dox+) **(E)** Comparative Western blot analysis for HMGA1 expression across selected cell lines (left panel) (n=3 independent experiments). Quantification of HMGA1 levels was done using the ImageJ software. See methods for details on statistical assessment. Note that one experiment used for quantification in **(E)**, was provided by Priya Ramani **(F)** Combined quantification plotted for HMGA proteins across three independent experiments for both HMGA2 and HMGA1. Error bars show SD. Statistical assessment done using two-way ANOVA (Sidak's multiple comparisons). ns not significant, *p < 0.05, ***p < 0.001, ****p < 0.0001. Taken from (Ahmed, Ramani et al. 2019).

To directly demonstrate that the differential sensitivity to SN38 is due to differences in HMGA2 expression levels, we complemented HMGA2 knockout cells with varying amounts of the HMGA2 expression vector (**Fig. 33A**). We found that low/moderate levels of HMGA2 generated increased amounts of the 30-100 kb DNA fragments, whereas high levels of HMGA2 (in this case 1.5 μ g HMGA2 expression vector transfected) generated lower amounts of the 30-100 kb fragments (**Fig. 33A**). We followed up on these findings with cell survival assays that strengthened the above observation. While high HMGA2 expressing cells were found to be resistant to SN38, the low/moderate HMGA2 expressing cells were sensitive to SN38-induced genotoxicity compared to mock controls (**Fig. 33B**).

Figure 33- HMGA2 levels determine the extent of 30-100 kb fragment fraction generation. (A) Various amounts of HMGA2 expression vector (ranging from 0.25 to 1.5 μ g), transiently transfected in H1299 HMGA2 knockout cells, followed by 2 μ M SN38 treatment for 48 h. DNA fragmentation was evaluated by PFGE (centre panel). Western blot confirming HMGA2 expression at the indicated amounts transfected (left panel). DNA fragmentation (both for the >1 Mb as well as the 30-100 kb fractions) was quantified using ImageJ (right) (n=4 independent experiments). See methods for details on statistical assessment. (B) Cell viability (CCK8) assay following transfection of H1299 HMGA2 knockout cells with 0.25 μ g (low) and 1.5 μ g (high) HMGA2 expression vector (mock used as control), and SN38 treatment (2 μ M) for 48 h (n=4 independent experiments with 3 technical replicates in each). See methods for details on statistical assessment. (C) Comparative PFGE of different cell lines that either express high (H1299 parental), moderate (P2) or no (HeLa) HMGA2 after SN38 treatment for 48 h with untreated cells used as control (n=2 independent experiments) (D) HMGA2 expression in H1299 cells analyzed after 2 μ M SN38 treatment for 48 h (A, B, D) taken from (Ahmed, Ramani et al. 2019).

Furthermore, a comparative PFGE analysis with cell systems that either lack HMGA2 (HeLa), express low/moderate levels of HMGA2 (P2) or high amounts of HMGA2 (H1299) directly revealed the differential sensitivity to SN38. The 30-100 kb fragments were found to be considerably elevated in the moderate HMGA2 expressing P2 cells whereas the high HMGA2 expressing H1299 cells had reduced breaks (**Fig. 33C**). Additionally, the possibility that the expression levels of HMGA2 are altered with drug treatment was also ruled out as we did not see any significant difference in expression (**Fig. 33D**).

3.2.5 Accumulation of TOP1cc correlates with HMGA2 levels *ex vivo*

Single molecule analysis found HMGA2 to scrunch supercoiled DNA, generating HMGA2-scDNA complexes that appear as elongated, rod-like structures (Zhao, Peter et al. 2017). To further confirm that HMGA2 binds to supercoiled (sc) DNA via its three AT-hooks and form these higher-order complex structures, we used *in vitro* gel retardation assays that allow us to examine protein-DNA interactions (Lane, Prentki et al. 1992). We observed that at various stoichiometries of HMGA2:DNA, purified wild-type HMGA2 binds to supercoiled DNA in a concentration dependent manner and reduces its electrophoretic mobility compared to protein-free input DNA as shown in **Fig. 34**. Such an effect was almost absent when the 2,3-hook mutant was used at identical stoichiometric ratios, indicating that HMGA2 requires multiple AT-hooks to bind and scrunch scDNA within higher-order complexes.

Figure 34- HMGA2 binds supercoiled plasmid DNA and forms higher order HMGA2-scDNA complexes. Representative gel retardation assay with renilla reporter plasmid (4 kb) incubated at different molar ratios of purified wild-type and 2,3-hook mutant HMGA2 purified proteins as indicated. Before electrophoresis in 0.8% agarose gel, samples were incubated at 37°C for 30 min (n=3 independent experiments). Note that the input (control) is supercoiled renilla plasmid alone, with the positions of open circular DNA (oc) and supercoiled DNA (sc) marked. Taken from (Ahmed, Ramani et al. 2019)

Independent *in vitro* experiments performed with purified HMGA2 and TOP1 have shown that moderate levels of HMGA2 increase the efficacy of SN38 in trapping TOP1 with scDNA. However, at higher HMGA2 stoichiometries, this effect was considerably reduced (Peter, Yu et al. 2016), pointing at a potential competition for TOP1 binding sites on scDNA by HMGA2 (Zechiedrich and Osheroff 1990). We decided to further test this *in vitro* observation with a more direct approach (see ICE bioassay; **methods**), utilizing a specific antibody that allows us to detect and quantify trapped TOP1cc in the presence of SN38 *in vivo*. Dot blot analysis following SN38 treatment revealed that cells expressing high levels of HMGA2 (parental H1299) had lower TOP1cc levels compared to cells that lack HMGA2 (HMGA2 knockout) (**Fig. 35A**). In contrast, cells expressing moderate amounts of HMGA2 (P2 clone) accumulated higher levels of TOP1cc compared to HeLa cells that lack HMGA2 (**Fig. 35B**).

Figure 35- Accumulation of TOP1cc correlates with HMGA2 levels. Dot blot analysis comparing the accumulation of TOP1cc between (A) H1299 (parental) and HMGA2 KO cells and (B) HeLa (parental) and its HMGA2-expressing clone P2, after SN38 treatment (top sections). Quantification of TOP1cc accumulation (bottom sections) after normalization to total DNA visualized by ethidium bromide staining (n=3 independent experiments; representative blot shown). DMSO-treated cells were used as controls. See methods for details on statistical assessment. Taken from (Ahmed, Ramani et al. 2019)

In addition, in collaboration with Prof. Yan's laboratory, we utilized single molecule analysis that allowed us to investigate supercoiled DNA relaxation mediated by TOP1 (data not shown) (Ahmed, Ramani et al. 2019). Plectonemic supercoiled DNA generated using magnetic tweezers were first exposed to 5 nM TOP1, which rapidly and effectively relaxed scDNA (**Fig. 36A**), indicating that the enzyme was active. Next, upon adding 5 μM SN38 to this mixture, we observed much slower relaxation times, highlighting the trapping of TOP1 in covalent DNA complexes (TOP1cc) by SN38 (**Fig. 36B**). 500 nM HMGA2 in the presence of TOP1 also appeared to partially interfere with DNA relaxation, most likely due to the protein effectively constraining supercoiled DNA (**Fig. 36C**). Surprisingly, however, HMGA2 and SN38, when incorporated together with TOP1, showed massive, more than additive impairments in DNA

relaxation, indicating that HMGA2 could potentially work synergistically and increase drug-TOP1 interactions (**Fig. 36D**).

Figure 36- Effect of HMGA2, either alone or in combination with SN38, on TOP1-mediated DNA relaxation. Graphical representation of magnetic tweezers used to apply torsional stress on constrained DNA molecules, followed by analysis of supercoiled DNA relaxation times in the presence of (A) TOP1, (B) TOP1 and SN38, (C) TOP1 and HMGA2 or (D) TOP1, HMGA2 and SN38.

Taken together, our combined data strengthens the hypothesis that moderate HMGA2 levels (found in P2/P8/P19 and HT1080 Dox-), increase the number of trapped complexes (TOP1cc) by increasing SN38-TOP1 interactions, leading to more frequent fork collapse, whereas high HMGA2 levels (found in parental H1299 and 1.3/1.5/1.6) decrease accumulation of TOP1cc as a result of DNA supercoil constraint and/or TOP1 exclusion from DNA binding, leading to reduced DNA damage.

3.2.6 HMGA2 expression influences subtelomere fragility induced by the TOP1 poison SN38

With the levels of 30-100 kb DNA fragments specifically dependent on drug treatment and HMGA2 expression, we decided to map these regions to the human genome in order to understand their prevalence and importance. In addition, since these fragments accumulate consistently with the two drug models used in our study (**sections 3.1 and 3.2**), they might indicate vulnerability of yet unknown regions to genotoxic stress. Following treatment with SN38, the 30-100 kb fragments were extracted from agarose gels, purified and processed by high-throughput next generation sequencing (data not shown; (Ahmed, Ramani et al. 2019)). The sequencing data revealed that the 30-100 kb fragments arise partly from the fragile subtelomeric/telomeric regions of the genome, with the majority of the chromosomes containing breakpoints in the subtelomeric domains (data not shown; (Ahmed, Ramani et al. 2019)). To confirm the high-throughput sequencing data, we decided to perform Southern blots using a probe that detects telomeric repeat regions. Following PFGE analysis of SN38 treated cells, the DNA transferred to nitrocellulose membranes was probed for telomeric DNA. We observed that the 30-100kb fragments were enriched for (sub)telomeric DNA, as shown in **Fig. 37A-C**, thereby confirming our sequencing data. Furthermore, complementing HMGA2 knockout cells with high amounts of HMGA2 expression vector revealed that HMGA2 protected these fragile regions from SN38-induced DSBs (**Fig. 37A**). We also confirmed that the subtelomeric regions are sensitized in the P2/P8/P19 clones that express moderate levels of HMGA2, indicating that HMGA2 levels can influence stability of subtelomeres and telomeres by controlling SN38-induced replication stress (**Fig. 37B**). Interestingly, DNA fragmentation showed a markedly different pattern in low/moderate HMGA2-expressing human embryonic stem cells treated with SN38 for 24 h (**Fig. 37C**), where a smaller smear ranging in sizes of below 40 kb was apparent, pointing at the extreme subtelomeric/telomeric vulnerability of these cells to topological stress induced by SN38.

Figure 37- HMGA2 expression influences subtelomere fragility induced by the TOP1 poison SN38. (A) Western blot analysis after HMGA2 expression vector transfection (1.5 μ g) in H1299 HMGA2 knockout cells (left panel) and Southern blot analysis probing for telomeric DNA in HMGA2 knockout cells, following transfection with mock/HMGA2 expression vector and treatment with 2 μ M SN38 for 48 h (right panel). DMSO treated cells were used as control (n=3 independent experiments). (B) Southern blot analysis probing for telomeric DNA in HeLa (parental) and its three HMGA2 expressing clones (P2/P8 and P19), following treatment with 2 μ M SN38 for 48 h. DMSO treated cells were used as control (n=3 independent experiments). (C) Western blot analysis of HMGA2 expression in indicated cell lines (left panel) and Southern blot analysis probing for telomeric DNA in non-transformed human embryonic stem cells following treatment with 0.1 μ M SN38 for 24 h. DMSO treated cells were used as control (n=3 independent experiments). (D) Southern blot analysis probing for telomeric DNA in HeLa (parental) and its three HMGA2 expressing clones

(P2/P8 and P19), following treatment with 100 mM hydroxyurea for 24 h (n=3 independent experiments). **(E)** PFGE analysis for DNA fragmentation (left panel) and corresponding Southern blot analysis probing for telomeric DNA in HeLa (parental) cells, following treatment with indicated doses of TMPyP4. Treatment with 100 mM hydroxyurea was used as a control for DNA fragmentation comparison. Taken from (Ahmed, Ramani et al. 2019)

Using the same method, we found subtelomeric regions to be protected from hydroxyurea-mediated DNA damage in the presence of HMGA2 (**Fig. 37D**). To further confirm the specificity of DNA damage at these specialized locations, we used TMPyP4, a porphyrin compound that specifically stabilizes G-quadruplexes, inducing replication fork stalling. These structures are formed at particular G-rich sequences and are a characteristic feature of subtelomeres and telomeres (Lin and Yang 2017) (Vannier, Pavicic-Kaltenbrunner et al. 2012). Following a dose titration with TMPyP4, we observed a dose-dependent increase in the 30-100kb fragments, which were enriched for subtelomeric/telomeric DNA (**Fig. 37E**).

3.2.7 HMGA2 expression influences chemosensitivity to irinotecan *in vivo*

To corroborate our *ex vivo* analysis and further strengthen our *in vitro* observations, we decided to utilize colorectal cancer patient-derived tumor xenografts (PDX) that were maintained and evaluated by Stephen Wong (**Fig. 38**). PDX mice were treated with irinotecan, a TOP1 poison that is currently used in clinical practice to treat colorectal cancers (Fuchs, Mitchell et al. 2006).

Figure 38- Model for generating tumor xenografts from colorectal cancer patients. Primary tumors from five colorectal cancer patients were collected and

inoculated subcutaneously into immune-compromised mice, with each PDX model (935, 1017, 1030, 1073 and 1414) divided into two treatment groups, either irinotecan or just the vehicle control.

Analysis of HMGA2 expression by Western blotting revealed that three of the five (935, 1073 and 1030) PDX models expressed low/moderate levels of HMGA2 compared to our standard cell lines (high HMGA2 expressing H1299 and low/moderate expressing HT1080), whereas models 1017 and 1414 had no detectable HMGA2 expression (**Fig. 39A**).

Strikingly, analysis of tumor volumes in mice treated with irinotecan over a period of six weeks revealed tumor regression in only the moderate HMGA2-expressing PDX models (935, 1073 and 1030), but not in PDX models 1017 and 1414 that do not express HMGA2 (**Fig. 39B**). We could not observe substantial differences in tumor volumes in the five models treated with vehicle control (**Fig. 39C**), indicating that the presence or absence of HMGA2 did not affect tumor growth, thereby ruling out the possibility of variable growth rates contributing to the observed differences in tumor regression. To further confirm this finding, we analyzed tumor growth inhibition (TGI), a measure of treatment efficacy (considering the physiological growth of each of the vehicle treated control groups) and found that only the low/moderate HMGA2 expressing xenografts had TGI above 100%, confirming tumor regression, as compared to xenografts that lack HMGA2 which have TGI lower than 100% (**Fig. 39D**). Taken together, the *in vivo* data supports our *ex vivo* analysis, as we found that the low/moderate levels of HMGA2 sensitized PDX tumors to irinotecan treatment. Unfortunately, none of the PDX models expressed high HMGA2 levels comparable to the SN38-resistant H1299 cell line.

Figure 39- HMGA2 expression controls chemosensitivity to irinotecan in a mouse xenograft model. (A) Representative Western blot analysis of HMGA2 across five different colorectal cancer patient-derived tumor xenograft (PDX) samples (n=3 independent experiments). Quantification (right panel) was done using ImageJ software. See methods for details on statistical assessment. **(B)** Analysis of tumor growth across the five PDX models (n=6 animals/model) treated with irinotecan. **(C)** Analysis of tumor growth across the five PDX models (n=6 animals/model) treated with vehicle control. **(B, C)** Error bars show SEM. Statistical assessment was done using two-way ANOVA (Tukey's multiple comparisons). ns not significant *p<0.05, **p<0.01, ***p<0.001 **(D)** Tumor growth inhibition percentage calculated across the five xenograft models at day 39. TGI <100% indicates that tumors do not regress after treatment whereas TGI>100% indicates tumor regression following drug treatment. Error bars show SEM. Statistical assessment was done using two-way ANOVA (Tukey's multiple comparisons). ns not significant *p<0.05, **p<0.01, ***p<0.001. Note

that tumor volume data was provided by Stephen Wong. Taken from (Ahmed, Ramani et al. 2019).

3.3 Induced replication stress - TOP2 inhibition

Apart from TOP1, we sought to inhibit topoisomerase 2 (TOP2), as it preferentially relaxes positive supercoils and resolves pre-catenanes formed ahead and behind of the replication fork, respectively (McClendon, Rodriguez et al. 2005). In particular, we utilized etoposide, a clinically highly relevant TOP2 poison that induces fork stalling (Allen, Ashley et al. 2011) due to the accumulation of toxic TOP2-DNA covalent complexes (TOP2cc). TOP2cc act as roadblocks to fork progression, leading to fork collapse and DSBs/apoptosis (Yan, Tammaro et al. 2016) (McClendon and Osheroff 2007) (Wilstermann and Osheroff 2003).

3.3.1 HMGA2 protects against DNA damage induced by the TOP2 poison etoposide

Analysis of DSBs by PFGE after 24 h exposure to etoposide revealed DNA fragmentation similar to that observed with DNA synthesis/TOP1 inhibitors. In the case of HT1080 C1 and C2 cells that allow downregulation of HMGA2 by doxycycline-inducible anti-HMGA2 shRNA (see **Fig. 22**), cells were exposed to doxycycline for four days with etoposide treatment starting on the fourth day.

HMGA2 expression, irrespective of its levels, reduced DNA fragmentation across the four different model systems, as we observed significant differences mainly in the 30-100 kb fragment fractions (**Fig. 40**).

A

B

C

D

E

Figure 40- HMGA2 expression safeguards against etoposide-induced DNA fragmentation. PFGE analysis of (A) parental H1299 and HMGA2 knockout cells, (B) HeLa cells (parental) and its three HMGA2 expressing clones (P2/P8/P19), (C) A549 (parental) cells and its three HMGA2 expressing clones (1.3/1.5/1.6), (D) HT1080 C1 cells (Dox-/Dox+) and (E) HT1080 C2 cells (Dox-/Dox+) after treatment with indicated doses of etoposide for 24 h (left panel). Notice the elevated levels of the 30-100 kb DNA fragment fraction observed in cells lacking HMGA2 across all the cancer cell models. DNA fragmentation (both for the >1 Mb as well as the 30-100 kb fractions) was quantified using ImageJ (right panel), from three independent experiments for each cancer cell model system. See methods for details on statistical assessment. Taken from (Ahmed and Droge 2019).

In addition, the induction of DSBs appears to be dose-dependent to etoposide in both H1299 and HeLa cell systems, with a clear increase observed in the 30-100 kb fragment fractions as shown in **Fig 41A-B**. Furthermore, we tested the HeLa model system as an example and found HMGA2 expression significantly reduced the occurrence of DSBs, in particular the 30-100 kb fragments, which start to appear as early as 6 h upon etoposide treatment (**Fig. 41C**). Interestingly, the occurrence of the 30-100 kb fragment fractions was not observed in human embryonic stem cells treated for 24 h across a concentration range of 0.6 μ M to 10 μ M etoposide (**Fig. 41D**). The extensive and extremely short DNA fragmentation (<40 kb) detected in these cells due to SN38 treatment (see **Fig. 37C**) was also not observed, indicating the possible existence of yet unknown resistance mechanisms to TOP2 poisons in hESCs which clearly deserve further investigation.

Figure 41- Dose-dependent induction of DNA fragmentation upon etoposide treatment (A) PFGE analysis of parental H1299 and H1299 HMGA2 knockout cells after 24 h exposure to etoposide across three different doses. Note that the highest fragmentation occurs at 30 μM etoposide dose in these cells (B) PFGE analysis of HeLa cells after 24 h exposure to etoposide across five different doses. Note that the highest fragmentation was observed at 10 μM etoposide dose. (C) PFGE analysis of HeLa cells (parental) and P2 (HMGA2 expressing clone) after treatment for 6 h with 10 μM etoposide. DNA fragmentation (both for the >1 Mb as well as the 30-100 kb fractions) was quantified using ImageJ (right panel) and normalized to total DNA loaded (n=3 independent experiments) (D) Etoposide dose titration for 24 h, followed

by PFGE analysis of human ES cells (n=2 independent experiments). (**A**, **B**) taken from (Ahmed and Droge 2019).

To directly confirm the fork protection against TOP2 poison is mediated by HMGA2, we transiently transfected HeLa cells (no detectable HMGA2 expression) with a HMGA2 expression vector and analyzed DSB induction after 24 h etoposide treatment. We observed a significant decrease in the 30-100 kb fragments in cells that were complemented with HMGA2. Furthermore, the protective effect of wild-type HMGA2 against etoposide-induced DSBs was abrogated when a mutant variant of HMGA2 (2,3M HMGA2) was used, indicating that the protective function involves DNA binding via the AT hooks (**Fig. 42**).

Figure 42- HMGA2 overexpression reduces etoposide-induced DNA damage in HeLa cells. Representative Western blot analysis (left panel) and PFGE analysis (centre panel) after complementing HeLa cells with mock (control), wild-type and 2,3M HMGA2 expression vectors. DNA fragmentation (both for the >1 Mb as well as the 30-100 kb fractions) was quantified using ImageJ (right panel), after normalization to total DNA from three independent experiments. Modified from (Ahmed and Droge 2019).

To confirm that the etoposide dose (10-30 μ M) employed in our cell-based assays is effective in trapping TOP2 in covalent DNA complexes (Deweese and Osheroff 2009), we used *in vitro* plasmid linearization assay (Nitiss, Soans et al. 2012). TOP2 alone (0 μ M) nearly completely relaxes supercoiled (sc) DNA, whereas a clear concentration-dependent increase in DNA cleavage (TOP2cc) was observed with etoposide, that is highlighted by the accumulation of increased linearized plasmid DNA and a concomitant reduction in covalently closed DNA topoisomers (**Fig. 43**), confirming the efficacy of the employed drug as TOP2 poison.

Figure 43- Etoposide effectively traps TOP2cc *in vitro*. Trapping of the topoisomerase 2-DNA complex (TOP2cc) by etoposide converts supercoiled DNA to linear DNA. Note the increase in the DNA cleavage mediated in the presence of TOP2A with increasing etoposide drug concentration, ranging from 12.5 μ M to 100 μ M as compared input (without TOP2). The position of open circular DNA and linear plasmid DNA is shown. $n=3$ independent experiments. Taken from (Ahmed and Droge 2019)

Taken together, we propose that the fork protection function of HMGA2 is closely related to its ability to attenuate the accumulation of double strand breaks at replication forks that are generated as a result of TOP2 poisoning and that this function is dependent on DNA binding via its three AT-hooks.

3.3.2 HMGA2 expression promotes cancer cell viability following etoposide treatment

In order to gain insights into the physiological consequences of the observed etoposide sensitivity, we evaluated the effect of HMGA2 expression on cell survival following etoposide treatment. Across the various cancer cell model systems, HMGA2-expressing cells were more resistant to etoposide-induced cell death (**Fig. 44A-E**), thus directly corroborating our PFGE analysis discussed in **section. 3.3.1** above.

Figure 44- HMGA2 expression promotes cell survival after etoposide treatment in all cancer models. CCK8 assay in (A) H1299 (parental) and HMGA2 knockout

cells, **(B)** HeLa (parental) and its three HMGA2-expressing clones (P2/P8/P19), **(C)** A549 (parental) and its three HMGA2-expressing clones (1.3/1.5/1.6), **(D)** HT1080 C1 (Dox-/Dox+) and **(E)** HT1080 C2 (Dox-/Dox+) after exposure to three different doses of etoposide for 24 h. Graphs plotted from two independent experiments. See methods for details on statistical assessment. Taken from (Ahmed and Droge 2019)

3.3.3 HMGA2 does not regulate TOP2A expression

Etoposide targets both topoisomerase 2 (TOP2) isoforms (TOP2A and TOP2B) found in eukaryotic cells (Willmore, Frank et al. 1998) (Canela, Maman et al. 2017).

Figure 45- Expression of etoposide targets TOP2A and TOP2B in the tested cancer cell models. Western blot evaluation of TOP2A/TOP2B in **(A)** H1299

(parental) and HMGA2 knockout cells, **(B)** HeLa (parental) and its three HMGA2 expressing clones (P2/P8/P19), **(C)** A549 (parental) and its three HMGA2 expressing clones (1.3/1.5/1.6) and **(D)** HT1080 C1 and C2 cells. **(E)** HMGA2 expression in H1299 (parental) cells after 30 μ M etoposide treatment for 24 h (n=3 independent experiments). β -actin was used as loading control. Taken from (Ahmed and Droge 2019)

Therefore, it is imperative to look at TOP2A and TOP2B expression levels across all tested cell lines, in order to rule out the possibility that the observed drug sensitivity differences arise as a result of HMGA2-altered topoisomerase levels. Western blot analysis revealed differential TOP2A expression in the tested cell lines with no differences observed for TOP2B (**Fig. 45A-D**). Interestingly, higher TOP2A levels were detected in cells expressing HMGA2, except for HT1080 C1 and C2 cells which exhibited an inverse correlation. Although higher topoisomerase levels have been previously reported to contribute to enhanced drug sensitivities (Burgess, Doles et al. 2008), our observations with etoposide show the opposite effect, suggesting that the sensitivity to TOP2 poisons might be independent of TOP2A/TOP2B expression. This is also in line with the fact that the sensitivity to TOP2 poisons does not always follow a linear correlation with TOP2 levels (Pommier 2013) (Fry, Chresta et al. 1991), pointing at the projected role of HMGA2 in protecting tumor cells against etoposide-induced replication stress. To rule out the possibility that changes in HMGA2 expression levels could lead to a protective effect after drug treatment, we treated H1299 cells with 30 μ M etoposide for 24 h and found no significant changes in HMGA2 expression, ruling out such a scenario (**Fig. 45E**).

We next asked if HMGA2 could upregulate TOP2A protein expression, as we had found TOP2A levels to not always correlate with HMGA2 expression, with reversed correlation in HT1080 C1 and C2 cells. Therefore, we performed complementation assays in HeLa cells that lack HMGA2, and using Western blot analysis found that the transient expression of HMGA2 did not alter TOP2A levels, as seen in **Fig. 46**.

Figure 46- HMGA2 does not regulate TOP2A expression in HeLa cells. Western blot of TOP2A (upper panel) after transfection with different amounts of wild-type and 2,3M HMGA2 expression vector in HeLa cells. Corresponding HMGA2 expression of both wild-type and 2,3-hook mutant is shown (lower panel), with β -actin used as a loading control. Taken from (Ahmed and Droge 2019).

In addition, we used the 2,3-hook mutant variant of HMGA2 (**Fig. 46**) (only one remaining fully functional AT-hook) and also found no changes in the expression levels of TOP2A, ruling out the possibility of TOP2A regulation by HMGA2 through its DNA binding (Reeves and Nissen 1990).

3.3.4 HMGA2 protects against catalytic TOP2 inactivation *ex vivo*

It is well documented that TOP2 poisons, such as etoposide, increase intracellular TOP2cc levels that are ultimately transformed to double strand breaks by replication run-off events (Deweese and Osheroff 2009) (Yan, Tammara et al. 2016) (Tammara, Barr et al. 2013). However, a diverse group of clinically increasingly relevant TOP2 catalytic inhibitors do not induce accumulation of cleavage complexes (TOP2cc), but rather directly inhibit the catalytic activity of TOP2 (Larsen, Escargueil et al. 2003) (Pommier, Leo et al. 2010). In particular, merbarone that inhibits the TOP2 catalytic activity (Fortune and Osheroff 1998) (Drake, Hofmann et al. 1989) was utilized in our study to explore the possibility of a more direct role for HMGA2 in regulating topological stress.

PFGE analysis following merbarone treatment revealed DNA fragmentation profiles similar to that observed with etoposide (see **Fig. 40A-E**), indicating that the loss of TOP2 supercoil relaxation function alone (i.e. without TOP2cc formation) can significantly contribute to fork stalling/collapse, most likely due to the build-up of (+)

supercoiling ahead of the fork (Wang, Roy et al. 2007) (Chen and Beck 1995). This is also supported by a recent study that found the mechanical properties of chromatin to drive positive supercoils to the front of the fork and that TOP2 preferentially acts on these single chromatin substrates with high affinity (Le, Gao et al. 2019).

Figure 47- HMGA2 protects against merbarone-induced DNA damage. PFGE analysis of (A) parental HeLa cells and one HMGA2 expressing clone (P2) (B) parental A549 and one HMGA2 expressing clone (1.3) and (C) parental H1299 and HMGA2 knockout cells, after treatment with indicated doses of merbarone for 48 h (left panel). Notice the rise in 30-100 kb DNA fragments with increasing drug concentrations in cells lacking HMGA2. DNA fragmentation (both for the >1 Mb as well as the 30-100

kb) was quantified using ImageJ (right panel), from three independent experiments for each cancer cell model system. See methods for details on statistical assessment. Taken from (Ahmed and Droge 2019).

Strikingly, expression of HMGA2 protected against merbarone-induced DNA damage with noticeably reduced genome fragmentation (30-100 kb) across the three cancer cell model systems tested (**Fig. 47A-C**).

3.3.5 HMGA2 expression promotes cell survival following TOP2 catalytic inhibition

In order to further verify if the observed differential sensitivity to merbarone (**Fig. 47A-C**) has physiological consequences, we performed cell survival assays and assessed the effect of HMGA2 expression on viability following merbarone treatment.

Figure 48- HMGA2 expression promotes cell survival after merbarone treatment in all cancer models. CCK8 assay in **(A)** parental HeLa and one HMGA2 expressing clone (P2), **(B)** parental A549 and one HMGA2 expressing clone (1.3), **(C)** parental H1299 and HMGA2 knockout cells, and **(D)** HT1080 C1 (HMGA2 positive Dox-/ HMGA2 negative Dox+) after exposure to two different doses of merbarone for 48 h. Graphs plotted from two independent experiments. See methods for details on statistical assessment. Taken from (Ahmed and Droge 2019).

We found HMGA2 expression to directly correlate with higher cell survival after merbarone treatment for 48 h across all four cancer cell models (**Fig. 48A-D**),

indicating HMGA2 can reduce induced topological stress and prevent fork collapse or DNA damage.

3.3.6 HMGA2 stimulates TOP2A catalytic activity *in vitro*

With the regulation of TOP2A expression by HMGA2 having been ruled out (see **Fig. 46A-B**), we asked if HMGA2 could function similarly to the chromatin binding proteins HMGB1 (Stros, Bacikova et al. 2007) and GapR (Guo, Haakonsen et al. 2018) that were found to stimulate TOP2 activity and thereby promote DNA replication. Our observations that HMGA2 associates with supercoiled DNA with high affinity *in vitro*, forming unique higher order complexes involving DNA supercoil scrunching (Zhao, Peter et al. 2017) (see **Fig. 34**), as well as its ability to manage excess topological stress induced by merbarone, prompted us to check if such a scenario exists. With limited *in vivo* experimental tools currently available to experimentally assess this hypothesis, we utilized canonical *in vitro* DNA relaxation assays with TOP2A, where partially relaxed supercoiled plasmid DNA was incubated with varying amounts of wild-type and 2,3M HMGA2 mutant proteins.

Figure 49- HMGA2 stimulates TOP2A catalytic activity to relax supercoiled DNA *in vitro*. Partially relaxed plasmid DNA (see no HMGA2 lane) was incubated with wild-type and 2,3-hook mutant HMGA2 at various HMGA2:DNA molar ratios as indicated, for 30 min at 37°C and then subjected to electrophoresis in 0.8% agarose gel. Input lane (control) is negatively supercoiled renilla plasmid alone. Different topological forms of DNA, corresponding to altered mobilities are indicated in the right. Representative image for HMGA2 mediated-DNA relaxation shown (left panel) with data from three independent experiments plotted as percentage of relaxed supercoiled DNA (right panel). Taken from (Ahmed and Droge 2019).

First, we set out to determine the concentration of human recombinant TOP2A enzyme that would only partially relax the substrate plasmid DNA (data not shown). The

partially relaxed DNA is now observed as topoisomers (differing in their linking number) compared to the input (compare lane “no HMGA2” vs “input”) (**Fig. 49**). Next, wild-type HMGA2 was included in the relaxation assays at increasing stoichiometric ratios (WT HMGA2:DNA), leading to DNA relaxation in a dose dependent manner (seen as complete disappearance of the highly supercoiled DNA substrate band and more relaxed topoisomers at the highest stoichiometric ratio of 125:1). This effect was markedly reduced with the 2,3-hook mutant HMGA2, indicating the importance of multiple AT-hooks/HMGA2 molecule in binding to supercoiled DNA and enhancing TOP2A enzymatic activity (**Fig. 49**).

3.3.7 Subtelomeric regions are protected against TOP2-induced DNA damage by HMGA2

As the fragile (sub)telomeric domains are particularly sensitive to replication stress induced by hydroxyurea, the TOP1 poison SN38 and the G-quadruplex stabilizer TMPyP4 that are known to pose formidable DNA topological barriers within the human genome (Vannier, Pavicic-Kaltenbrunner et al. 2012) (Zhang, Zhang et al. 2017) (see **section 3.2.6**), we decided to examine if TOP2 inhibition by either etoposide or merbarone would also induce DNA damage at these sites. By probing for severed telomeric DNA using Southern blot analysis, we identified similar 30-100 kb fragment fractions with both etoposide (**Fig. 50A**) and merbarone (**Fig. 50B**) hence also implicating HMGA2 expression to play a crucial role in regulating topological stress at these sites.

Figure 50- Subtelomeric regions are guarded against TOP2 induced DNA damage by HMGA2. (A) Southern blot analysis probing for telomeric DNA in H1299 (parental) and HMGA2 KO cells, treated with etoposide for 24 h, followed by PFGE separation. See **Fig. 40A** for the corresponding EtBr stained PFGE image (B)

Southern blot analysis probing for telomeric DNA in HeLa (parental) cells and its HMGA2 expressing clone P2, treated with three different doses of merbarone for 48 h, followed by PFGE separation. Taken from (Ahmed and Droge 2019).

Taken together, I have provided here mechanistic insights as to how the oncofetal chromatin structuring protein HMGA2 can act as a modulator of transient DNA supercoiling. Using a variety of cancer cell models that allow modulation of HMGA2 expression, we elucidated the cellular response to replication/topological stress and the role of HMGA2 in genome stability. In addition, the use of clinically relevant inhibitors for both TOP1 and TOP2 pinpointed human subtelomeric chromosome regions as novel therapeutic targets that are ultra-sensitive to DNA topological challenges.

4. Discussion

DNA synthesis requires efficient replisome translocation, which necessitates the coordinated action of a large number of proteins (Leman and Noguchi 2013). This integrated functional assembly is constantly challenged as replication with or without DNA topological stress can lead to replication fork stalling and collapse (Morafraila, Hanni et al. 2019). However, cells have evolved diverse protective mechanisms to rescue stalled forks, and thereby prevent DNA damage (Branzei and Foiani 2010). Interestingly, some of these mechanisms, that are yet elusive, could aid cancer cells in circumventing genotoxic insults, thereby promoting chemoresistance and tumor relapse.

The chromatin structuring HMGA2, an oncofetal protein, has been previously reported to chaperone stalled forks, reducing the frequencies of their collapse (Yu, Lim et al. 2014). Although, it was proposed that the protein forms a physical scaffold around branched DNA structures during replication fork stalling mediated by hydroxyurea, further studies were required to discover the precise mechanism behind this fork protection function. It was also revealed that heterologously expressed human HMGA2 prevents the topologically-driven phenomenon of fork regression in yeast cells lacking *mec1*, a crucial yeast protein required to maintain fork integrity (Yu, Lim et al. 2014).

Subsequent work from our laboratory using single molecule DNA analysis, along with AFM imaging, revealed strong binding affinities of HMGA2 to unconstrained negatively and positively supercoiled DNA (scDNA) (Zhao, Peter et al. 2017), thereby, scrunching one of the many transient DNA structures/domains to occur at stalled forks (Waga and Stillman 1998), hinting at a possible mechanism of HMGA2-mediated fork chaperoning. Uncontrolled levels of DNA supercoiling, that arise when topoisomerase action is compromised, can cause replication stress and is considered a major threat to fork stability (Wang 2009) (Ahmed, Ramani et al. 2019). Using a multidisciplinary approach, my study identified possible regulatory mechanisms of replication stress, mediated by HMGA2, mitigating the consequences of DNA topological changes.

I will now discuss the identified role of HMGA2 in modulating cellular responses against commonly used chemotherapeutic drugs that target topoisomerases (TOP1/2)

or DNA polymerase and highlight the immediate clinical significance that could potentially be used to overcome therapy-related challenges.

4.1 HMGA2 influences the efficacy of TOP1 poisons

Identifying the clinical determinants of response to TOP1 poisons has been a challenging process as a network of DNA repair or stress response pathways potentially hamper this pursuit, leading to futile treatment efficacies (Pommier 2013). Here I reported that the endogenous expression levels of HMGA2 are critical factors, as certain cancer cells are resistant to SN38 treatment whereas others are sensitized, despite having similar TOP1 levels.

In fact, based on *in vitro* DNA nicking experiments with purified proteins, HMGA2 was previously reported to increase the efficacy of irinotecan in trapping TOP1 in a cleaved complex with DNA, and that the trapping was dependent on the stoichiometry of HMGA2 bound to DNA with moderate amounts increasing the nicking efficiency whereas high amounts reduced nicking efficiency mediated by TOP1 (Peter, Yu et al. 2016). This suggested that the apparent chemosensitivity differences arise from different HMGA2 levels, with low/moderate HMGA2 expression sensitizing cancer cells to TOP1 poisons, whereas high HMGA2 levels protecting cells against SN38-induced genotoxicity. Such an observation was more directly confirmed by transiently expressing varying amounts of HMGA2 in HMGA2 knockout cells. Both DNA fragmentation analysis as well as cell survival assays pointed to the fact that HMGA2 levels are critical in determining the cellular response to TOP1 poison.

Furthermore, utilizing a highly specific antibody that provides an insight into the accumulation of trapped TOP1cc in cancer cells following drug exposure, revealed high HMGA2 expressing cells (H1299) to have lower TOP1-DNA trapped complexes compared to cells that lack HMGA2 (HMGA2 KO). On the contrary, moderate HMGA2 expressing cells (P2) had higher TOP1cc compared to cells that lack HMGA2 (HeLa), further strengthening our conclusions. The accumulation of these complexes on parental DNA can lead to replication run-off, which causes DNA damage as a result of clashes between protein-DNA adducts and replisomes. When such collisions occur at the topologically strained 20-200 kb sub-telomeric and less than 20 kb long telomeric regions, nucleolytic degradation occurs, that would truncate the

chromosomal DNA ends generating the 30-100 kb telomere rich fragments (**Fig. 51**) as observed in our DNA fragmentation and Southern blot analysis.

Figure 51- Graphical representation of replication stress at subtelomeric and/or telomeric domains. Replication or topological stress induced by TOP poisons generates DSBs either at telomeres (red) or subtelomeres (blue).

We identified subtelomeres to be extremely sensitive to topological stress that is induced by TOP1 poison. Telomeres containing the 'TTAGGG' conserved repeats protect chromosomal ends, as loss of these specialized structures can activate cell cycle arrest, DNA end degradation and apoptosis (de Lange 2002). Heterochromatic sub-telomeric regions that represent a mosaic of repetitive sequences are transitional regions located upstream that is, between specific chromosomal sequences and telomere repeats (Gilson and Geli 2007), and host unidirectional replisomes that replicate the chromosomal ends (Drosopoulos, Kosiyatrakul et al. 2012). Both the telomeres and sub-telomeres can contribute to chromosomal instability as these regions represent common fragile sites (CFSs) (Murnane 2010).

CFSs are specific loci that challenge replication and are prone to DNA damage as a consequence of stalled replication (Durkin and Glover 2007), particularly sub-telomeric regions as they are somewhat impaired in double strand break repair activity (Miller, Reynolds et al. 2011). The sub-telomeric and telomeric regions pose several barriers to replication forks hindering smooth fork progression leading to topological stress and chromosomal instability (Higa, Fujita et al. 2017) (Ivessa, Zhou et al. 2002). Repetitive sequences, shelterin complexes and formation of higher order secondary

structures such as G-quadruplexes (Rhodes and Lipps 2015), T-loops (Griffith, Comeau et al. 1999) and R-loops (Higa, Fujita et al. 2017) interfere with replication fork progression when unresolved. In addition, the association of telomeres with nuclear matrix (Burla, La Torre et al. 2016) and lack of origin activation at the ends of the chromosome that could potentially rescue a stalled fork, can also contribute to fork collapse and DSBs at these sites (Ozeri-Galai, Lebofsky et al. 2011) (Debatisse, Le Tallec et al. 2012). Here, I showed that HMGA2 levels influence the stability of specific sub-telomeric regions that are vulnerable to topological stress induced by TOP1 poisoning. Furthermore, *in vitro* as well as *ex vivo* data indicate that the observed differential sensitivity is owed mainly to the different HMGA2 expression levels and is most likely linked to the protein's ability to constrain sc DNA (**Fig. 52**).

Figure 52- Proposed model for the regulation of subtelomere stability by HMGA2 expression levels following TOP1 poisoning. (A) Low/moderate HMGA2 levels promote the accumulation of TOP1cc that can lead to increased replication run-off events, thereby, generating DNA breaks. **(B)** High HMGA2 levels prevent TOP1cc formation as a result of constraining of (+) sc DNA and preventing the binding of TOP1

to its DNA substrate, leading to reduced fork collapse at the subtelomeres. Taken from (Ahmed, Ramani et al. 2019)

At low/moderate levels, HMGA2 stabilizes the TOP1-DNA cleavage complexes most likely by increasing drug binding to TOP1 as evident from *in vitro* magnetic tweezer experiments, which found HMGA2 to severely impact DNA relaxation in the presence of TOP1 and SN38 (Ahmed, Ramani et al. 2019). Such an observation points to HMGA2 binding and constraining scDNA domains in a manner that impedes the rotation of the cleaved single strand, generated by TOP1, around the intact DNA strand. This would resemble a loaded spring which would unwind on its own over time (supercoiling energy) but any constraint (in this case mediated by HMGA2) would block rotational movements (**Fig.53A**). Furthermore, this impediment to strand rotation could lead to more frequent misalignments of the tyrosyl-DNA phosphodiester backbone and the freely-rotating cleaved DNA containing the 5'-hydroxyl DNA terminus, preventing religation and therefore stabilizing the intermediate TOP1-DNA cleavage complexes (**Fig. 53A**). These trapped complexes with single strand nicked DNA can then be converted into double strand breaks upon fork collisions leading to increased genome instability (**Fig. 52A**).

Figure 53- Proposed mechanism behind altered SN38 efficacy in the presence of low/moderate and high HMGA2 expression levels. Segment of scDNA depicting the mechanism of trapping TOP1 in cleavage complex in the presence of drug with (A) low or moderate HMGA2 levels and (B) high HMGA2 levels.

At very high levels of HMGA2, however, the protein binds sc DNA, perhaps in a co-operative manner, and forms protein bridges shielding the DNA supercoils (Zhao, Peter et al. 2017), which likely prevents TOP1 from binding to the supercoiled DNA substrate. This effect will indirectly interfere with drug action (**Fig. 52B and Fig. 53B**) as TOP1-induced DNA cleavage is reduced and, therefore, the trapping of TOP1 in covalent complexes by SN38 is attenuated, leading to reduced sensitivity to TOP1 poison (Burgess, Doles et al. 2008). However, in the absence of HMGA2, the accumulation of TOP1cc as well as excess unconstrained supercoils can lead to increased replication run-off events and fork regression respectively, ultimately leading to cell death.

Apart from replication induced DNA damage, the possibility of DNA damage occurring as a result of transcription machinery colliding with TOP1cc cannot be excluded, as topoisomerases also resolve (+) and (-) sc DNA ahead and behind the RNA polymerase complex (Wu, Shyy et al. 1988) (Sakasai and Iwabuchi 2016). However, additional experimental evaluation would be required to exactly pinpoint the role of HMGA proteins, if any, in the context of transcription-induced supercoiling and TOP1cc formation.

Furthermore, several other mechanisms could potentially serve to explain the observed differential chemosensitivity such as differential expression of multi-drug resistance genes (and associated proteins) (Ma, Maliepaard et al. 1998), mutations in TOP1 enzyme (Tsurutani, Nitta et al. 2002), increased TOP1 poison degradation (drug-metabolizing enzymes) (Khanna, Morton et al. 2000), drug inactivation (Cummings, Boyd et al. 2002), differential subcellular redistribution of the drug (Edwards, Saleem et al. 2000), increased or decreased drug interaction with TOP1 (Mao, Sun et al. 2000), increased/decreased expression of DNA repair proteins (Arnaudeau, Lundin et al. 2001) and mutations of cell-cycle control proteins including p16, Rb protein and ATR (Lai, Visser-Grieve et al. 2012) (Cliby, Lewis et al. 2002) (Rasheed and Rubin 2003). Additionally, the differences of protein expression (HMGA and TOP1) in tumor tissues could possibly explain these observations, although they seemed to be similar, ruling out such a possibility (Uhlen, Bjorling et al. 2005).

Figure 54- Observed tumor responses in PDX models. Streamlined model indicating the relationship of HMGA2 expression with tumor growth/response following irinotecan treatment across five different colorectal patient-derived xenograft models.

A potential clinical implication arose from our study, although only based on a relatively small set of human colorectal tumor samples. In a collaborative effort, we found that low or moderate HMGA2 expressing human colorectal tumors regressed with irinotecan treatment (**Fig. 54**), as compared to tumors that do not express HMGA2. Our model presented in **Figs. 52** and **53** now provide mechanistic explanation for such an observation, which implicates HMGA2 expression as a predictive molecular marker.

4.2 HMGA2 attenuates DNA damage induced by TOP2 poisons or inhibitors

Although both topoisomerase enzymes (TOP1/TOP2) alleviate torsional stress efficiently, albeit through different mechanisms, regulation of their enzymatic activity *in vivo* is not completely understood. It is not until recently that chromatin associated factors such as HMGB1 (Stros, Bacikova et al. 2007), HMGB2 (Stros, Polanska et al. 2009) and GapR (Guo, Haakonsen et al. 2018) have been shown to contribute to smooth replication progression by stimulating TOP function, pointing at the existence of additional factors also at play in maintaining chromosome stability. Furthermore, several pleiotropic factors such as p53 (Kwon, Shin et al. 2000), YB-1 (Wu, Wang et al. 2014) and ARF (Karayan, Riou et al. 2001) were also found to stimulate the activity of these ubiquitous enzymes by either increasing ATP hydrolysis or interacting directly with TOPs. In this regard, our study showed for the first time that the structuring protein HMGA2 has a similar stimulatory effect on TOP2, thus contributing to the rising interest in understanding how topoisomerase activity is regulated. Such findings could provide probable mechanistic explanations for observed chemotherapeutic failures in cancer

patients treated with TOP2 poisons (Bansal, Bajaj et al. 2017) (Ganapathi and Ganapathi 2013).

Changes in treatment efficacies brought about by diverse patient genetic profiles as well as cellular pharmacokinetics have increased the incidences of TOP2 targeted chemotherapeutic failures (Ganapathi and Ganapathi 2013) (N and Gabrielli 2017). Our work provides mechanistic insights into the regulation of TOP2-mediated DNA damage by HMGA2, which could be crucial to improve chemotherapeutic response. Indeed, an extensive study that includes samples from more than 350 human AML patients treated with TOP2 inhibitors alone or in combination with replicative DNA polymerase inhibitors implicated HMGA2 expression to poor clinical outcomes (Marquis, Beaubois et al. 2018). The independent study which was also validated in another AML patient cohort comprising of more than 200 patients, directly correlates with our findings that identified HMGA2 to confer a broad overall protection against fork collapse induced by TOP2 poison/inhibitor, across different cancer cell model systems, irrespective of its levels. We propose that the resistance to standard AML treatment that lead to more than half of the AML patients succumbing to the disease, is due in part to the ability of HMGA2 to stimulate TOP2 activity as well as protect forks that stall due to DNA synthesis inhibition. Such a protection against TOP2 targeting drugs could also guard transformed hematopoietic stem or progenitor cells (HSCs), that express high levels of HMGA2 (Copley, Babovic et al. 2013) (Kumar, Beck et al. 2019) and are key in the progression of AML (Corces, Chang et al. 2017) (Sanchez-Aguilera and Mendez-Ferrer 2017), to escape genotoxicity leading to relapse or recurrence. This was the case in more than 70% AML patients that initially showed signs of complete remission (Marquis, Beaubois et al. 2018).

The stimulation of TOP2 catalytic activity by HMGA2 can be attributed to the binding and constraining of sc DNA by the AT hooks, as evident from *in vitro* experiments that found the 2,3-hook mutant protein to have considerably reduced activation function. Our data excluded the possibility of TOP2A regulation by HMGA2 as transient transfection experiments with wild-type and mutant HMGA2 vectors did not show any significant changes in the TOP2A protein levels. We cannot, however, exclude the possibility that a direct HMGA2-TOP2A interaction could contribute to this catalytic activation function of HMGA2, as HMGA2-TOP2A interaction were identified in a recent interactome study in mouse cells (Singh, Ozturk et al. 2015). In addition, HMGA

proteins and TOP2 were found to colocalize at AT-rich chromatin domains (Saitoh and Laemmli 1994) (Martelli, Riccio et al. 1998) (Reeves 2010), thus strengthening the scenario in which HMGA2 can work together with TOP2A to bind and recognize specific domains within the entangled chromatin network. Taken together, we speculate that at a given time, the enhanced TOP2A-mediated relaxation in the presence of HMGA2, due to scDNA constraintment and/or direct TOP2A-HMGA2 protein-protein interactions, could affect drug interactions leading to reduced TOP2 trapping (Fig. 55). On the contrary, etoposide-TOP2A interactions would be higher in the absence of HMGA2, as TOP2 is not stimulated, with comparatively slower relaxation kinetics which would increase the likelihood of drug activity and higher trapping of TOP2 in cleavage complexes.

Figure 55- Proposed mechanism behind reduced efficacy of etoposide in the presence of HMGA2. A combination of events at the supercoiled DNA domains mediated by HMGA2 can lead to enhanced TOP2 mediated scDNA relaxation, thereby influencing cellular response to etoposide. Note that the AFM image (inset) is taken from (Zhao, Peter et al. 2017).

Using merbarone, a catalytic inhibitor of TOP2, that does not form stable cleaved DNA complexes (TOP2cc), we found the drug to generate DNA damage profiles similar to the TOP2 poison etoposide, validating that excess topological stress alone, due to the lack of TOP2 activity, can cause replication forks to stall and collapse, generating double strand breaks. Identified here for the first time, our study contests the broadly accepted notion that the formation of TOP2cc on the parental strands and subsequent

collision with replisomes is solely responsible for fork collapse (Larsen, Escargueil et al. 2003) (Nadas and Sun 2006).

We also identified subtelomeres to be particularly vulnerable to TOP2 poisons/inhibitors with HMGA2 influencing DNA damage at these sites as shown previously with TOP1 or DNA polymerase inhibitors. We postulate that the mechanism of supercoil constraintment and TOP2A stimulation by HMGA2 which is critical for fork stability, is similar to a recent report that showed GapR, a bacterial chromatin structuring protein that activates gyrase within the bacterial nucleoid, promoting replication progression (Guo, Haakonsen et al. 2018).

Figure 56 - Proposed model depicting the role of HMGA2 in protecting stalled forks at subtelomeres following TOP2 poisoning or inhibition. (A) In the absence of HMGA2, targeting TOP2 with etoposide generates high levels of the toxic TOP2cc that lead to replication run-off events generating DSBs. Targeting TOP2 activity by merbarone generate excess (+) sc DNA that eventually lead to fork stalling and

collapse (**B**) In the presence of HMGA2, fork collapses are reduced as the protein constrains (+) sc DNA and activates TOP2A within the transient ternary complexes to attenuate topological stress. Modified from (Ahmed and Droge 2019).

In the absence of HMGA2, etoposide will lead to accumulation of cellular TOP2cc, which enhance replication run-off events generating unscheduled chromosomal breakage especially at the subtelomeres (**Fig. 56A**). A similar consequence is observed with merbarone that induces fork stalling or collapse into DSBs as a result of generating excess (+) sc DNA ahead of the replication fork (**Fig. 56A**). Interestingly, our models were supported by independent studies that implicate TOP2A to play a crucial role at telomeres. TOP2 poisoning lead to the increased excision of telomeric DNA called t-circle tails from stalled forks as a result of trapped TOP2-DNA complex and fork collapse (Zhang, Zhang et al. 2017). Furthermore, catalytic inhibition of TOP2A lead to increased telomere fragility, possibly due to unresolved catenated sister chromatids (d'Alcontres, Palacios et al. 2014). In the presence of HMGA2, however, the protein attenuates the consequences of both TOP2 poison/inhibitors as it constrains sc DNA as well as catalytically activates TOP2A, to prevent fork collapse and promote subtelomere stability (**Fig. 56B and Fig. 55**).

4.3 HMGA2 attenuates DNA damage induced by DNA synthesis inhibitors

In the context of The DNA synthesis inhibitor hydroxyurea, we saw HMGA2 expression to have a broad overall protection, similar to the one observed against TOP2 poisons/inhibitors. The hydroxyurea-induced replication stress generated at topologically strained subtelomeres can lead to fork stalling or endonucleolytic cleavage as a result of regressed forks, leading to increased DNA breaks (**Fig. 57A**). However, it is plausible that the constraint of DNA supercoiling at least temporarily by HMGA2 could protect stalled forks from collapse (**Fig. 57B**).

Figure 57- Proposed Model for HMGA2 protecting stalled forks at subtelomeres after hydroxyurea induced replication stress. (A) In the absence of HMGA2, HU induces fork stalling and increased fork regression events that lead to fork collapse or DSBs. **(B)** In the presence of HMGA2, fork collapses are considerably reduced as the protein forms a protective scaffold that promotes genome stability. Modified from (Ahmed, Ramani et al. 2019).

Additionally, the protein could form a protective scaffold, that protects ssDNA from nucleolytic attack, generated when a fork stalls due to helicase uncoupling from the replisome (Droge, Sogo et al. 1985) (Byun, Pacek et al. 2005) (Toledo, Altmeyer et al. 2013). With HMGA2 also able to bind secondary DNA structures such as hairpins (Zhao, Peter et al. 2017), there is reason to suggest that the protein could aid single-stranded DNA binding proteins such as the prominent RPA proteins in stabilizing these structures, that could form within excess ssDNA.

These findings are in line with a recent study that identified poor overall survival in pancreatic cancer patients that express HMGA2, succumbing to the disease following treatment with DNA synthesis inhibitors (Strell, Norberg et al. 2017). Additionally, the use of DNA synthesis inhibitors in a combinatorial chemotherapeutic regimen is quite

common, with HMGA2 implicated in reduced patient survival as well as increased tumor recurrence (Marquis, Beaubois et al. 2018).

4.4 Limitations of the study

Due to the nature of research questions asked, potential limitations exist. First, DNA supercoils are highly transient, making them extremely difficult to study inside living cells. In addition, limited tools and complex nature of eukaryotic genomes prevent the precise determination of topological stress *in vivo*. Second, the double strand breaks that arise directly from exposure to genotoxic agents which in turn trigger programmed cell death, could also arise indirectly from apoptosis alone. We do not yet completely understand such an influence, thereby, warranting the need for further investigation. Finally, PDX models used in this study were limited to a small sample size of five colorectal cancer patients which either lacked HMGA2 or expressed HMGA2 at moderate levels. Therefore, a larger PDX sample size that potentially includes high HMGA2 expressing colorectal tumors needs to be tested to further strengthen our proposed model. In relation to this, we analysed (in collaboration with Charité–Universitätsmedizin Berlin) a recently developed large-scale colorectal cancer biobank (Schutte, Risch et al. 2017) that includes close to 1200 human colorectal PDX models tested against clinically relevant compounds and found only 90 of these colorectal cancer-derived models to be treated with irinotecan. We aimed at correlating the published RNAseq data (HMGA expression) with irinotecan-mediated treatment outcomes in these PDX tumor models (Schutte, Risch et al. 2017) (**Fig. 58**) and found a trend that was similar to our findings with irinotecan (SN38), indicating that our study has broader clinical implications.

Figure 58- Comparing available HMGA transcriptome profiles and irinotecan-mediated treatment outcomes in 21 PDX tumor models. Tumors expressing moderate HMGA levels respond better to irinotecan treatment (either complete response (CR) or partial response (PR) to treatment). Unpaired two tailed t-test was used to calculate statistical significance. * $P < 0.05$. Data analysed from (Schutte, Risch et al. 2017).

5. Conclusion

Although topoisomerase inhibitors are extensively incorporated in several chemotherapeutic anticancer regimens, current evidence suggest several challenges to their efficacy that include drug inactivation, resistance and toxicity (Pommier 2013). With integrated approaches and technical advances providing mechanistic insights into the observed chemotherapeutic failures, focusing on identifying new drug targets and developing personalized therapeutic interventions might be promising in the future (Ahmad, Ahmed et al. 2018). Implementation of these prevention strategies are already underway, especially for breast cancer patients carrying BRCA mutations, significantly reducing risk of cancer (Phillips, Milne et al. 2013) (Murthy and Muggia 2019). We identified HMGA to be a cancer cell-specific determinant of the efficacy of clinically important topoisomerase-targeting drugs both *ex vivo* or *in vivo* with HMGA2 expression (low/moderate and high) particularly influencing drug sensitivity to TOP1 poisons and as an independent prognostic marker for a wide array of tumors (Marquis, Beaubois et al. 2018) (Nie, Zhang et al. 2018) (Binabaj, Soleimani et al. 2019) (Zhang, Mo et al. 2019), thereby, necessitating HMGA expression profiling that grades cancer patients before finalizing clinical treatment regimes. In addition, identifying HMGA antagonists that could be used in a combinatorial setting with clinically approved TOP targeting drugs, could pave way for better treatment outcomes in the near future especially for HMGA2 positive cancer patients. In relation to this, our laboratory has developed a high throughput cell-based compound screening system for HMGA2 antagonists (Peter, Yu et al. 2016) and using two independent screens, has identified a handful of antagonists (data not shown). One of the identified small molecules, appears to bind directly to HMGA2, most likely to the C-terminus, and interferes with transcriptional activator function of HMGA2. Further studies are required before the

identified drug could be trialed in the clinic in a combinatorial setting, to specifically target and improve overall survival in HMGA2 positive cancer patients.

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7. Appendix

Author's publications related to this thesis during the PhD period Jan 2016- Jan 2020

Research Articles

1. **Ahmed, S. M.**, P. D. Ramani, S. Q. R. Wong, X. Zhao, R. Ivanyi-Nagy, T. C. Leong, C. Chua, Z. Li, H. Hentze, I. B. Tan, J. Yan, R. DasGupta and P. Droge (2019). "The chromatin structuring protein HMGA2 influences human subtelomere stability and cancer chemosensitivity." PLoS One 14(5): e0215696.
2. **Ahmed, S. M.** and P. Droge (2019). "Oncofetal HMGA2 attenuates genotoxic damage induced by topoisomerase II target compounds through the regulation of local DNA topology." Mol Oncol.

Scientific Blogs

1. **Ahmed, S. M.** 'A novel mechanism of combating inherent DNA replication problems?' July 2018 Kerafast, Inc. Boston, MA 02210
2. **Ahmed, S. M.** 'Natural molecule derivatives as Topoisomerase Poisons - Insights into chemotherapeutic failure' Dec 2018 International Natural Product Sciences Taskforce (INPST), Poland

Poster Presentation

1. **Mechanisms of Recombination** 'HMGA2 modulates topological stress at (sub)telomeres revealing therapeutic potential' May 20-22, 2018 The Francis Crick Institute, London.
2. **EMBO Workshop on Nuclear Mechanogenomics** April 17-20, 2018 National University of Singapore, Singapore.
3. **Frontiers in Cancer Science** 'HMGA2 modulates topological stress at (sub)telomeres revealing therapeutic potential' 6-8 Nov 2017 Biopolis, Singapore.

**Author's publications not related to this thesis during the PhD period Jan 2016-
Jan 2020**

Research Articles

1. R. Ivanyi-Nagy, **Ahmed, S.M.**, S. Peter, P. D. Ramani, P.F. Ong, O. Dreesen, P. Dröge. (2018) "The RNA interactome of human telomerase RNA reveals a coding-independent role for histone 1C mRNA in telomere homeostasis". [eLIFE](#).