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CMG Helicase Assembly and Activation: Regulation by c-Myc through Chromatin Decondensation and Novel Therapeutic Avenues for Cancer Treatment

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CMG Helicase Assembly and Activation:
Regulation by c-Myc through Chromatin Decondensation
and
Novel Therapeutic Avenues for Cancer Treatment

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
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DEDICATION

This dissertation is dedicated to my mother, Elaine Bryant, who sadly died too young from cancer. Although she never had the chance to see me enter graduate school, she is the reason I decided to do a cancer research Ph.D. She always encouraged me to excel in school and made sure I stayed at university even when I wanted to quit so I could become the first person in my family to get a degree. I hope my work here has contributed in some small way to the cancer research field and to improving cancer patient survival.

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ABSTRACT

The CMG (Cdc45, MCM, GINS) helicase is required for cellular proliferation and functions to unwind double-stranded DNA to allow the replication machinery to duplicate the genome. Cancer cells mismanage helicase activation through a variety of mechanisms, leading to the potential for the development of novel anti-cancer treatments. Mammalian cells load an excess of MCM complexes that act as reserves for new replication origins to be created when replication forks stall due to stress conditions, such as drug treatment. Targeting the helicase through inhibition of the MCM complex has sensitized cancer cells to drugs that inhibit DNA replication, such as aphidicolin and hydroxyurea. However, these drugs are not used in the clinical management of cancer. We hypothesized that the effectiveness of the clinically relevant drugs gemcitabine and 5-FU against pancreatic cancer cells, and oxaliplatin and etoposide against colorectal cells, could be increased through co-suppression of the MCM complex. The oncogene *c-Myc* also leads to the mismanagement of CMG helicases in part due to a non-transcriptional role in overactivating replication origins and causing DNA damage. We sought to elucidate the mechanism by which *Myc* causes overactivation of CMG helicases.

Herein we demonstrate that co-suppression of reserve MCM complexes in pancreatic or colorectal cancer cell lines treated with clinically applicable

chemotherapeutic compounds causes significant loss of proliferative capacity compared with cells containing the full complement of reserve MCMs. This is in part due to an inability to recover DNA replication following drug exposure, leading to an increase in apoptosis. Targeting of Myc to genomic sites induced large-scale decondensation of higher order chromatin that was required for CMG helicase assembly and activation at reserve MCM complexes. The physiological mediators of Myc, GCN5 and Tip60, are required for the chromatin unfolding and Cdc45 recruitment.

We conclude that depletion of the reserve MCM complexes causes chemosensitization of multiple human tumor cell types to several chemotherapeutic drugs used in the clinical management of human cancer. This argues for the development and use of anti-MCM drugs in combination with chemotherapeutic compounds, which has the potential to increase the therapeutic index of existing clinical compounds. We have also identified a previously unknown role for Myc in normal cell cycle progression whereby DNA replication initiation is regulated through the assembly and activation of CMG helicases on Myc-mediated open chromatin regions. Our results also provide new mechanistic insight into Myc oncogenic transformation in which overstimulation of DNA replication could result in genomic instability and provide an explanation for Myc driven oncogenic transformation.

CHAPTER ONE: INTRODUCTION

Cancer

Cancer is a group of over 100 distinct diseases characterized by uncontrolled growth and spread of abnormal body cells. Cancer is caused by both external factors, such as exposure to carcinogens, and internal factors such as inherited genetic mutations and immune conditions. Cancer is the second most common cause of death in the US, with a lifetime risk of 1 in 2 for men and 1 in 3 for women, and is strongly associated with aging, with 78% of all cancers occurring in people aged 55 years or older [American Cancer Society 2015]. Cancer prevention and early detection become keys to managing this disease and reducing cancer-related mortality. While the 5-year survival rate for all cancer has improved from 49% in 1975-1977 to 68% in 2004-2010, due to both earlier detection and improved treatments, 1 in 4 deaths in 2015 were attributable to cancer [American Cancer Society 2015], indicating the great need for more research into novel treatment strategies. The majority of cancer related deaths occur due to metastasis from the initial tumor site. The extra cellular burden of the tumor results in less nutrients and oxygen being available for normal tissue and organ function.

Cancer is a genetic disease resulting from the accumulation of mutations within the DNA of a cell that result in sustained cellular proliferation. For a disease to be classified as a cancer, it must share characteristics known as the “hallmarks of cancer” [1]. These hallmarks are acquired during the multistep generation of a tumor, and include sustained proliferative signaling, evasion of growth suppressive signals, resistance to cell death, induction of angiogenesis, enabling of replicative immortality, evasion of immune destruction, reprogramming of cellular metabolism, and activation of invasion and metastasis. Two other enabling characteristics that facilitate the acquisition of these hallmarks are genomic instability and tumor-promoting inflammation.

Loss of Proliferative Control

The most defining characteristic of cancer is sustained chronic cellular proliferation. Growth promoting signals released by normal tissues dictate entry and progression through the cell cycle and ensure that a balance is maintained between proliferation and quiescence [1]. Cancer cells lose control over this balance, and constantly emit growth-promoting signals to enable their own sustained proliferation, while simultaneously ignoring any growth suppressive signals released by normal cells. Somatic acquired mutations and gene amplifications in proto-oncogenes, which are genes involved in normal cell growth and division, lead to this sustained proliferative signaling. An example of a proto-oncogene is *MYC*, which can become upregulated through gene amplification, retroviral insertion or translocation to a constitutively active promoter. Up to 70% of all cancers have Myc overexpression, an oncoprotein involved in activating the transcription of genes that promote entry into the cell cycle [2]. But

other mechanisms of growth promotion by Myc are being discovered. One of the main focuses of this dissertation is a novel role Myc plays in promoting oncogenesis through manipulation of DNA replication, a necessary event for tumor cells to maintain their oncogenicity.

Genomic Instability in Cancer

The maintenance of genomic stability through detection and resolution of mutations and aberrant DNA structures is key for successful DNA replication and maintenance of a normal cellular phenotype. Errors in DNA replication or mutations caused by carcinogens occur in normal cells, and regulatory surveillance systems have evolved to detect these genetic alterations and halt the cell cycle while the defects are repaired. If they cannot be repaired, cell death is triggered. The acquisition of tumor enabling mutations in the lifespan of a cancer cell often leads to increased mutability, through breakdowns in the genetic surveillance systems or increased sensitivity to carcinogens [1]. A large number of defects have been identified in genes involved in genome maintenance and repair in tumors, leading to loss of control of genomic integrity that favors cancer progression [1]. Genomic instability in cancer cells is visible through large deletions, amplifications or rearrangements of chromosomal material.

Current Cancer Treatments

The most common current treatment strategies for cancer are surgical removal, chemotherapy and radiotherapy. Early detection and surgical removal of cancer often offers the greatest chance of a cure for cancer [American Cancer Society 2015]. Many

cancers are not eligible for surgical removal, for example blood cancers, or the cancers are suspected to have spread to distal body regions. Chemotherapy treatment involves giving medicines or drugs that target the highly proliferative neoplastic cells. However, it often affects normal highly proliferative body cells as well as cancer cells and gives rise to side effects such as nausea, hair loss and leukopenia. Radiation therapy uses high-energy particles to destroy cancer cells, and is often given in combination with surgery or chemotherapy. While radiation therapy is more targeted than chemotherapy, it can still damage normal surrounding cells and lead to secondary cancer development later on. In the past few decades, more targeted therapies have emerged due to a detailed understanding of the underlying biology surrounding each specific cancer disease. One of the aims of targeted therapies is to minimize the damage to normal cells, while maintaining maximum anti-neoplastic efficacy. In Chapter 2 we demonstrate the clinical need for a targeted anti-MCM complex drug, a protein complex necessary for DNA replication, to increase the efficacy of current chemotherapy treatments.

The Cell Cycle

For both normal cells and cancerous cells to duplicate and create the next generation, they must undergo a series of coordinated events known as the cell cycle [3]. The fundamental steps of the cell cycle are the duplication of the parental genome, and the division of the parental cell into two daughter cells through cytokinesis [4]. The majority of body cells are terminally differentiated in a quiescent or senescent state called G_0 , in which they are no longer actively cycling through the cell cycle. However,

actively cycling cells such as tumor cells and highly proliferative normal cells, or cells stimulated by growth factors to re-enter the cell cycle, traverse through a series of highly regulated cell cycle stages. The order of the stages of the mammalian cell cycle is Gap Phase 1 (G1), Synthesis-Phase (S), Gap Phase 2 (G2) and Mitosis (M), (Figure 1.1).

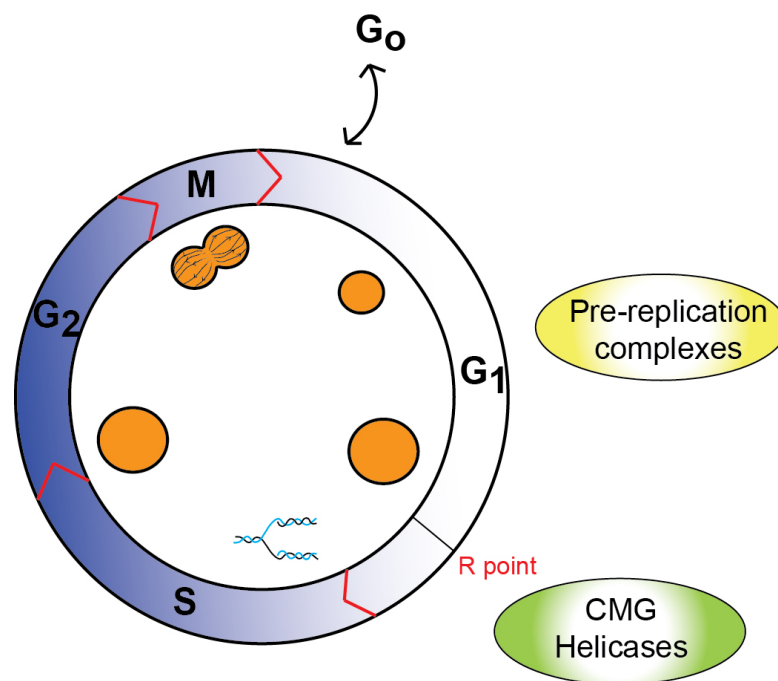


Figure 1.1: The Cell Cycle. The stages of the mammalian cell cycle. The key protein complexes involved in preparation for the initiation of DNA replication are the pre-Replication Complex and the CMG Helicase, which form during G₁-phase.

During G1 phase, cells increase protein supplies and grow, but importantly they also prepare for entry into S phase, where the DNA is replicated. In this project we will look into more detail about the events occurring in G1 phase that prepare a cell to replicate its 3 billion base pairs of DNA, by examining the formation of the pre-Replication Complex (pre-RC) and the replicative CMG Helicase. Once cells reach a point in late-G1 phase, called the Restriction Point (R-point), the cells no longer need to receive growth factors in order to complete the cell cycle and are committed to enter S phase, which marks a point of no return for the cell [5]. The G1-S transition is when the initiation of DNA replication begins, and is a tightly regulated event, deregulation of which promotes oncogenesis [6]. The genomic DNA is replicated in S phase, which results in a doubling of the DNA content of the cell. G2 phase allows continued cell growth and preparation for cellular division. Mitosis then ensues in which the DNA and cellular content are evenly divided into two daughter cells, thus creating the next generation.

Regulatory molecules called cyclins and cyclin-dependent kinases (CDKs) coordinate the cells progress through the cell cycle [7]. Cyclins are the regulatory molecules and CDKs are the catalytically active molecules, that when activated by a bound cyclin, perform phosphorylation reactions on other proteins to activate or repress them to allow the progression through the various stages of the cell cycle [7]. Levels of the various cyclins increase or decrease throughout the cell cycle, as different cyclin-CDK combinations are required during different stages. Cell cycle progression is also controlled through three main checkpoint controls, which are points at which the conditions of the cell are assessed, defects are detected, and the cell cycle delayed

until favorable conditions are met [8]. The R-Point is one such checkpoint, which operates 2-3 hours before the G1-S transition [5]. There is also a G2-M phase DNA damage checkpoint, which checks that the DNA has been replicated and the cell is prepared for entry into mitosis [9]. The final main checkpoint is the metaphase or spindle checkpoint point, which operates in mitosis and ensures that the chromosomes have aligned at the mitotic plate prior to anaphase [9]. Checkpoints ensure that any replication errors are detected and repaired, and prevent any flawed chromosomal segregations, with the ultimate goal of preserving genomic stability. Cancer cells differ from normal cells in that they have lost control over some of the regulatory systems that control cell cycle progression, and accumulate damage and chromosomal segregation errors that cause genomic instability and promote tumorigenesis [10].

DNA Replication

DNA replication is one of the most important events in the life of a proliferating cell, whether normal or cancerous. Complete and accurate DNA replication is crucial to maintain genomic stability in normal cells, and mistakes in this process can significantly contribute to carcinogenesis.

Replication Origins

Replication origins are sites of initiation of DNA replication. In prokaryotic bacteria, there is a single, well defined replication origin on a circular chromosome [11]. In contrast, due to the larger size of their genomes, eukaryotes have hundreds to

thousands of such replication origins on multiple chromosomes and are activated throughout S phase in a cell-type-specific manner [11]. The sequence specificity of these sites varies widely between different eukaryotic species. In the unicellular *Saccharomyces cerevisiae*, origin sequences are 10-15 base pair (bp) long, and 3-4 such sequences are spread over a 100-150 bp region. These sequences contain the highly conserved autonomously replicating sequence consensus sequence (ACS) [12]. In *Schizosaccharomyces pombe* yeast, replication origins contain 20-50 bp AT-rich sequences spread over 800-1000 bp [12]. Metazoan origins are less well defined, the replication origins present in the early embryonic stages of *Drosophila melanogaster* and *Xenopus laevis* require little to no sequence specificity [12]. It appears that in eukaryotic cells, while some sites have been demonstrated to consistently act as replication origins, the vast majority of replication origins display little to no sequence specificity and take the form of broad initiation zones, representing an array of potential replication initiation sites, in which in a typical S phase only one or two will be used [12-15]. DNA that is replicated from a single origin is known as a replicon [16]. Mammalian replicons vary in size from 50-500 kb, which contrasts with *Xenopus laevis* replicons that are spaced only 10-15 kb apart, and appear to replicate in staggered clusters [17]. A number of protein complexes are assembled at eukaryotic replication origins, which lead to the formation of a bi-directional replication fork.

The Pre-Replication Complex

The first such highly evolutionary conserved protein complex is the pre-Replication Complex, or pre-RC (Figure 1.2), which in cycling cells is assembled in late

mitosis and early G1 phase of the cell cycle [18, 19]. However, in this project we are using mammalian cells that are released from quiescence (therefore not cycling), and evidence shows that in these cells pre-RC assembly occurs in late-G1 phase [20, 21].

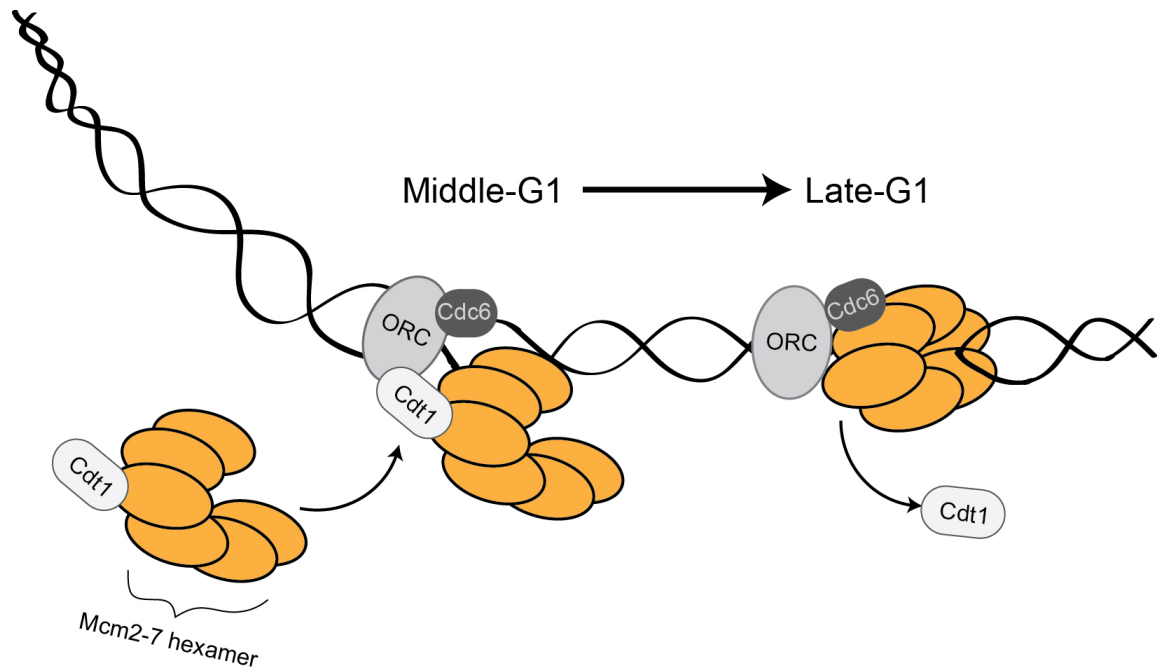


Figure 1.2: The Pre-Replication Complex. This multi-protein complex forms on chromatin in cells released from quiescence from middle to late G1-phase, and involves the recruitment and loading of the MCM2-7 complex in a process called licensing, limiting replication to one round per cell cycle.

First, the hetero-hexameric origin recognition complex (ORC) 1-6 binds to the future sites of replication origins. ORC acts as the initiator of origins by selecting the future replication sites [12]. ORC then recruits Cell division cycle 6 (Cdc6) and Cell

division cycle 10-dependent transcript 1 (Cdt1) proteins, which are required to recruit and load the hetero-hexameric Mini-Chromosome Maintenance (MCM) 2-7 complex, the final step in pre-RC assembly. The genes that encode the MCM2-7 subunits were originally found in genetic screens for proteins involved in plasmid maintenance, cell cycle progression and chromosomal mis-segregation [12]. The MCM complex is the catalytic core of the future CMG (Cdc45, MCM, and GINS) helicase, which is the replicative helicase that provides the mechanical action to unwind the DNA at the initiation of S phase [22, 23]. Each MCM subunit is an ATPase and member of the AAA+ family [24]. The MCM complexes are loaded as a double-hexamer onto DNA, providing a configuration capable of initiating a bi-directional replication fork [22]. MCM complexes remain stably tethered to DNA for many hours, and this is important as they remain dormant until selected to become functional CMG helicases at the G1-S transition [11]. To ensure that no replication origin initiates more than once per cell cycle, a mechanism has evolved called 'licensing', in which MCM complexes can only be loaded onto DNA in G1 phase. Licensing inhibits MCM loading outside of G1 phase through the three inhibitors CDKs, a Cdt1 inhibitor called geminin, and the Cul4-containing ubiquitin ligase [11]. By preventing MCM loading in S phase, this ensures that the newly synthesized DNA is not accidentally re-replicated and due to origin firing [11]. Once the MCM complexes have been loaded onto chromatin, certain MCM complexes are then chosen to become CMG helicases.

The CMG Helicase

Replicative helicases are motor proteins that use ATP hydrolysis to unwind double-stranded DNA into single-stranded DNA to provide access for the replication machinery to enter and begin DNA synthesis [25]. The MCM complex was implicated as the putative replicative helicase due to its isolation and demonstration of limited helicase activity *in vitro* [26, 27]. All Mcm proteins were determined equally important for entry into S phase and activation of late-firing origins after the activation of early-firing origins [28]. MCM complexes were required both for replication initiation and elongation of nascent DNA molecules [28]. Dormant MCM complexes are displaced from chromatin as the replication fork progresses, and are prevented from loading onto the newly synthesized DNA through licensing [28]. As mentioned earlier, each Mcm protein has ATPase activity, and the ATPase activity of the whole complex depends on the coordinated interactions between all six subunits [29]. The Mcm4/6/7 subcomplex comprises the catalytic core, whereas Mcm2/3/5 serve a regulatory function [29]. The eukaryotic helicase contains both subcomplexes, i.e. Mcm2-7, for full functionality, and a gate exists between Mcm2/5 for it to load onto chromatin [25]. The first indication that the MCM complex wasn't the complete replicative helicase came from a study analyzing the role of Cdc45 in replication initiation and elongation. Cdc45 was purified and found to be in a complex with the MCM2-7 hexamer and the GINS ('Go, Ichi, Nii and San'; 'five, one, two and three' in Japanese) tetramer [30]. Cdc45 and GINS were necessary for the G1-S transition and the whole complex had ATP-dependent helicase activity [30]. GINS is required for the progression of replication forks by maintaining the association between MCMs and Cdc45 within replisome progression complexes [31].

Thus the replicative helicase is now known as the CMG (Cdc45, MCM and GINS) helicase (Figure 1.3).

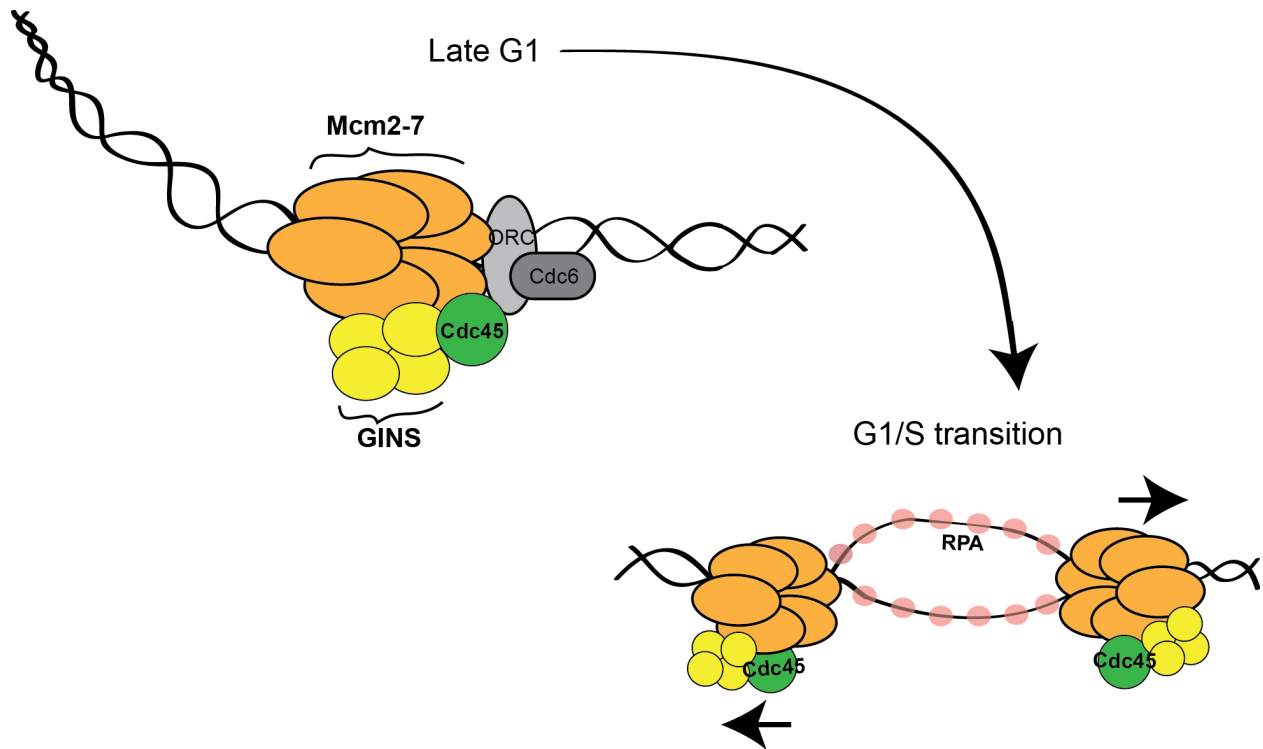


Figure 1.3: The CMG Helicase. Cdc45 and GINS join the pre-loaded MCM complexes to form the CMG Helicase, the multi-protein complex responsible for unwinding DNA at the G1-S transition. Once the helicase is activated, it creates a bi-directional replication fork, with RPA protein binding to the single-stranded DNA.

The CMG helicase components are essential for initiation of DNA replication, and for the elongation phase of replication [23, 31]. The MCM complex is activated upon addition of Cdc45 and GINS, and enhances the ATP-hydrolysis of the MCM hexamer

and enhances DNA substrate recognition [23]. At the G1-S transition, Cyclin A-Cdk2 and Dbf4-Cdc7 kinases activate the CMG helicase, which begin to unwind DNA and form a bidirectional replication fork [32]. DNA polymerase α /primase and ϵ along with Mcm10 are recruited and DNA is synthesized in a 5' to 3' direction. As the replicative helicase unwinds DNA, Replication Protein A (RPA) binds to single stranded DNA, preventing it from winding back onto itself or forming secondary structures and allowing DNA polymerase to replicate it [33].

The Pre-Replication Complex and CMG Helicase Stoichiometry

A typical replicon in *Xenopus laevis* embryonic extracts, which is 10-15 kb long, contains one ORC hexamer, two Cdc6 molecules, 1-2 Cdc45 molecules, and 20-50 MCM hexamers, which equates to one pre-RC to initiate DNA replication from [17]. The vast excess of MCM complexes likely leads to the efficiency of DNA replication in these extracts, and results in dormant MCM complexes that are utilized in replicative stress, as will be discussed later. The typical mammalian replicon is far larger than the *Xenopus laevis* embryonic extracts, ranging from 50-500 kb, with an average of 100 kb [17]. There are a predicted 10^4 to 10^6 replicons per mammalian cell [34]. The pre-RC and CMG helicase stoichiometry is also significantly different, with mammalian ORC and MCM complexes far less abundant, and Cdc45 extremely low and rate-limiting for DNA replication. For every 100 kb in the mammalian genome, there is one ORC hexamer, 2 Cdc6 molecules, 4-5 MCM hexamers, and only 0.35 Cdc45 molecules [17]. The chromatin bound complement of many of these proteins is even lower, with high-affinity (chromatin-bound) Cdc45 present as 2 molecules per 1400 kb [17]. Cdc45 has

been shown to be a replication-limiting factor in *S. pombe* yeast, *Xenopus* extracts, and in mammalian cells [17, 35, 36]. The Cdc45 protein in mammalian cells is very stable, the cell enters and exits S phase with the same number of Cdc45 molecules [17]. The staggering of replication firing in mammalian cells may be due to the availability of Cdc45 molecules in those regions, and the same Cdc45 molecules must be reutilized throughout S phase. Cdc45 levels cannot be manipulated in cells through over- or under-expression, as this renders cells unviable [17]. However, microinjections of purified Cdc45 protein into S phase nuclei causes a three-fold increase in replication foci, indicating the induction of dormant MCM complexes into active CMG helicases [17]. The limiting nature of the Cdc45 protein provides a molecular explanation for the larger size of the mammalian replicons compared to those of the *Xenopus*, as a lower density of MCM hexamers are competing for more limited Cdc45 proteins to become active origins. Mammalian cells take ~10-fold longer to replicate their DNA compared with *Xenopus* extracts, which is explained by the larger replicons and lack of Cdc45 protein [17]. Quantification of the GINS protein complex has yet to be determined, and so we cannot speculate on whether it too is rate-limiting like Cdc45, or whether there is an abundant like with the MCM complexes.

There has been some debate in the literature whether tumor cells up-regulate pre-RC proteins as a consequence of cellular transformation. In a study from our group, we compared on an equal cell-to-cell basis the pre-RC and Cdc45 protein levels in the normal highly proliferative Chinese Hamster Ovary (CHO) cell line and mouse 3T3 fibroblast cell line, to the tumor cell lines HeLa and Wilson [17]. We found no difference in protein levels between these specific cell types, and used multiple antibodies for each

protein, and compared the total protein levels in the cells to bacterially-purified human or CHO proteins. This extremely thorough study is not typical in the literature, and studies which have concluded there is a difference between normal and tumor cells use less strict testing criteria, such as loading equal protein levels between cell types, and using slow growing primary cells lines for comparison [37]. The overall up-regulation seen in studies like this could be due to the overall proliferative nature of the cancer cells and protein level increases due to this rapid cell growth and division. Cells need to be analyzed on a cell-to-cell basis, as we did, to determine if pre-RC protein levels are up-regulated in tumor cells, which we did not see. However, individual Mcm proteins have been shown to be up-regulated in human tumors. *MCM7* is a known oncogene and is overexpressed in several tumor types [38, 39]. It has been demonstrated that Mcm7 interacts with the tumor suppressor protein Retinoblastoma (Rb) [40-42], and overexpressed Mcm7 likely sequesters Rb away from binding with the CMG helicase and preventing its activation, thus driving cell replication and cellular proliferation.

MCM Complexes as Tumor Suppressors

Eukaryotic cells license more origins than are necessary for a normal S phase by loading extra MCM complexes [43]. Functional MCM complexes consist of two heterohexameric MCM2-7 protein complexes. With 4-5 MCM hexamers per average mammalian replicon size, this results in one complex needed for replication initiation in that region, and approximately 2 extra complexes with the potential to become functional (Figure 1.4) [17]. These dormant or back-up MCM complexes are suppressed by normal S phase checkpoint kinase activity, but become functional when replication

forks stall due to the action of replication inhibitor drugs such as hydroxyurea (HU), a ribonucleotide reductase inhibitor, or aphidicolin, a DNA polymerase inhibitor [15]. This activation of back-up origins during drug treatment allows maintenance of DNA replication rates, but can be diminished by targeting of MCM subunits with siRNA, which leads to a reduction in MCM2-7 levels on chromatin and a reduction in the ability to recover DNA replication during replicative stress [15]. MCM complexes mark DNA that has not yet been replicated, and due to replication licensing, MCM complexes can only be loaded onto DNA in G1 phase of the cell cycle and cannot load more MCM complexes on DNA in S phase if replicative stress causes forks to stall [15]. The need to be able to recover DNA replication during S phase stresses would explain why cells license more replication origins than are needed for a normal replication (Figure 1.4). A four-fold reduction in one MCM subunit reduces the ability of the whole complement of back-up MCM complexes to activate origins and recover DNA replication following fork stalling drug treatment, however, in the absence of replicative stress, short-term replication is unaffected [15, 44, 45]. Activation of back-up origins does not require checkpoint activation, they become active from the slowing down of replication forks leading to an increase in the time before the dormant origins are passively replicated, as seen by an increase in origin density [15]. While reduction of back-up MCM complexes allowed normal short-term replication rates, long-term viability was affected following transient HU or camptothecin treatment [15]. The replication stressing drugs used in this study are not clinically relevant for cancer treatment. We demonstrate in Chapter Two that reduction of back-up MCM complexes sensitizes tumor cells to chemotherapy drugs used in the clinical management of these tumors, through inability to recover DNA

replication and activation of apoptosis. We also show that tumor cells and normal cells display differential sensitivity to MCM loss in combination with chemotherapy treatment [44].

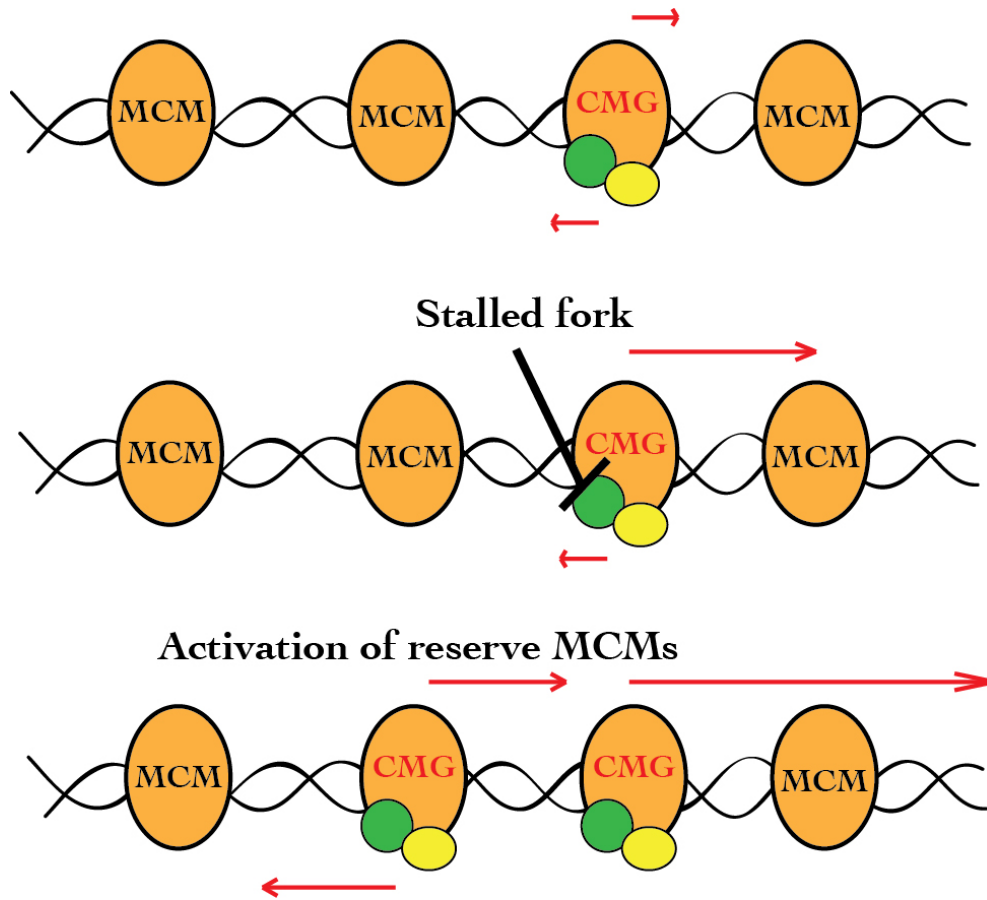


Figure 1.4: MCM Complexes act as Tumor Suppressors. Excess MCM complexes are loaded onto chromatin to be turned into new CMG helicases when replication forks stall in S phase due to replicative stresses such as DNA topology or drug treatment.

Shima et al. demonstrated that back-up MCM complexes/dormant origins were not just required to recover from the replicative stress of fork stalling drug treatment, but were also crucial to the cells ability to traverse a normal, unperturbed S phase. They used a *Mcm4*^{Chaos3} allele in homozygous mouse fibroblasts, which has a Phe345Ile amino acid change that compromises the stability of MCM complexes loaded onto chromatin and leads to a 60% reduction in the total number loaded (hence reducing the ~2/3rds back-up complement) [43]. This reduction in the back-up MCM complement lead to the accumulation of stalled replication forks in a normal, unperturbed S phase, despite normal helicase activity of the remaining MCM complexes, and resulted in incomplete DNA replication. Replication forks encounter stresses in S phase, such as the topology of DNA, or fluctuations in nucleotide levels, which stall replication forks and require activation of a dormant replication origin to rescue the replication of that region (Figure 1.4). Despite a normal ATR-CHK1 checkpoint pathway, the level of stalled replication forks observed was not enough to trigger a checkpoint response [43]. Multiple DNA repair pathways become activated in the *Mcm4*^{Chaos3} cells; however, these stalled forks persist into M phase and lead to replication intermediates that interfered with chromosomal segregation [43]. This lead to increased aneuploidy, chromosomal breaks, translocations and tetraploidy, all of which are characteristics of the genomic instability seen in cancer cells, and mice with this mutation were prone to develop a large variety of cancers [43]. This study indicates that back-up MCM complexes function as tumor suppressors in a normal, unperturbed S phase, and loss of these dormant origins may occur at an early stage of carcinogenesis and drive genomic instability [43]

Chromatin

Eukaryotic double-stranded DNA does not exist in a naked state in the cell nucleus. The DNA is tightly wrapped around proteins called histones, which then package the DNA into structural units called nucleosomes [46]. DNA wrapped around histones is referred to as chromatin, and this structural arrangement allows a genome that would normally stretch to 2 meters (m) long to be tightly packaged into the nucleus of a cell the size of 6 μ m. Each histone unit is a positively charged complex of eight histone core proteins, comprised of two histone H2A-H2B dimers and a histone H3-H4 tetramer [47]. Core histones interact as dimers in a head-to-tail fashion and they all possess the histone fold domain, which is three alpha helices linked by two loops [48]. The N-terminal histone tails provide sites for multiple types of post-translational modifications, such as methylation, acetylation, phosphorylation and ubiquitination [49]. The tails constitute 25-30% of the mass of the histone and provide a large exposed surface for interaction with other proteins, such as those containing bromodomains [49].

A bromodomain is a 110 amino acid protein domain that recognized acetylated lysine residues, such as those found on histone tails, and transduce this chromatin mark to elicit a biological effect [50]. Combinations of histone modifications are read by other proteins and constitute the 'histone code', a language read by these other proteins that leads to downstream functions [49]. In a single nucleosome, 146-bp of DNA is wrapped around a histone core, with the histone H1 linker binding the entry and exit sites to the histone core, securing the DNA in place [51]. Nucleosomes are arranged in different 'beads-on-a-string' higher-order conformations depending on the variable length of the

linker DNA in between each unit, the most common higher-order structures being the 30nm fiber and the 100nm fiber [52]. The tight packaging of DNA into chromatin structures presents physical barriers to accessing DNA for purposes of transcription, replication and repair [46]. Areas of the genome that are less tightly compacted are called euchromatin, and these regions are more transcriptionally active and replicate first in S phase [46]. Areas of the genome that are more condensed are referred to as heterochromatin, are less transcriptionally active and replicate later in S phase after the euchromatin [46].

The manipulation of chromatin structure represents an important regulatory event for transcription and DNA replication to dictate DNA accessibility. Chromatin remodeling and the role this plays in DNA replication control has been a very understudied topic, the attention has been focused on the role in transcriptional activation. Modifications that cause a chromatin decondensation event lead to increased access of the DNA for the transcriptional machinery, but it can easily be imagined that this accessibility will also be beneficial for the DNA replication machinery to begin the process of DNA synthesis. Two key classes of proteins that are studied in this project manipulate histone tails through the post-translational modification acetylation. Histone Acetyltransferases (HATs) add an acetyl group ($-COCH_3$) to histone tails. This results in partial neutralization of the positive histone charge and decreases the association of histones with the negatively charged DNA substrate [53]. Chromatin regions containing acetylated histones are generally associated with increased transcriptional activity. During DNA replication, histones are synthesized and assembled onto the newly replicated DNA in a pre-acetylated state, which becomes erased as the new chromatin

matures [49]. Proteins that remove the acetyl group from histone tails are called Histone Deacetylases (HDACs). These proteins oppose the reaction caused by HATs, and the interplay between HAT and HDAC activity plays a fundamental role in many biological processes that involve acetylation.

The Oncogene Myc

One of the first oncogenes discovered was *MYC*. In the 30 plus years since its discovery, over 30,000 articles have been published, and even to this day we are learning new functions this oncogene performs both in normal cells and on the road to carcinogenesis. Deregulation of Myc expression may be the driving force in up to 70% of human cancers [2]. The Myc oncoprotein plays critical roles in cellular proliferation, cell growth, apoptosis, differentiation, metabolism, and stem cell renewal [2]. Myc also plays key roles in chromatin structure and DNA replication, the main themes of this research project. We will explore the history, structure, and functions of this enigmatic protein, and show how our work contributes new knowledge that is highly relevant for further understanding the biological roles of Myc.

The Discovery of Myc

In 1911 Peyton Rous isolated a retrovirus that could cause cancerous sarcomas in chickens. This was the first oncogenic retrovirus discovered, and it was discovered that the gene within the virus responsible for oncogenesis was *SRC*, a tyrosine kinase involved in regulating cell growth and differentiation [54]. When non-infected normal

cells showed they had the same gene sequences as the viral transforming gene, this led to the notion that retroviruses could steal oncogenes from other species and use them to drive cellular growth, and thus viral proliferation. Using information learnt from the isolation of *SRC*, the search for other viruses that could cause cancer began, with the aim of identifying the human homologues of the transforming sequences within the virus. An avian acute leukemia virus (MC29) caused a spectrum of cancers, including sarcomas and carcinomas [54, 55]. The transforming sequence in MC29 was isolated in 1979 and called *v-MYC*, after the myelocytomatosis virus from which it was originally isolated [56]. The human homologue of *MYC* was discovered in 1982 [56].

The Myc Oncogene Family

MYC is a member of a family of oncogenes. The majority of studies have been conducted on the 454 amino acid long oncoprotein c-Myc (herein referred to as Myc), named after the cellular homologue of the *v-MYC* gene was found, and coded by the *MYC* gene found on human chromosome 8 [55]. The gene has a large noncoding first exon, and two additional coding exons [55]. Two major Myc isoforms are present in vertebrate species, Myc-1 and Myc-2 [57]. Myc-1 is translated from a CUG initiation codon at the end of exon 1, and gives rise to a 67-kDa protein [55]. Myc-2 is translated from an AUG codon in exon 2 and produces a 64-kDa protein [55]. The majority of growing cells express the Myc-2 protein [57], but during development of certain tissues types, or during oncogenesis of those tissues, other Myc family members are overexpressed. c-Myc is crucial for development and the c-Myc null phenotype is embryonic lethal [58].

The N-Myc oncoprotein is 464 amino acids long and is coded by the *MYCN* gene, found on human chromosome 2. It is normally only expressed during development [54]. It was originally discovered in a panel of neuroblastoma cell lines and tumor samples [54]. N-Myc overexpression is associated with aggressive disease and poor outcomes [55]. The gene *MYCN* can replace *c-MYC* during mouse development and allow survival into adulthood and reproduction [59]. Also, when placed under the *c-MYC* promoter, *MYCN* is similarly regulated and is functionally complementary to c-Myc for the biological processes of cellular growth and differentiation [59].

The L-Myc oncoprotein is 364 amino acids long and is encoded by the *MYCL1* gene on human chromosome 1, and is also only normally expressed in development, but is found amplified in certain small-cell lung cancers and ovarian carcinomas [55]. c-Myc, N-Myc and L-Myc interact with Max, a necessary interaction for transcriptional activation by the Myc proteins [60]. All three also cooperate with mutant the *H-RAS* gene to transform rat embryo fibroblasts and to generate cancers in transgenic mice [58].

Human, mouse and avian cells transiently express a Myc protein called c-Myc S, which results from translation from a downstream AUG initiation codon [57]. This protein has a truncated N-terminal transactivation domain lacking the first 100 amino acids, but an intact C-terminal DNA binding and protein dimerization domain [57]. This protein would be expected to be a dominant negative inhibitor of c-Myc proteins, through binding with Myc-binding partners but not eliciting transactivational events [57]. However, c-Myc S high levels in certain tumors and its transient synthesis during rapid

cell growth suggest that it does not inhibit the cellular proliferation functions of full-length c-Myc [57]. The function and regulatory role of this Myc protein is still largely unknown.

The Myc Oncoprotein

Serum stimulation of quiescent cells in culture lead to rapid upregulation of Myc mRNA, establishing Myc as an immediate early gene [55]. Myc mRNA and protein have an extremely short half-life (15-30 min), and both are expressed at constant levels in cycling cells [54, 61]. Myc expression is acutely responsive to extracellular signaling and is one of the most tightly regulated cellular genes [55]. Many of Myc's regulators are themselves oncogenes or tumor suppressor genes [55]. The main and most studied function of Myc is as a transcriptional activator and repressor. Myc is a nuclear protein that binds to double-stranded DNA through the basic region (BR) in the C-terminus [62]. Also in the C-terminus is the helix-loop-helix-leucine zipper (HLH-LZ) domain, which is the dimerization domain for Max-Myc interactions, crucial for the ability of Myc to act as a transcription factor [54]. There are several highly conserved Myc-box (MB) domains (Figure 1.5) between c-Myc, N-Myc and L-Myc.

The transactivation domain in the N-terminus allows activation of gene transcription when the C-terminus is bound to DNA, and it contains MBI and MBII [54]. The MBI domain (amino acids 44-63) contains the Ser62 and Thr58 post-translational modification sites, and is crucial for Myc and Ras cooperated oncogenic transformation of rat embryonic fibroblasts (REFs) [54]. Deletion of this domain however still allows transformation of Rat-1A cells [54]. The MBII domain (amino acids 128-143) is essential for transformation of both REFs and Rat-1A cells, is crucial for transcriptional activation

and repression [54], activation of DNA replication (discussed later) and is a domain necessary for interaction with chromatin modifiers (as discussed later).

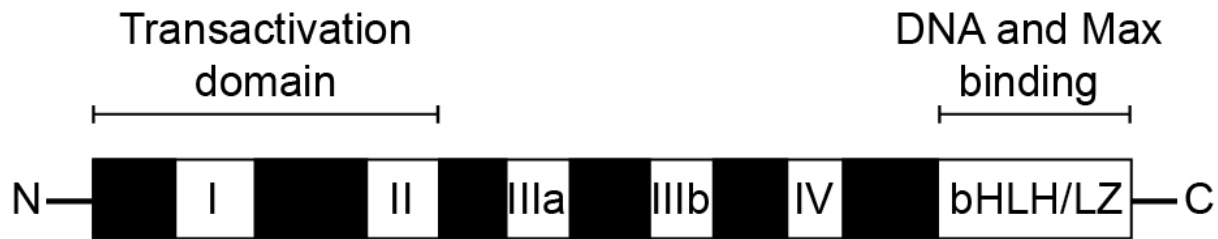


Figure 1.5: c-Myc Protein Domains. The c-Myc protein contains several conserved Myc-box (MB) domains, of which MBI and MBII are contained within the transactivation N-terminal domain, a basic region for binding to DNA, and a helix-loop-helix-leucine zipper domain for dimerization with Max in the C-terminus.

The MBIIIa domain (amino acids 188-199) is not conserved in L-Myc, and is essential for Rat-1A transformation but only has intermediate transforming ability compared with MBII deletion mutants [54]. The MBIIIb domain (amino acids 259-270) is conserved but has no function currently assigned to it [54]. The MBIV domain (amino acids 304-324) contains part of the nuclear localization signal (amino acids 320-328) and is required for Rat-1A and RK3E focus formation, but not REFs, and is dispensable for Rat-1A soft agar assays [54]. The BR domain (amino acids 355-369) is the site of Myc binding to E-box and non-E box sites present in DNA and is necessary for transformation of primary and immortal cells [54]. The HLH-LZ domain (amino acids

370-439) is the interaction domain for Max, and is essential for transformation of primary and immortal cells [54].

Specific serine and threonine residues are phosphorylated on the Myc protein to regulate its stability and activity, the most important being Thr58 and Ser62 [54]. Phosphorylation of Ser62 stabilizes and promotes Myc activation, but this post-translational modification then allows phosphorylation of Thr58, promoting the binding of the tumor suppressor FBW7 to recruit the SCF complex, leading to Myc ubiquitination and proteosomal degradation [55]. Point mutations at and around the Ser62 and Thr58 residues have been found in some Burkitt and AIDS-associated lymphomas, possibly driving Myc stability and subsequent oncogenesis [55].

Deregulation of Myc in Cancer

Deregulation of Myc transcription can occur by both direct and indirect mechanism, leading to cellular proliferation and oncogenic transformation [54]. Unlike some other common oncogenes, the mechanisms of *MYC* deregulation that promote oncogenesis are based on overexpression of the Myc protein product, rather than activating mutations in the *MYC* gene sequence [54]. The three mechanisms of Myc deregulation are insertional mutagenesis, chromosomal translocation, and gene amplification [55]. Retroviral transduction is the result of the *v-GAG-MYC* gene driving oncogenesis [54]. Insertional mutagenesis occurs when a retrovirus integrates into the genome in a promoter or enhancer region of an oncogene and causes the genes transcriptional activation. Myc was the first cellular oncogene demonstrated to be

activated by retroviral promoter insertion [54]. Myc deregulation can occur through activation of *MYC* by chromosomal translocation, a common event in hematopoietic malignancies. In Burkitt Lymphoma, which is driven by activated expression of Myc, one of the chromosomes containing the Ig heavy and light chains (14, 2 or 22), are translocated with chromosome 8, containing the *MYC* gene. Due to the genetic rearrangements of the immunoglobulin genes to provide an enormous variety of antigen recognition sites, these chromosomal regions are susceptible to translocations, and the juxtaposition of *MYC* to the Ig loci drives lymphomagenesis [54]. The first Myc transgenic mouse, E μ -Myc, was developed to model Burkitt's lymphoma [55]. Through studying homogeneously staining regions and double-minute chromosomes in cancer cell lines, it was discovered that these contained multiple copies of the *MYC* gene, and lead to the discovery of *MYCN* in neuroblastoma cell lines and tumors [54]. Gene amplification of *MYC* is commonly detected in solid tumors, unlike chromosomal *MYC* translocation which are found in hematopoietic cancers [54]. All three of these mechanisms of *MYC* deregulation lead to overexpression of the oncoprotein Myc, which drives cellular proliferation through increased expression of genes that drive cellular growth and proliferation, and decreased expression of genes involved in cell cycle arrest. Overactivation of upstream signaling pathways also leads to increased *MYC* mRNA production, creating abnormally high Myc protein levels. This could be due to activation of hormones or growth factors, the receptors they bind to, or activated secondary intracellular messengers such as Ras, Src, MEK or ERK, that all converge on and cause Myc expression [54].

Transcriptional Activation and Repression by Myc

Myc acts downstream of many cellular signaling pathways, interacts with dozens of cofactors, and controls the expression of hundreds of specific Myc target genes [2, 63, 64]. The cofactors recruited by Myc modify chromatin to allow access to the DNA, and engage the transcriptional machinery allowing gene expression [2]. Myc was found to bind to specific sites in the genome called canonical E-boxes (CACGTG) through interacting with Max. Max (Myc-associated factor X) is a bHLH-LZ protein that binds to the Myc bHLH-LZ domain to form heterodimers, and this interaction is critical for both transcriptional activation of target genes and cellular transformation by Myc (Figure 1.6) [2, 55, 65]. Max also forms heterodimers with the Mxd (formally Mad) family members, which compete with Myc binding to repress Myc-Max gene activation and mediate transcriptional silencing (Figure 1.6) [2]. Myc-Max heterodimers bind to DNA and activate transcription through the transactivation domain of Myc (amino acids 1-143), by associating with a variety of cofactors, such as TRRAP [2]. Up to 15% of all genes are predicted to be in the Myc target gene network [66]. Target genes transcriptionally activated by Myc are typically involved in cell growth, protein synthesis, ribosomal biogenesis, metabolism, glycolysis, mitochondrial function and cell cycle progression [67]. Targets genes that are transcriptionally repressed by Myc are involved in cell cycle arrest, communication between cells, cell adhesion or inhibitors of Myc-induced cellular transformation [67]. It became clear that Myc does not act as a classical transcription factor. The maximal gene induction is only two-fold, which is considerably lower than other classical transcription factors [66, 68, 69]. Myc has important biological

roles that extend beyond transactivation, including global influences on chromatin structure and non-transcriptional roles in DNA replication initiation.

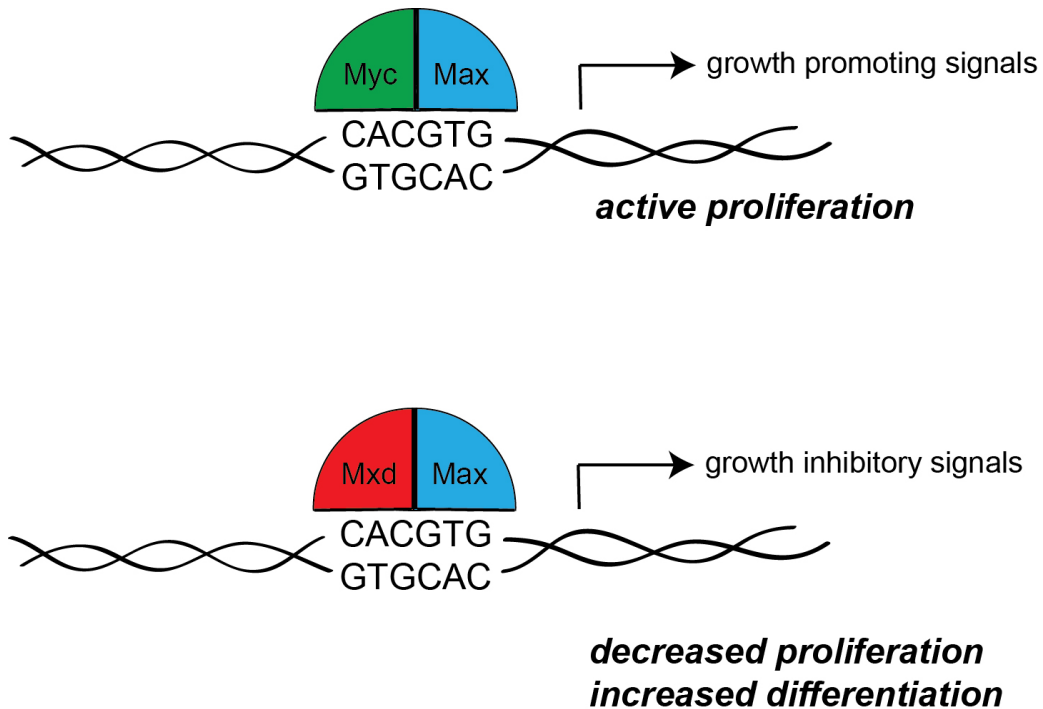


Figure 1.6: Myc, Max and Mxd Transcriptional Functions. Myc heterodimerizes with Max to promote proliferation, whereas Max can also heterodimerize with Mxd to inhibit growth.

The Role of Myc in DNA Replication

In the early days of Myc research, it was speculated that Myc was directly regulating DNA replication. Several studies in the late 1980's indicated that Myc had an important role in replication, however, the model of Myc being a regulator of gene

transcription yielded more consistent and comprehensive results and the majority of effort was placed in this field [54]. More recently, the interest of understanding the direct role of Myc in DNA replication has been reawakened, largely due to the efforts of the Gautier Laboratory who conduct many of their experiments in a transcriptionally inert system. In Chapter 3, we will demonstrate a novel mechanism for Myc-mediated control over DNA replication.

Two reports from Ariga and Ariga, published in 1987, were the first to suggest a direct role for Myc in DNA replication. They showed that a plasmid containing mouse liver DNA could replicate *in vivo* and *in vitro* only if c-Myc was present, and that by transfecting an anti-c-Myc antibody, this replication could be inhibited [70]. They also detected c-Myc binding activity at the replication initiation site in the plasmid, and suggested that c-Myc promotes DNA replication through binding to origin sequences [70]. In a separate study this group then looked at whether the DNA replication function of c-Myc was similar to that of SV40 T antigen, and showed that c-Myc could be substituted for the T antigen for SV40 DNA replication [71]. SV40 Large T antigen acts as a helicase to unwind DNA containing the SV40 replication origin [72]. This could suggest that c-Myc is present at replication origins due to it being in a helicase complex.

In 1989 there was a report of an intriguing correlative observation in early embryonic *Xenopus laevis* relating to c-Myc. Vast quantities of unusually stable maternal c-Myc protein were found in *Xenopus laevis* oocytes, and located in the cytoplasm, in contrast with somatic cells in which it is located in the nucleus [73]. Upon fertilization, the c-Myc protein is rapidly translocated to the nucleus as it prepares to initiate DNA replication, and the level on a per cell basis decreases exponentially as

rapid cell division occurs exponentially [73]. Cell cycle progression and DNA replication in the early *Xenopus* embryos do not rely upon transcription, indicating that Myc is providing an unknown non-transcriptional function during the replicative period. Once the maternal c-Myc supplies are depleted, the embryos are capable of transcription and translation of its own Myc mRNA.

To facilitate the study of the functions of the Myc oncoprotein, a hormone-activated Myc fusion protein was created by M. Eilers in 1989 [74]. The hormone binding domain of the estrogen receptor gene was fused to the 3' end of *MYC*, creating a *MYCER* gene that produced a 100kDa MycER protein. This protein was demonstrated to be activated by estrogen or hydroxytamoxifen (4-OHT) addition, to migrate to the nucleus, and caused soft agar colony growth, an indication of transformation [74]. The mutant Δ MycER protein missing MBII did not cause soft agar colony growth. Transformation by MycER was reversible through removal of hormone activation [74]. Cells infected with retroviruses containing MycER plasmids are grown in media lacking phenol red and containing charcoal-stripped serum, in an attempt to remove any estrogen hormone or hormone-like chemicals that could inadvertently activate the fusion protein during normal cell culture. The ER domain was shown to not contribute to transcriptional activation on its own, so this was not contributing to the observed transformation phenotype elicited solely by Myc [74]. In chapter 3 we utilize the MycER and Δ MycER system as described here. A report using the chimeric MycER and Δ MycER proteins in non-transformed, EGF-dependent, immortalized BALB mouse keratinocyte (MK) cells, demonstrated super-incorporation of radioactively labeled thymidine during S phase upon MycER induction in early G1 phase, but not upon

Δ MycER induction [75]. Expression of several Myc sensitive genes involved in cell cycle progression, cyclins A, E and D1, were unaffected by Myc induction [75]. Histone H1 kinase activity associated with an E2F-1 complex containing cyclin A and Cdk-2 is increased in late-G1 and early-S phase [75]. The MBII domain has now been shown to be crucial for transformation, transactivation and DNA replication initiation.

Gautier et al. published a Nature paper in 2007 conclusively demonstrating a non-transcriptional role for c-Myc in control of DNA replication [14]. Both c-Myc and N-Myc were shown to be in complexes with many pre-RC proteins, which were the MCM2-7, Orc2, Cdc6, Cdt1, and Myc's binding partner Max was also present in these complexes [14]. At the time of this study, the full complement of the replicative helicase was unknown, so Cdc45 and GINS were not examined. Proteins involved in DNA elongation, Mcm10, PCNA and RPA32, were absent from these complexes [14]. Myc was found co-localized with BrdU foci in early-S phase only, an indication that Myc is at sites of DNA synthesis. Myc was found at two replication origins, lamin B2 and c-Myc, for which it is known that Myc does not cause transcriptional activation of these genes [14]. It was known that Myc was necessary for the G1-S transition to occur, but whether this was because Myc was needed to transcriptionally induce S phase promoting factors or because Myc was needed to associate with the replication apparatus was not known [14]. By fusing G1 cells to S phase cells, which could provide the necessary cyclin-cdk complexes, and using cyclohexamide to inhibit further protein synthesis, it was shown that *c-MYC*^{-/-} cells could not progress into S phase, but *c-MYC*^{+/-} cells could [14]. This finding suggests Myc controls mammalian DNA replication initiation in part due to a non-transcriptional role. In *Xenopus* cell-free extracts, which are transcriptionally inert, full-

length Myc was also necessary for DNA replication to occur, and depletion of Myc caused a decrease in the overall number of active replication origins but not a decrease in the replication fork progression rate [14]. In both mammalian cells and *Xenopus* extracts, the pre-RC complex loaded onto chromatin, suggesting Myc is having an effect after pre-RC assembly but before helicase activation [14]. Expression of MycER caused an increase in the number of DNA replication foci in early-S phase, which was not affected by α -amanitin, a transcriptional inhibitor [14]. Myc overexpression in *Xenopus* extracts caused an increase in the amount of endogenous Cdc45 localized on chromatin, and in mammalian cells caused an increase in the amount of HA-tagged exogenous Cdc45 on chromatin, partially indicating there could be more CMG helicases assembled [14]. While this study clearly demonstrates overexpressed Myc causes an increase in DNA replication in S-phase, the mechanism behind how this occurs is not known.

This study also looked at the effect overexpressed Myc has on DNA damage. Increased origin firing is a source of replicative stress, and Gautier et al showed that exogenous Myc caused an increase in histone H2A.X Ser 139 phosphorylation, an indicator of DNA damage in mammalian cells and *Xenopus* extracts [14]. This effect was inhibited by Geminin, which blocks MCM loading of the pre-RC. Overexpressed Myc activated the ATM/ATR-Chk1-dependent checkpoint in *Xenopus* extracts as DNA damage appeared [14]. Myc induced DNA damage could be a contributing factor to the genomic instability of Myc amplified tumors, another study showed that in as few as 2 cell cycles, Myc can induce genomic instability [76]. Myc overexpression may be an

early step in oncogenesis that allows further mutations to accumulate through the induction of genomic instability [77].

Gautier's group published another report in 2013 in which they used DNA strand stretching to visualize replication initiation sites and the effect Myc overexpression had on them. Myc overexpression caused earlier firing of origins, a reduction in the interorigin distance and an increase in the number of origins firing, indicating over-usage of origins (presumably by using the back-up MCM complexes discussed earlier) [78]. This result matches the previous results in the literature showing that overexpressed Myc caused an increase in the number of BrdU foci and increased thymidine incorporation [14, 75]. Replication elongation following origin firing is normally symmetrical, however, with Myc overexpression, the number of asymmetrical origins increased, suggesting Myc is triggering replication fork stalling events [78]. Based on their previous finding that Myc overexpression caused an increase in chromatin-bound Cdc45, they overexpressed Cdc45 and saw that it recapitulated the Myc phenotype of increased origin usage and asymmetrical forks [78]. Co-overexpression of both Myc and Cdc45 did not have an additive effect, suggesting they function on the same pathway. Similar effects were seen with GINS overexpression. Myc induced replicative stress and DNA damage was dependent on Cdc45 presence, suggesting Myc is acting upstream of CMG helicase functionality [78]. Origin activation requires the activity of cyclin E/Cdk2 complexes, and Myc is known to antagonize p27, an inhibitor of cyclin E/Cdk2 complexes [78]. They found that Myc lowers the threshold of CDK activity required for Cdc45 recruitment and origin activation [78]. Whether this is the sole mechanism for how Myc promotes CMG helicase assembly or activation was not known at this point,

and in Chapter 3 we act a new mechanism that could also be involved in origin activation.

Myc has been shown to directly interact with Mcm7, presumably through the C-terminus since N-terminal deletion did not affect the interaction, which required the presence of the MBII domain [79]. Myc may be directly interacting with the pre-RC and CMG helicase through Mcm7 binding, but the biological effect this interaction would have is unknown. The tumor suppressor protein Retinoblastoma (Rb) is known to interact with and inhibit the CMG helicase through binding to the C-terminal of Mcm7 [42]. It could be possible that Myc and Rb are competing with each other for the same binding location on the CMG helicase, and whereas Rb binds to inhibit its function, Myc could be binding to promote helicase activity and thus DNA replication. The *Mcm7* gene is also a transcriptional target of N-Myc, and neuroblastoma tumors with N-Myc amplification have a 10-fold higher Mcm7 protein level [80]. Overexpression of Mcm7 may sequester Rb and prevent its inhibitory binding to the CMG helicase, while concurrent N-Myc amplification allows Myc to bind to and activate the helicase instead.

The Role of Myc in Chromatin Modifications

For transcription factors to facilitate the binding of RNA polymerase to transcription initiation sites, the condensed chromatin must be remodeled via ATP-dependent restructuring of nucleosomes to provide access to the DNA, and histone modifications for other proteins to read to recruit transcriptional activators or repressors [2]. Myc had long been thought to act solely as a classic transcription factor, regulating a small set of target genes through discrete chromatin modifications at their promoters

[81]. A large number of genomic studies have now demonstrated that Myc binds to tens of thousand of sites throughout the genome, as many as half these sites are significant distances from promoters, and Myc regulates global chromatin modifications, far more than a classic transcription factor [81]. More than a thousand putative target genes have been identified for Myc, and Myc is thought to regulate the gene expression of up to 15% of all genes [81].

Myc binds to genomic sites that already contain certain histone acetylation and methylation marks, namely, H3K4me2, H3K4me3, H3K79me2, H3K9ac and H3K18ac [82]. Once bound to chromatin, through interactions with chromatin modifiers Myc induces further acetylation of histones H3 and H4, specifically H3K9, H3K14, H3K18, H4K5, H4K12, H4K8, H4K91 and H2AK5 [82]. Most Myc targets display multiple histone marks. Ablation of N-Myc in neuronal progenitor cells results in histone hypoacetylation of H3 and H4 and loss of methylation marks such as H3-triMeK4, a sign of chromatin inactivation, and condensed nuclei [83]. This was rescued by expression of exogenous N-Myc, but depended on the presence of the MBII domain [83]. Loss of Myc also negatively affects DNA accessibility, as measured by MNase accessibility assays, leading to loss of euchromatin [83]. Myc was found present at the lamin B2 replication origin in early G1-phase and this was associated with nucleosome remodeling, indicating that Myc affects chromatin to allow greater accessibility to cellular machinery, potentially involved in processes such as DNA replication [84]

Myc Associated Histone Acetyltransferases

Histone acetylation is catalyzed by a variety of histone acetyltransferases (HATs). HATs enzymatically transfer an acetyl group from acetyl-coenzyme A to specific lysine residues found on histones [82]. Myc interacts with a number of different chromatin modifying enzymes, but the majority of studies have focused on the interaction with the HATs GCN5 and Tip60 [2]. Myc interacts with a nuclear cofactor called TRRAP (transformation-transactivation domain associated protein) through the MBII domain, and TRRAP is essential for oncogenic transformation by Myc [85, 86]. TRRAP is a large protein that lacks a kinase domain, but serves as a scaffold for assembling multiprotein complexes including HATs [2]. *GCN5* is a direct Myc target gene, and also recruited to promoter regions by Myc [2, 83]. GCN5 preferentially acetylates H3 (K9/14/18) [2]. Myc recruits both TRRAP and GCN5 through direct physical interaction of the MBII domain with the human STAGA (SPT3-TAF-GCN5) coactivator complex [87]. GCN5 protein expression is required for Myc to acetylate histones, suggesting one of the main ways Myc modifies chromatin is through recruitment of GCN5 to acetylate histone tails [83]. TRRAP can also contain the HAT Tip60 instead of GCN5, and Tip60 is recruited to chromatin by Myc [2, 88]. Tip60 preferentially acetylates H4 (K5/8/12) and H2AK5 [2]. Overexpression of an enzymatically mutant Tip60 delays Myc induced acetylation of histone H4 and reduces the amount of Myc binding to chromatin [88]. Both GCN5 and Tip60 are Myc cofactors that are recruited to chromatin by Myc, and cause acetylation of histones that are associated with open, accessible chromatin, which would benefit both transcriptional activation, and potentially DNA replication initiation, as discussed in Chapter 3.

Myc Associated Histone Deacetylases

Histone deacetylases (HDACs) remove acetyl groups from histones. Myc-Max function is antagonized by another transcription factor complex containing Mxd-Max, which block Myc-Max function by recruitment of either mSin3A or mSin3B, which in turn recruit either HDAC1 or 2 [86]. Overexpression of Mxd results in a reduction in global acetylated H3 and H4, which depends on the presence of the mSin3-HDAC binding domain [83]. The global deacetylation of nucleosomal histones results in a closed, transcriptionally inert chromatin state. Overexpression of Max does not affect global chromatin structure, as expected since Max is required for the opposing functions of both Myc and Mxd [83]. By using a HDAC inhibitor, the histone hypoacetylation in N-Myc null cells was reversed; suggesting the role of Myc in chromatin modifications is to shift the balance towards HATs and away from HDACs [83].

Targeting Myc for Cancer Therapeutics

With up to 70% of cancers displaying deregulation of Myc expression, strategies are being explored to target Myc therapeutically. Oncogene addiction is the phenomenon displayed by some cancers that depend on a single oncogene for sustained proliferative growth. Experimental models of cancers driven by Myc have suggested that some tumors are addicted to Myc, as activation of Myc elicits genomic instability or tumor formation, but subsequent inactivation of Myc causes regression of established tumors and a loss of the transformative phenotype [74, 76, 89]. Targeting Myc therapeutically has been challenging due to its necessary role in normal cells, the

diverse mechanisms driving its overexpression, and the challenge of disrupting protein-DNA interactions [90]. Pharmaceutical strategies so far have attempted to inhibit Myc expression, interrupt Myc-Max binding to each other and to DNA, and interfere with Myc target genes [91].

BET (bromodomain and extra-terminal) bromodomain family members, such as BRD2, BRD3 and BRD4, recognize and associate with acetylated chromatin and modulate transcriptional activation through recruitment of transcriptional regulators [90, 92]. Myc recognizes pre-acetylated histones and binds to such regions and increases the histone lysine acetylation. A selective small molecule inhibitor of BET bromodomains, JQ1, displaces BET bromodomains from chromatin by competitively binding to the acetyl-lysine recognition pocket [92]. JQ1 was used in multiple myeloma, leukemia and lymphoma cell lines, which often has a translocation of Myc, and it inhibited Myc transcription and Myc protein levels and lead to downregulation of the Myc transcriptional program, causing cell cycle arrest and senescence [90, 92]. These studies demonstrate that inhibition of Myc through targeting BET bromodomains might be of therapeutic value for the treatment of cancer patients. A small molecule inhibitor of Myc-Max dimerization called Mycro3 was found shrink pancreatic tumors in mice with KRAS* mutations dependent on Myc activity, and also saw increases in apoptosis and a reduction in proliferation [93]. Other therapeutic strategies have focused on targeting Myc target genes, such as viral expression of a Myc-repressed microRNA called miR-26a which yielded remarkable responses in a liver cancer model [91]. Sumoylation of Myc is essential for tumor growth, so perhaps this can be exploited therapeutically [91]. Myc induces replicative stress, which can make cells more sensitive to a variety of

drugs such as chemotherapeutic agents, replication inhibitors and checkpoint inhibitors, as discussed in Chapter 3. There is a drug in phase 1 of clinical trials in patients with solid tumors called DCR-MYC is a synthetic double-stranded RNA that targets Myc, but whether this proves to be beneficial to patients is currently unknown ([Clinicaltrials.gov](https://clinicaltrials.gov)).

CHAPTER TWO:
SUPPRESSION OF RESERVE MCM COMPLEXES CHEMOSENSITIZES TO
GEMCITABINE AND 5-FLUOROURACIL

Note to Reader

Portions of this chapter have been previously published in *Molecular Cancer Research*, 2015, Sep;13(9):1296-305, and have been reproduced with permission from AACR publishing (see Appendix).

Abstract

Pancreatic ductal adenocarcinoma (PDAC) has one of the poorest cancer survival rates due to its late diagnosis and lack of effective treatment regimens. Chemotherapeutic drugs such as gemcitabine and 5-fluorouracil (5-FU) are used to treat PDAC patients. These drugs act as indirect blockers of DNA replication through causing the stalling of replication forks and thus inability to successfully complete S phase. However, these drugs display only modest efficacy and do not stop the progression of this deadly disease. There is a strong need to develop novel and innovative therapeutic approaches to improve PDAC patient survival. Recent studies

have indicated that suppression of the MCM helicase may provide a novel means to sensitize cancer cells to chemotherapeutic drugs that inhibit replicative fork progression. Mammalian cells assemble an excess of MCM complexes onto DNA, greater than the amount needed to initiate and complete S phase. The reserve MCM complexes are utilized in conditions of replicative stress, in which they are converted into active replication origins to recover from stalled replication forks. Herein we provide definitive evidence that co-suppression of the reserve MCM complexes sensitizes PDAC tumor cell lines to both gemcitabine and 5-FU, leading to increased loss of proliferative capacity compared to drugs alone. This is due to an inability to recover replication following chemotherapy drug exposure due to reduced levels of reserve MCM complexes, leading to an increase in apoptosis. PDAC tumor cells are more sensitive to MCM loss in the presence of gemcitabine than are non-tumor, immortalized epithelial cells. Our results apply to other cancer types, as colon tumor cells experienced loss of proliferative capacity when exposed to the crosslinking agent oxaliplatin or topoisomerase inhibitor etoposide when the reserve MCM complexes were depleted. Neither of these chemotherapy drugs alone is effective at treating colorectal cancer, but our strategy has increased their efficacy. These studies demonstrate that suppressing the reserve complement of MCM complexes provides an effective sensitizing approach with the potential to increase the therapeutic index of drugs used in the clinical management of PDAC and other cancers.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the deadliest cancers. The 5-year survival rate is only 7% due to its symptomless early stages, and thus late diagnosis. In the US in 2015 an estimated 48,960 new cases were diagnosed, and 40,560 deaths were expected [American Cancer Society 2015]. It affects men and women equally, is the eighth most common cancer but the fourth most common cause of cancer-related death [American Cancer Society 2015]. There are no reliable early detection screening programs, and less than 20% of patients at diagnosis are surgical candidates due to the fact that it has already spread [American Cancer Society 2015]. Such patients are usually treated with chemotherapeutic agents to extend their lives. Chemotherapeutic interventions for advanced pancreatic cancer often include gemcitabine, or a combination chemotherapy regimen including 5-fluorouracil (5-FU) [94, 95]. The mechanism of action of many currently used chemotherapeutic agents targets DNA replication, leading to cellular death. 5-Fluorouracil is an inhibitor of nucleotide synthesis. The nucleoside analog Gemcitabine indirectly inhibits DNA polymerase through incorporation into replicating DNA, resulting in partial chain termination and stalling of replication forks [96]. These drugs cause replication forks to stall and thus indirectly suppress DNA replication and S phase. However, these drugs only demonstrate modest efficacy against PDAC disease progression and are associated with toxic side effects, which make them unsuitable for certain patients [95].

Colorectal cancer is the third most common cancer in men and women, accounting for 8% of the total new cancer cases in the US in 2015 [American Cancer

Society 2015]. It is also the third most common cause of cancer related death, due to slow uptake on the colorectal screening programs and relatively symptomless early stages of the disease [American Cancer Society 2015]. Etoposide is an anthracycline that intercalates into the DNA and inhibits topoisomerase II-DNA complexes, causing double-stranded DNA breaks [96]. Thus the development of novel innovative strategies to increase the efficacy of the existing chemotherapeutic compounds used to treat PDAC and colorectal cancer, and at lowered doses to reduce their toxicity, would be beneficial to cancer patients due to increased therapeutic index of current drugs in clinical practice.

Suppression of the MCM complex has been demonstrated to increase the efficacy of drugs that slow or inhibit DNA replication through stalling of replication forks [15, 45, 97]. The MCM complex is a heterohexameric ATPase complex comprised of Mcm2-7 subunits. The MCM complex forms the core of the replicative helicase and it is loaded onto DNA in excess during G1 phase of the cell cycle in a process called licensing, which limits DNA replication to a single round per cell cycle [98-100]. In late-G1 phase, certain MCM complexes are chosen to become active replicative helicases when Cdc45 and the GINS protein complex are recruited to the core MCM complexes, forming the CMG helicase (Cdc45, MCMs, GINS) [23, 101, 102]. The active CMG helicases represent the functional origins of DNA replication and proceed with the replication forks during elongation [23, 102-104]. In mammalian cells an excess of MCM complexes is loaded onto DNA during G1 phase than are necessary to initiate and complete S phase. This is because MCM complexes can only load in G1 phase and cannot be loaded in S phase if replicative stresses occur [15, 45, 97-99, 105, 106].

These are called dormant or reserve MCM complexes, and several studies have estimated that this excess is 3-10 times the number needed for a successful replication [15, 17, 45]. The excess of MCM complexes serves as back-ups for cells when they are subjected to replication stresses during S phase, such as topological issues, nucleotide fluctuations, or after exposure to replication-fork slowing drugs including chemotherapeutic agents [15, 45].

Several studies have shown that loss of reserve MCM complexes causes tumor cells to become chemosensitized to replication fork stalling drugs. The MCM complex works as a hexamer, reduction of one subunit reduces the ability of the whole complex to load and function on chromatin [43]. The depletion of Mcm5 in U2OS osteosarcoma tumor cells by two-thirds of its normal level reduces the reserve complexes of normal levels, and while this does not effect proliferative capacity alone, it causes sensitivity to aphidicolin (a DNA polymerase inhibitor) and hydroxyurea (HU; a ribonucleotide reductase inhibitor) [15]. However neither of these drugs is used clinically in the clinical management of cancer. A similar anti-proliferative sensitizing effect was observed when Mcm5 suppression occurred in combination with exposure to the chemotherapy drug camptothecin (a topoisomerase inhibitor) [15]. Hypersensitivity to Aphidicolin and HU is observed in HeLa cells with severely reduced (5-10% of normal levels) Mcm2 or Mcm3 to ~5-10% of normal levels [45]. Drug sensitization in U2OS, HeLa, and MDA-MB-231 tumor cells to HU and hydrogen peroxide is also seen by reducing proteins involved in the assembly and loading of the MCM complex, ORC (origin recognition complex) or Cdc6 proteins, via loss of viability and proliferative capacity [107].

The studies described above indicate that a full complement of MCM complexes are required for cells to remain viable after exposure to certain drugs that slow or block replication fork progression and thereby cause S phase stress. Reduction of the reserve MCM levels produces hypersensitivity to replication inhibiting drugs through a decrease in the proliferative capacity of the cellular population. These findings could have applications to the study of anti-tumorigenic therapies. As a hypothesis, co-suppression of the MCM complex offers the opportunity to augment the efficacy and anti-tumor activity of existing chemotherapeutic agents that negatively affect replication fork activity. However, with the exception of camptothecin, the drugs used so far to investigate this chemosensitization concept are aphidicolin and HU, which are not suitable for use in the clinical management of human cancer [15, 45]. The chemotherapeutic agents gemcitabine and 5-FU are used in clinical management of PDAC, oxaliplatin and etoposide are used to treat colorectal cancer, and these drugs function by indirectly blocking replication fork activity [95]. From the previously mentioned studies, it can be inferred that the efficacy of gemcitabine and 5-FU against PDAC cells, and oxaliplatin and etoposide against colorectal cells, could be increased through co-suppression of the MCM complex. However, this concept has yet to be conclusively demonstrated.

In this study we utilized PDAC and colorectal tumor lines as a model system to explore a method for increasing the therapeutic index of clinical drugs. Herein we present definitive evidence that co-suppression of the reserve MCM complexes chemosensitizes PDAC cell lines to gemcitabine and 5-FU, and colorectal cells to oxaliplatin and etoposide, all of which are indirect inhibitors of DNA replication. Partial

loss of MCMs through siRNA treatment reduces the reserve complement of MCMs significantly reduces the proliferative capacity of PDAC cells beyond that achieved through the drug treatment of gemcitabine or 5-FU alone. The mechanism behind this chemosensitivity is the inability of the tumor cells with reduced MCM levels to recover DNA replication following exposures to gemcitabine. We compared the chemosensitivity to gemcitabine of non-transformed epithelial cell line to PDAC cells in the context of depleted reserve MCM complexes and found that the normal cells were not sensitized to the same drug dose as the tumor cells were. We also show that MCM co-suppression causes an increase in the apoptotic index of colorectal cancer cells exposed to oxaliplatin or etoposide, two chemotherapeutic agents that act indirectly on DNA replication and are used in the clinical management of colorectal cancer and other tumor types, but with toxic side effects [108]. The chemotherapeutic compounds we tested only have modest anti-tumor activity and can cause toxic side effects that limit patient tolerance [94, 95, 108-111]. This study provides a proof-of-principle that the development of anti-MCM drugs in the future has the potential to increase the therapeutic index of existing anti-neoplastic drugs and may apply to many tumor types other than those tested.

Materials and Methods

Cell Culture, Transfections and Drugs

Panc1, Colo357, and HaCaT [112] cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (Hyclone), and SW480 cells were cultured in RPMI medium containing 10% fetal bovine serum. Transfections utilized Dharmafect reagent according to manufacturer methods (Thermo Scientific). For clonogenic and apoptosis assays, cells were seeded at 50% density and transfected the next day (time 0-hr) with 1-3nM of siRNA targeting HsMcm7 or HsMcm4 (siGenome Smartpools, Thermo Scientific), or with non-specific control siRNA (siGenome control, Thermo Scientific). Exposure to siRNA pools occurred for 120 hrs (for clonogenic assays), or for 72 hrs (for SW480 apoptosis assays and Panc1 DNA replication recovery assays). Exposure to drugs occurred concurrent with siRNA treatment from 72-120 hrs (for clonogenic assays), 24-72 hrs (for SW480 apoptosis assays), or 72-78 hrs (for DNA replication recovery assays). Aphidicolin, gemcitabine, 5-fluorouracil, oxaliplatin, and etoposide were purchased from Sigma-Aldrich and used at concentrations indicated.

Immunoblotting and Antibodies

For verifying successful siRNA knockdowns of MCM proteins by immunoblotting, equal numbers of cells were scraped off plates into cold PBS, lysed and boiled in 1X Laemmli loading dye (for total lysates) and analyzed by standard immunoblotting techniques [17]. An aliquot of scraped cells were removed and resuspended in a

HEPES-buffered pH 7.5 solution containing 10mM EDTA to perform cell counts on a hemocytometer without using trypsin. Chromatin enrichment of samples was conducted as follows. Cells were resuspended in buffer A (10mM HEPES pH 7.9, 10mM KCl, 1.5mM MgCl₂, 0.34 M sucrose, 10% glycerol, 1 mM DTT, 5 µg of aprotinin per ml, 5 µg of leupeptin per ml, 0.5 µg of pepstatin A per ml, 0.1 mM phenylmethylsulfonyl fluoride) containing 0.1% Triton X-100 for 5 mins on ice. Nuclei were isolated by low-speed centrifugation (5 min, 1,300 x g 4°C), washed in buffer A without TX-100, and then lysed in buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, and previously described protease inhibitors) for 30 mins on ice. Insoluble chromatin was collected by low-speed centrifugation (5 min, 1,700 x g 4°C) and resuspended in an equal volume of buffer B and 2X Laemmli loading dye.

To determine the degree of partiality of siRNA knockdown for Mcm7 and Mcm4, samples from 72 hrs of knockdown were compared to serially titrated samples from non-transfected control cells. Percentages of the control lysates loaded are indicated in the figures, with 100% control lysate representing an equal amount of cell-number lysate relative to the si-Mcm7 or si-Mcm4 knockdown samples. Antibodies and concentrations used for immunoblots were mouse monoclonal anti-Actin (1:10,000; Sigma), mouse monoclonal anti-Mcm7 (1:1,000; Santa Cruz), and chicken polyclonal anti-Mcm4 (1:1,000) generated by Aves Labs [17]. Mouse monoclonal anti-Orc4 (1:500) was from BD Transduction Laboratories.

Cell Proliferation and Apoptosis Assays

For clonogenic assays, cells were seeded at 50% density and transfected the next day (time 0-hr) with 1-3nM of siRNA targeting HsMcm7 or HsMcm4 (siGenome Smartpools, Thermo Scientific), or with non-specific control siRNA (siGenome control, Thermo Scientific). Prior to drug exposures, cells were reduced in density 48 hrs after siRNA transfections by splitting 1:1000 into new duplicate 35mm plates (to allow better colony visualization). Transfected and non-transfected cells were exposed to drugs concurrently with siRNA treatment from 72-120 hrs, and allowed to culture for the remainder of two weeks. Cells were then fixed and stained with Giemsa to quantify surviving colony numbers.

For apoptosis assays, SW480 cells were not split after siRNA transfections. Transfected and non-transfected cells were exposed to drugs concurrently with siRNA treatment from 24-72 hrs. Apoptosis assays were then conducted using an Annexin-V staining kit (BD Biosciences) and flow cytometric analyses according to manufacturer methods.

DNA Replication Recovery Assays

Equal cell numbers of asynchronous Panc1 cells plated in 24-well dishes were transfected with 100nM siRNA against Mcm7, Mcm4, or si-control for 72 hrs. This achieved an ~ 90-95% partial reduction of Mcm7 protein for acute testing of DNA replication recovery. Parallel plates were not transfected as comparisons. Cells were exposed to 100nM gemcitabine for 6 hrs, and medium was changed (at time 0) to remove drug and siRNAs. Cultures were pulsed for 30 minutes in duplicate or triplicate

with 3 μ Ci/mL tritiated-thymidine at the times indicated, and subsequently fixed with 1 M ascorbic acid. Trichloroacetic acid (TCA) precipitable material was processed for scintillation counting as described [113]. The average counts per minute (cpm) were determined from one-third of each sample, and plotted to measure DNA replication rates for each condition and time after drug removal.

Results

Depletion of Reserve MCM Complexes Sensitizes Panc1 PDAC Cells to Gemcitabine and 5-Fluorouracil

Suppression of the reserve MCM complexes in osteosarcoma and HeLa tumor cell lines causes chemosensitization to the anti-proliferative effects of aphidicolin and hydroxyurea [15, 45]. These drugs act by directly or indirectly blocking DNA replication through inhibiting the progress of the replicative forks during S phase. A hypothesis from these studies is that chemotherapeutic drugs that also indirectly inhibit replication forks and are used in the clinical management of cancer could be made more effective in their anti-proliferative effects when used in combination with a reduction of reserve MCM complexes. To test this idea we used pancreatic ductal adenocarcinoma (PDAC) tumor cell lines as a model system, due to the need for novel experimental strategies to treat this deadly disease. The chemotherapeutic drugs gemcitabine and 5-fluorouracil (5-FU) are often used to treat PDAC patients, although they only display limited anti-proliferative efficacy for this disease [95]. We determined if reduction of the reserve MCM complexes could hypersensitize the PDAC tumor lines Panc1 and Colo-357 to the

anti-proliferative effects of gemcitabine and/or 5-FU, used clinically to manage these cancers.

Several studies have indicated that there is a 3-10X excess of MCM complexes loaded onto chromatin than are needed for a normal progress in S phase, which indicates that 70-90% of the total MCMs act as back-ups in the form of dormant origins [15, 17, 45]. Reducing the level of a single MCM subunit beyond the reserve level causes a severe reduction in the ability of mammalian cells to proliferate [45], consistent with the fact that all six MCM genes are essential for yeast viability [114-116]. In this study we wanted to cause a partial knockdown of the reserve MCM complexes, while leaving enough MCM complexes present for a normal S phase to occur and leave cell proliferation unaffected. We tried using the manufacturers recommended 200nM concentration of siRNA against Mcm7, but this caused a severe loss of Mcm7 protein beyond the reserve levels and lead to suppression of cell growth (data not shown). We titrated down the level of siRNA and found that concentrations between 1-100nM produced partial reductions in Mcm7 or Mcm4 ranging from 70-95% reductions, which did not affect cell growth on its own and was consistent with the loss of only the reserve complement of MCMs. Figure 2.1A shows that siRNA treatment of 3nM against Mcm7 reduces the total Mcm7 protein level to ~30% of the normal level in Panc1 cells. To establish the level of knockdown we used immunoblotting to compare the Mcm7 protein level following 72 hrs of siRNA treatment to serially titrated dilutions of untreated Panc1 whole cell lysates. The reduction of 70% of Mcm7 in Panc1 cells indicates that we have significantly suppressed the reserve MCM complement.

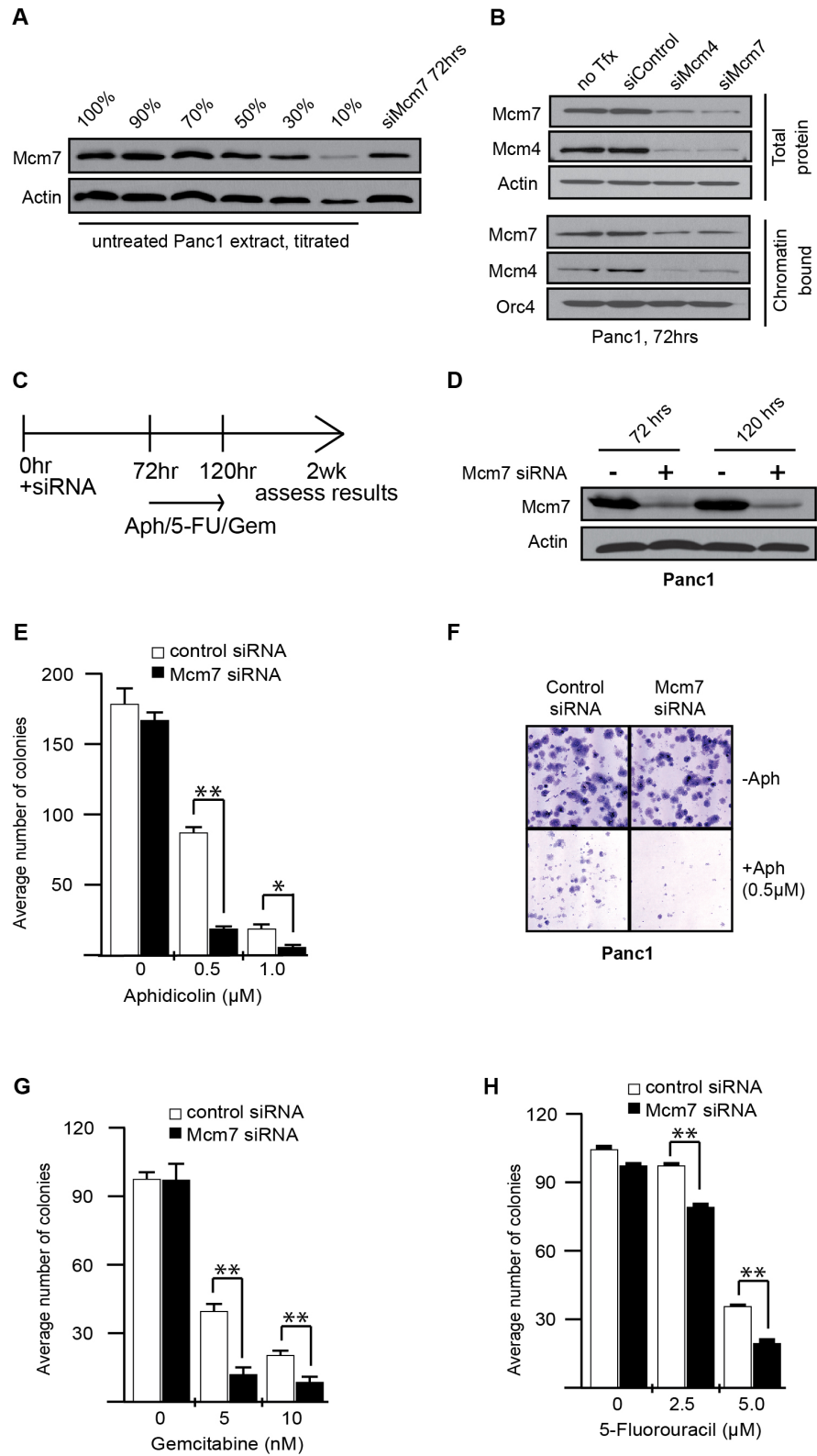


Figure 2.1: Depletion of Reserve MCM Complexes Through Mcm7 Reduction Sensitizes Panc1 PDAC Cells to Gemcitabine and 5-Fluorouracil.

Figure 2.1 (continued). In all panels, the Panc1 tumor cell line was used. (A) Immunoblot of serially titrated non-transfected total cell extract to the amounts indicated, to compare siRNA against Mcm7 (3 nmol/L) level. si-treatment was for 72 hours, and showed a reduction to 30% of the normal Mcm7 level. (B) Immunoblot of siRNA against Mcm4 or Mcm7 showed reduction of total protein levels and chromatin-bound levels of *both* Mcm4 and Mcm7. Actin and Orc4 are loading controls. (C) Diagram showing experimental strategy for cell proliferation assays in the rest of the study. (D) Immunoblot to show partial reduction in Mcm7 levels 72-120hrs after siRNA treatment and during the drug treatment in panels (E-H). (E) Cell proliferation assay in cells with Mcm7 partially depleted and treated with aphidicolin. si-Mcm7 treatment did not affect proliferative capacity alone, but in combination with aphidicolin treatment caused significant sensitization. Three fields in duplicate plates were counted for each condition, the results averaged ± 1 S.D. For statistics, * = $P \leq 0.05$, ** = $P \leq 0.01$. (F) Representative fields from (E), note that si-Control and si-Mcm7 treatment in the absence of drug shows similar colony size and number. (G) Cell proliferation assay showing chemosensitization of cells to gemcitabine treatment in the presence of co-suppression of Mcm7. Three fields in duplicate plates were counted for each condition, the results averaged ± 1 S.D. (H) Cell proliferation assay showing chemosensitization of cells to 5-FU treatment in the presence of co-suppression of Mcm7. Three fields in duplicate plates were counted for each condition, the results averaged ± 1 S.D.

The MCM complex acts as a hexamer for replicative helicase functionality, and by reducing a single MCM subunit by siRNA targeting, the whole complex is reduced on chromatin, and thus the reserve complement is depleted [15, 23, 45, 102]. Cells also appear to have a sensing mechanism that maintains the MCM subunits in equal stoichiometry to each other [17, 45]. We confirmed this was the case in Panc1 cells in which partial siRNA-mediated reduction of either Mcm7 or Mcm4 lead to a partial reduction in the other subunit (Figure 2.1B, *top*). Partial reduction of Mcm7 or Mcm4 also results in both subunits being depleted on chromatin, indicating that suppression of a single MCM subunit causes a partial reduction of the whole MCM hexamer on chromatin (Figure 2.1B, *bottom*).

We next looked at whether partial depletion of Mcm7 (and thus the reserve MCM complement) in Panc1 cells could be used to sensitize them to replication inhibiting drugs. Importantly, we did not want the depletion of the MCM complexes to be detrimental to proliferative capacity on its own. We performed cell proliferation (clonogenic) assays to assess the ability of Panc1 cells to survive and form colonies in the presence of partial Mcm7 reductions and concurrent treatment with replicative stress-generating drugs. Panc1 cells were transfected with 3nM of siRNA against Mcm7 or control (Figure 2.1C). The knockdown of Mcm7 was achieved by 24 hrs and persisted as long as 120 hrs. For the cell proliferation assays, cells were transfected with siRNA and after 48 hrs were transferred to dishes at a lower density. At 72 hrs, Panc1 cells were treated with various levels of drugs for 2 days, then allowed to culture for the remainder of two weeks. Cells were then fixed, stained, and the colonies were counted for each condition. We conducted immunoblotting to verify that Mcm7 was depleted by siRNA during the period of drug treatment with replicative stressors, 72-120 hrs (Figure 2.1D).

To relate our work to previously published studies that used aphidicolin to suppress DNA replication we conducted clonogenic assays in Panc1 cells with suppressed Mcm7 levels [15, 45]. We found that Panc1 cells were significantly sensitized to the anti-proliferative effects of aphidicolin via suppression of the reserve MCM complexes through Mcm7 targeting (Figure 2.1E). Targeting of Mcm7 alone did not affect proliferative capacity in the absence of replicative stress. Figure 2.1F shows example fields for 0.5 μ M aphidicolin exposure +/- siRNA against Mcm7 or control siRNA. Using the same experimental strategy we asked whether we could see a similar

hypersensitization to clinically relevant compounds used in the management of PDAC cancers. We tested our approach using both gemcitabine and 5-FU exposure during co-suppression of the reserve MCM complexes. In both cases, co-suppression of the excess back-up MCM complement alongside drug exposure increased the anti-proliferative effects of the chemotherapy drugs (Figure 2.1G&H). Of particular interest is that co-suppression of the reserve MCM complexes with 5 μ M gemcitabine treatment reduced proliferative capacity of the population more than gemcitabine alone at double the dose of 10 μ M (Figure 2.1G). This would help reduce the toxic side effects of high chemotherapeutic drug doses.

To confirm and validate the above findings, we next checked whether co-reduction of a different MCM subunit, Mcm4, could also chemosensitize Panc1 cells to 5-FU. We achieved the same protein knockdown with Mcm4 as we did with Mcm7, 3nM of si-Mcm4 caused a 70% reduction in the total protein level. We established this through comparing the level of Mcm4 knockdown after 72 hrs of siRNA treatment to that of serially titrated non-transfected Panc1 whole cell extract (Figure 2.2A). Our experimental strategy for the cell proliferation assays was similar to that described above for Mcm7 (Figure 2.2B). Immunoblotting verified that partial Mcm4 knockdown was achieved in Panc1 lysates at 72 hrs and 120 hrs, the time span these cells were exposed to 5-FU exposure (Figure 2.2C). Reduction of Mcm4 alone did not adversely affect proliferative capacity, as was seen with Mcm7 depletion, however, suppression of Mcm4 in the presence of 5-FU caused a significant sensitizing effect (Figure 2.2D). Representative fields for the conditions of 2 μ M 5-FU exposure +/- siRNA against Mcm4 or control siRNA are shown in Figure 2.2E. We conclude from these experiments that

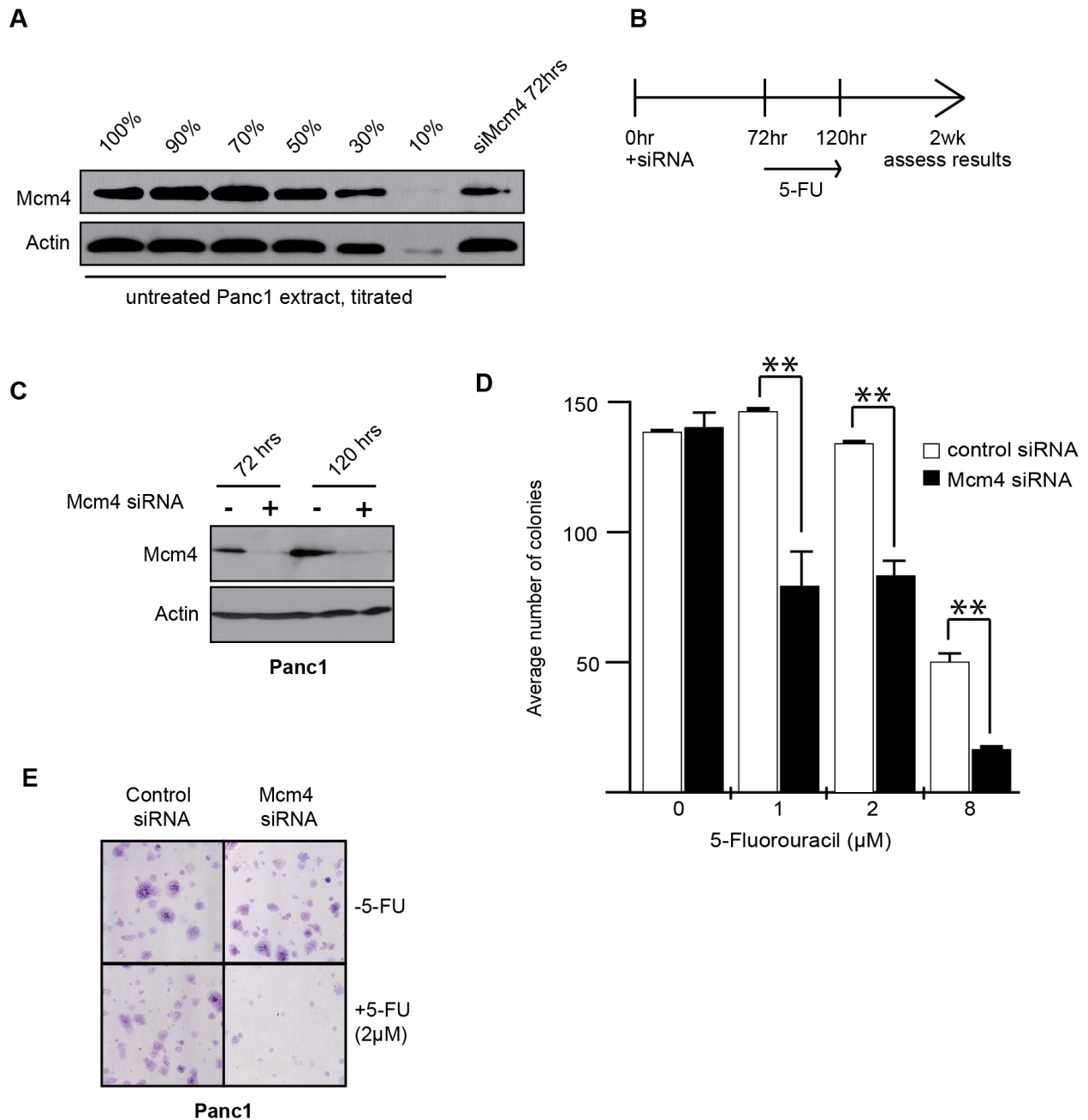


Figure 2.2: Depletion of Reserve MCM Complexes Through Mcm4 Reduction Sensitizes Panc1 PDAC Cells to 5-Fluorouracil. (A) Immunoblot of serially titrated non-transfected total cell extract to the amounts indicated, to compare siRNA against Mcm4 (3 nmol/L) level. si-treatment was for 72 hours, and showed a reduction to 30% of the normal Mcm4 level. (B) Diagram showing experimental strategy for cell proliferation assays.

Figure 2.2 (continued). (C) Immunoblot to show partial reduction in Mcm4 levels 72-120hrs after siRNA treatment and during the drug treatment in panels (D&E). (D) Cell proliferation assay in cells with Mcm4 partially depleted and treated with 5-FU. si-Mcm4 treatment did not affect proliferative capacity alone, but in combination with 5-FU treatment caused significant sensitization. Three fields in duplicate plates were counted for each condition, the results averaged ± 1 S.D. (E) Representative fields from (D), showing sensitization to 2 $\mu\text{mol/L}$ 5-FU with si-Mcm4 knockdown. si-Control and si-Mcm4 treatment in the absence of drug shows similar colony size and number.

co-suppression of the back-up MCM complement by reduction of Mcm7 or Mcm4 during exposure to gemcitabine or 5-FU chemosensitizes Panc1 PDAC tumor cells to the anti-proliferative effects of both drugs. This provides a novel strategy for increasing the therapeutic index of existing chemotherapy drugs.

Depletion of Reserve MCM Complexes Sensitizes Colo-357 PDAC Cells to 5-FU

We wanted to confirm that the results we saw in Panc1 cells were not cell-type specific and could also be observed in a different PDAC tumor cell line. We used the cell line Colo-357 and conducted cell proliferation assays in which the Mcm7 subunit was partially suppressed to the same level as had been in the Panc1 cell line (a 70% protein reduction, data not shown) concurrently with 5-FU treatment (Figure 2.3A). The partial depletion of Mcm7 was verified during the time of drug treatment, from 72-120hrs (Figure 2.3B), effectively removing the reserve MCM complexes. Proliferative capacity of Colo-357 cells was unaffected by partial suppression of Mcm7 in the absence of replicative stress (Figure 2.3C). However, co-suppression of Mcm7 in conjunction with 5-FU exposure resulted in a significant sensitization of the cell population to the replicative stress drug (Figure 2.3C). Representative fields for the dose of 5 μM 5-FU

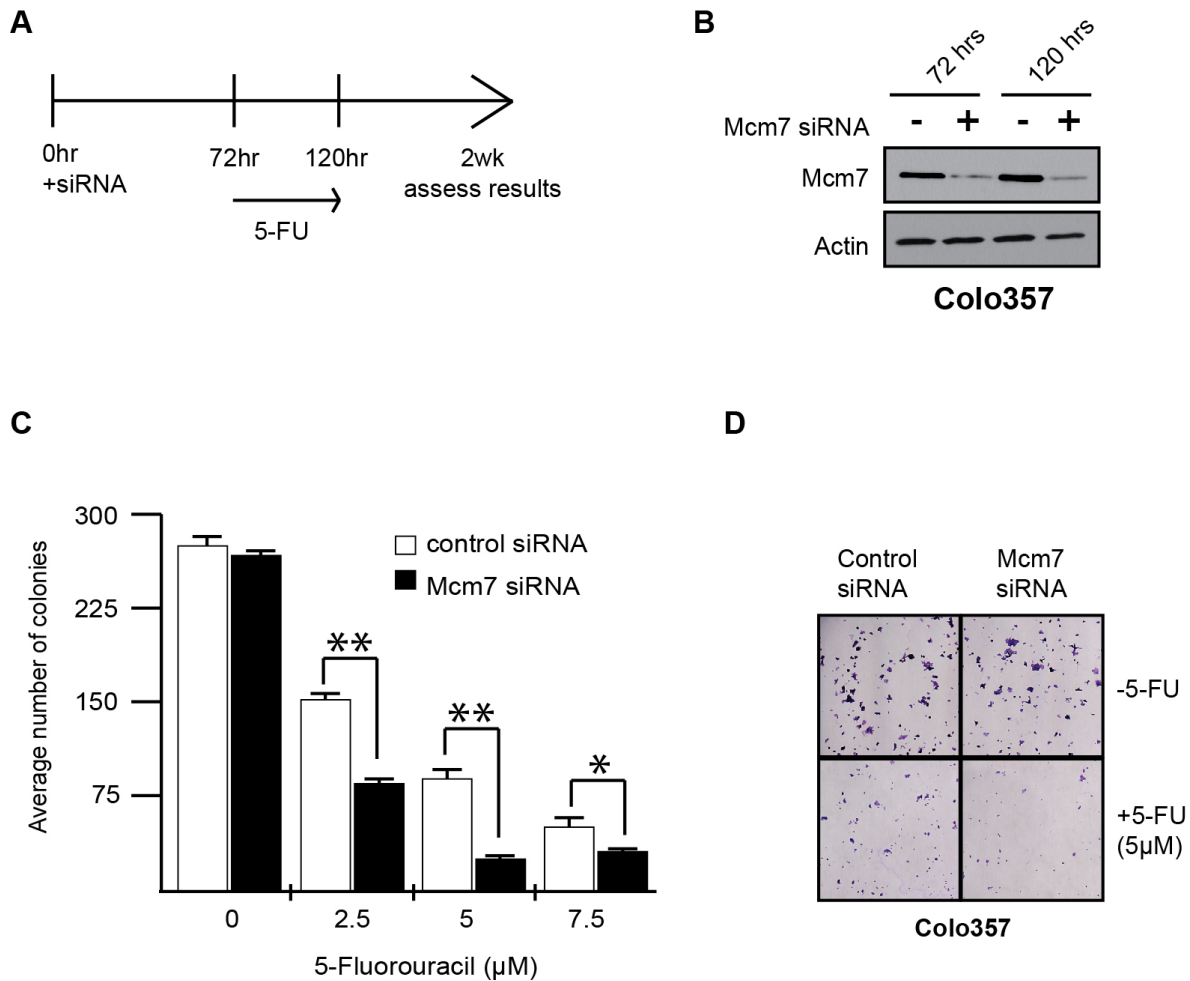


Figure 2.3: Depletion of Reserve MCM Complexes Sensitizes Colo-357 PDAC Cells to 5-FU. (A) Experimental design for cell proliferation assays. (B) Immunoblot verifying partial Mcm7 knockdown in Colo-357 PDAC cell line during the period of drug treatment, 72 hr and 120hr, with 3nmol/L siRNA. (C) Results of cell proliferation assay showing loss of Mcm7 sensitizes Colo-357 cells to 5-FU. si-Mcm7 treatment alone did not affect cellular proliferation. Mcm7 depletion in combination with 5-FU exposure causes significant sensitization. (D) Example fields from (C) showing si-Mcm7 or si-Control treatment alone did not affect colony size and number, but si-Mcm7 treatment with 5μM 5-FU caused loss of colony number and smaller colony size.

with or without siRNA against Mcm7 or control siRNA are shown in Figure 2.3D.

Unfortunately and unexpectedly, the Colo-357 cell line was resistant to the anti-

proliferative effects of gemcitabine, so we were unable to assess the effects of MCM co-suppression with gemcitabine in these tumor cells. We conclude from these experiments that Colo-357 PDAC tumor cells can also be chemosensitized to 5-FU exposure in the background of co-suppression of the reserve MCM complement.

Depletion of Reserve MCM Complexes Suppresses Recovery of DNA Replication Following Chemotherapy Treatment

We sought to determine how loss of reserve MCM complexes causes loss of proliferative capacity in PDAC cells exposed to gemcitabine. Other studies have shown that co-suppression of MCM complexes in HeLa and U2OS cells exposed to aphidicolin or HU show evidence of checkpoint activation and DNA damage responses. This was evident from increased levels of Chk1 and Chk2 activation, H2AX phosphorylation, or p53 phosphorylation, all of which have been associated with replication fork stress [15, 45]. We attempted to detect similar changes indicating DNA damage and fork stress in our PDAC cell lines using multiple levels of MCM depletion, replicative stress drug doses, and timing of drug exposures and MCM co-suppressions. However, we were unable to detect changes in Chk1, p53, p53-p, H2AX-p, or the BLM protein, a fork stress responder [43]. Similar results were obtained by others in U2OS cells, where Chk1 activation and H2AX-p changes are not observed even though p53 is altered upon MCM loss and HU exposure [15]. It has been demonstrated that certain cancer cells are defective in their ATR-mediated sensing mechanisms for the ability to detect DNA damage and fork stress, causing a sensitization of such cells to functional MCM loss in the absence of replicative stress [117]. This indicates that tumor cells may respond

differentially to co-suppression of MCM complexes in combination with drug treatment, which depends on the presence or absence of sensor pathways that ultimately cause indicators of replication stress through fork stalling and DNA damage.

Although we did not observe any biochemical indicators of replication fork stress, we hypothesized that PDAC cells with reduced MCM levels could still be having problems in recovering DNA replication after gemcitabine exposure. We reasoned this because reserve MCM complexes have been shown to be required for dormant origin activation during replicative stress by single-fiber DNA strand analyses [15, 45]. We designed an experiment in which Mcm4 or Mcm7 were partially depleted in Panc1 cells for 72 hrs, were then treated acutely with gemcitabine for 6 hrs, the drug was then removed and the DNA replication rates were measured every hour for the next 4 hrs (Figure 2.4A). We verified partial Mcm4 and Mcm7 suppression by immunoblotting (Figure 2.4B). Partial suppression of Mcm7 for 72 hrs in the absence of drug did not reduce the base levels of DNA replication, as measured by tritiated-thymidine incorporation (Figure 2.4C). This is consistent with idea that the reserve MCM complexes are not required for ongoing DNA replication in an unperturbed situation, rather, they function as back-ups. However, following exposure to gemcitabine for 6 hrs and subsequent removal, Panc1 cells with depleted Mcm7 (Figure 2.4D) or Mcm4 (Figure 2.4E) suffered significant reduction in DNA replication as measured through tritiated-thymidine incorporation. Interestingly, although the total amounts of DNA replication were reduced by MCM suppression, the slope of the curves are similar, suggesting that existing replication forks were likely progressing at similar rates, but that there were fewer overall forks in the cells. This is consistent with previous studies that

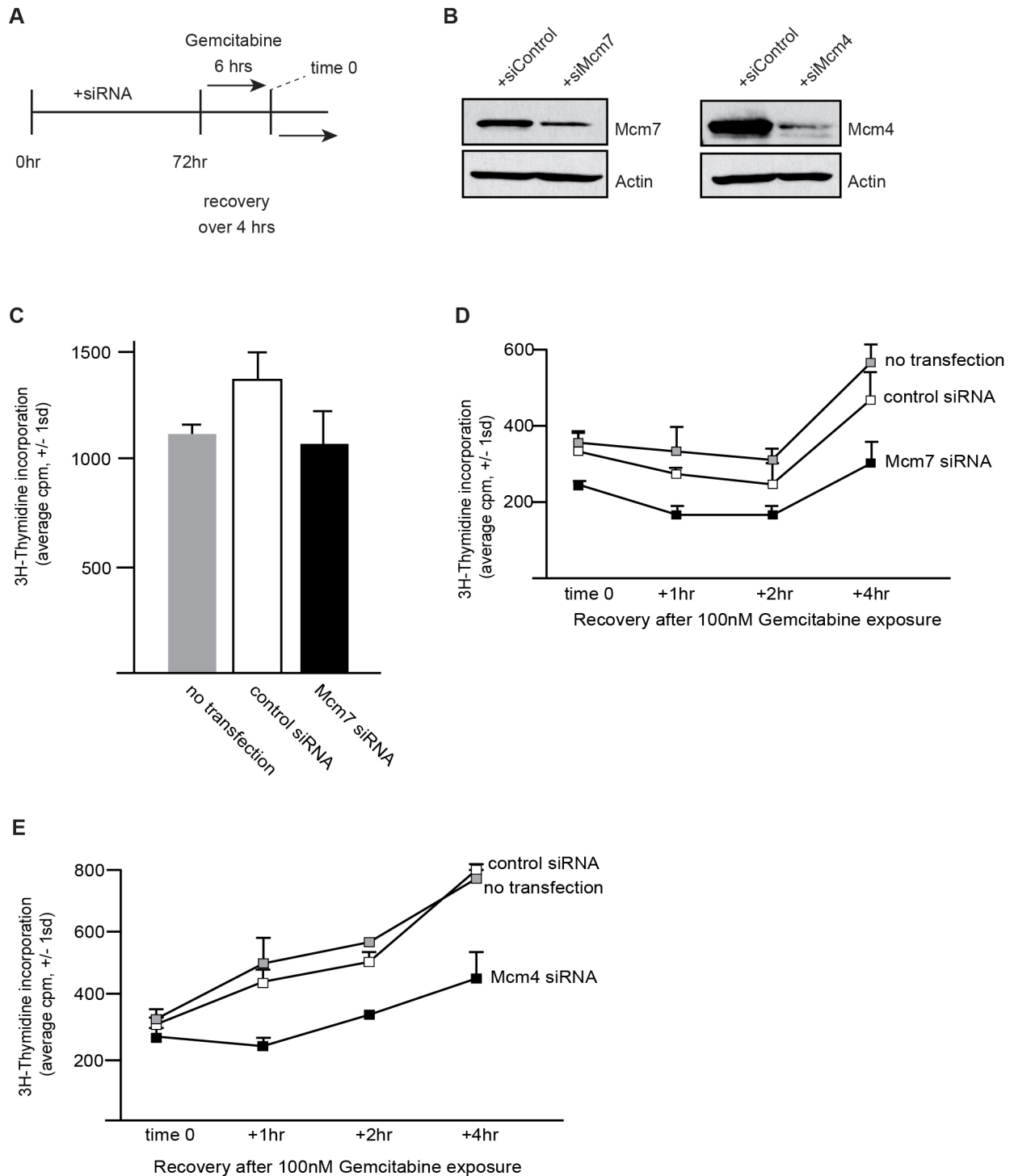


Figure 2.4: Depletion of Reserve MCM Complexes Suppresses Recovery of DNA Replication Following Chemotherapy Treatment. Panc1 cells used in panels (A-E). (A) Experimental design showing si-Mcm7 or si-Control treatment followed 72 hrs later by an acute gemcitabine treatment for 6 hrs, and then sequential measurements of DNA

Figure 2.4 (continued) replication activity over the next 4 hrs. (B) Immunoblots showing partial reduction of Mcm4 and Mcm7 at 72 hrs, the time of gemcitabine treatment. (C) The basal DNA replication rates prior to gemcitabine exposure in non-transfected, si-Control and si-Mcm7 treated cells at 72 hrs. Tritiated thymidine pulse for 30 mins, analyzed via scintillation counting, triplicate wells for each condition, averaged ± 1 S.D. (D) Partial suppression of Mcm7, no transfection or si-Control DNA replication rates measured via tritiated thymidine pulses at indicated times after 100nmol/L gemcitabine exposure for 6 hrs. Triplicate wells for each condition, averaged ± 1 S.D. (E) Partial suppression of Mcm4, no transfection or si-Control DNA replication rates measured via tritiated thymidine pulses at indicated times after 100nmol/L gemcitabine exposure for 6 hrs. Triplicate wells for each condition, averaged ± 1 S.D.

demonstrated loss of MCM complexes blocks activation of dormant origins on DNA fiber analysis [15, 45]. We conclude that PDAC cells exposed to gemcitabine are debilitated in their ability to recover DNA replication under conditions of reserve MCM suppression.

Tumor Cells are Differentially Sensitized to Reserve MCM Complex Loss During Gemcitabine Exposure than Nontransformed Cells

It has been indicated that tumor cells respond differentially to MCM depletion than normal, untransformed cells. Through overexpression of geminin, an inhibitor of MCM loading, Mcm2 levels were decreased on chromatin in U2OS and Saos2 tumor and normal primary cells lines, and normal cells appeared to sense the lack of sufficient MCM complexes and arrested in G1 phase with no apoptosis, whereas tumor cells progressed into a unsuccessful S phase, arrested and apoptosed [118]. We determined whether under our conditions of partial Mcm7 depletion, which does not affect cellular proliferation, we could see differential sensitivity to gemcitabine between Panc1 cells, and an immortalized and non-tumor derived cell line called HaCaT [112, 119]. We wanted to compare PDAC cells to this highly proliferative, non-transformed epithelial

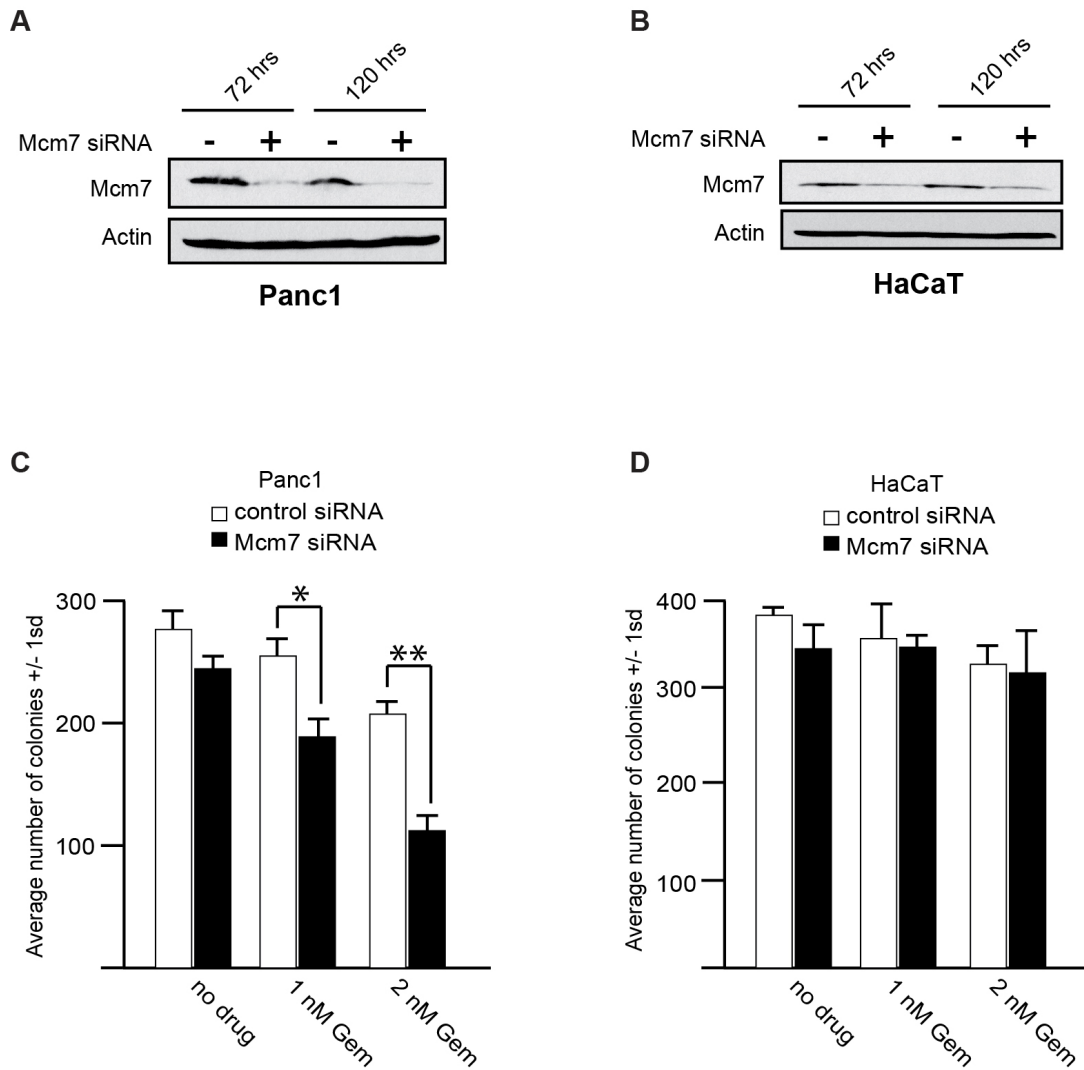


Figure 2.5: Tumor Cells are Differentially Sensitized to Reserve MCM Complex Loss During Gemcitabine Exposure than Nontransformed Cells. (A) Immunoblot showing partial Mcm7 reduction in Panc1 cells following 3nmol/L siRNA for 72 hrs and 120 hrs. (B) Immunoblot showing partial Mcm7 reduction in HaCaT cells following 3nmol/L siRNA for 72 hrs and 120 hrs. (C) Cell proliferation assays in Panc1 cells with partial Mcm7 suppression and the indicated doses of gemcitabine. Significant sensitization to drug was seen with Mcm7 co-suppression. (D) Cell proliferation assays in HaCaT cells with partial Mcm7 suppression and the indicated doses of gemcitabine. No sensitization to drug seen with Mcm7 co-suppression. For panels (C-D) three fields in duplicate plates were counted and averaged \pm 1 S.D.

cell type to remove any bias due to slow-proliferating issues (such as with primary cells), and because chemotherapeutic agents tend to cause adverse side effects epithelial cells in patients. We performed cell proliferation assays as before, and verified that Mcm7 protein levels were partially and similarly depleted in both Panc1 (Figure 2.5A) and HaCaT (Figure 2.5B) during the 72-120 hrs time period of gemcitabine treatment. We determined that Panc1 cells were significantly sensitized to the gemcitabine doses tested (Figure 2.5C) when MCM complexes were co-suppressed, however, at the same gemcitabine doses and same level of MCM suppression, HaCaT cells were not sensitized (Figure 2.5D). This suggests that suppression of MCM function might provide differential sensitivity of tumor cells to exposure with replicative stress drugs, while leaving normal cells relatively unaffected. This would be of benefit to patients, as an anti-MCM drug in conjunction with chemotherapy treatment would show anti-tumorigenic activity, but would not cause adverse side effects to normal, epithelial cells.

Depletion of Reserve MCM Complexes Chemosensitizes Colon Carcinoma Cells to Oxaliplatin and Etoposide

To establish whether our results extended beyond PDAC cancers into other cancer type, we utilized a colorectal cell line to determine whether co-suppression of MCMs causes chemosensitization. We tested SW480 colon carcinoma cells in the presence or absence of partial MCM suppression for their sensitivity to the two

chemotherapeutic drugs oxaliplatin and etoposide that have been used to treat colorectal cancer, but produce toxic side effects which reduce their indication for patient use. Both oxaliplatin and etoposide are indirect replication fork blocking drugs, oxaliplatin is a DNA crosslinking agent and etoposide is a topoisomerase inhibitor. We partially depleted Mcm7 by siRNA treatment for 72 hrs to approximately 30% of its normal levels in SW480 cells (Figure 2.6A). We performed cell proliferation assays as outlined (Figure 2.6B) and verified that Mcm7 was partially reduced during the 72-120 hr drug exposure time (Figure 2.6C). Exposure of SW480 cells to oxaliplatin or etoposide with partial MCM co-suppression significantly reduced the proliferative capacity of the colorectal tumor population at the indicated doses (Figure 2.6D&E).

We next determined if exposure to oxaliplatin or etoposide with co-suppression of MCM complexes caused an increase in the apoptotic index of the SW480 cells. We partially co-suppressed Mcm7 with siRNA for 72 hrs, and added either drug during the last 48 hrs, followed by the analysis of apoptotic index using Annexin-V staining and flow cytometry (Figure 2.6F). Reduction of Mcm7 protein occurred from 24-72 hrs (data not shown, but 72 hrs shown in Figure 2.6G). Treatment with both control and Mcm7 siRNA in the absence of chemotherapy drug produced a slight increase in apoptotic levels of the SW480 population (Figure 2.6H&I). However, exposure to oxaliplatin (Figure 2.6H) or etoposide (Figure 2.6I) in the background of partially reduced MCM complexes caused a significant sensitization to both drugs, resulting in higher apoptotic indices compared to no siRNA or si-Control treatment. Importantly, co-suppression of MCM complexes almost doubled the anti-proliferative effects of both oxaliplatin and etoposide at the highest concentrations tested (Figure 2.6H&I). Our conclusion from

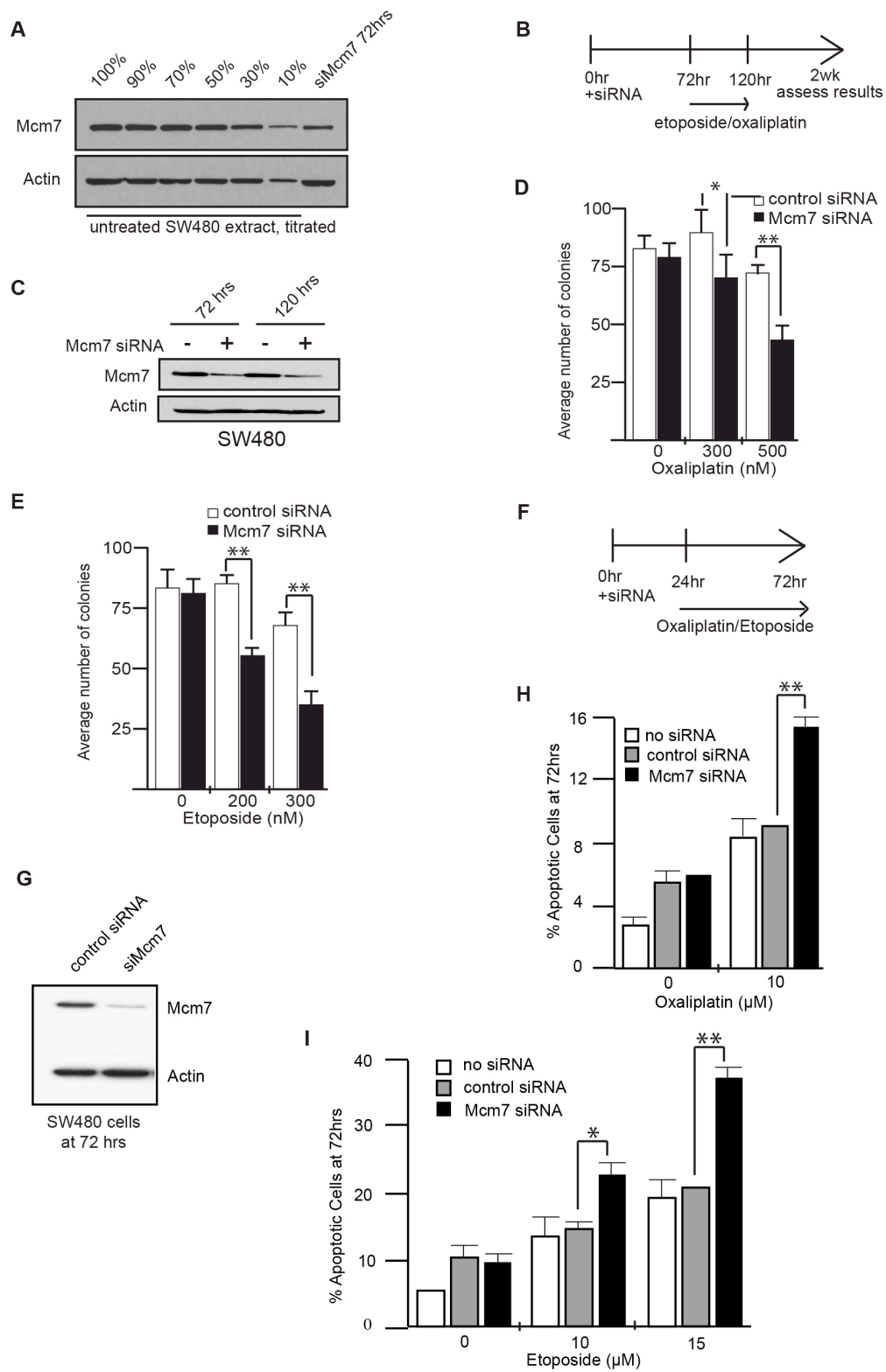


Figure 2.6: Depletion of Reserve MCM Complexes Chemosensitizes Colon Carcinoma Cells to Oxaliplatin and Etoposide.

Figure 2.6 (continued). The colorectal cell line SW480 was used in panels (A-I). (A) Immunoblot of serially titrated non-transfected total cell extract to the amounts indicated, to compare siRNA against Mcm7 (1 nmol/L) level. si-treatment was for 72 hours, and showed a reduction to 30% of the normal Mcm7 level. (B) Diagram showing experimental strategy for cell proliferation assays in panels (C-E). (C) Immunoblot to show partial reduction in Mcm7 levels 72-120hrs after siRNA treatment and during the drug treatment. (D) Cell proliferation assay in cells with Mcm7 partially depleted and treated with the indicated doses of oxaliplatin. si-Mcm7 treatment did not affect proliferative capacity alone, but in combination with oxaliplatin treatment caused significant sensitization. Three fields in duplicate plates were counted for each condition, the results averaged ± 1 S.D. (E) Cell proliferation assay in cells with Mcm7 partially depleted and treated with the indicated doses of etoposide. si-Mcm7 treatment did not affect proliferative capacity alone, but in combination with oxaliplatin treatment caused significant sensitization. Three fields in duplicate plates were counted for each condition, the results averaged ± 1 S.D. (F) Systematic for the apoptosis assays in panels (G-I). (G) Immunoblot showing partial Mcm7 depletion at time of apoptosis assay, 72 hrs post-transfection. (H) Apoptosis assay using annexin V staining and flow cytometry to measure apoptotic index of cells with partial Mcm7 depletion treated with oxaliplatin at 10 μ mol/L. Conducted in duplicate, averaged ± 1 S.D. (I) Apoptosis assay using annexin V staining and flow cytometry to measure apoptotic index of cells with partial Mcm7 depletion treated with etoposide at 10 and 15 μ mol/L. Conducted in duplicate, averaged ± 1 S.D.

these experiments is that co-suppression of MCM complexes also chemosensitizes colorectal carcinoma cells to the anti-proliferative effects of oxaliplatin and etoposide, similar to what we observed in PDAC cells with gemcitabine and 5-FU treatment.

Discussion

We provide here definitive evidence that depletion of the reserve MCM complexes causes chemosensitization of multiple human tumor cell types to several chemotherapeutic drugs used in the clinical management of human cancer. Partial suppression of MCM complexes utilizing siRNA against Mcm4 or Mcm7 causes PDAC

tumor cell lines to increase their sensitivity to the anti-tumor effects of both gemcitabine and 5-FU. We observed a similar effect in a colorectal tumor cell line treated with oxaliplatin or etoposide in the background of co-suppression of the MCM complexes. The chemotherapeutic agents tested act directly or indirectly to block DNA replication fork activity, and prior studies have suggested that reserve MCM complexes are necessary to recover replication following drug exposure that causes replicative stress [15, 45]. The prior studies that identified that MCM suppression could chemosensitize cells primarily used aphidicolin or hydroxyurea, which are not clinically relevant drugs [15, 45]. We have extended this important concept by testing clinically relevant drugs in cancer cell lines that recapitulate the cancers these drugs are used to treat. We demonstrated that following acute gemcitabine exposure, PDAC cells required reserve MCM complexes to recover DNA replication (see our model in Figure 2.7). Interestingly, PDAC cells were more sensitive to MCM depletion following gemcitabine exposure than a non-transformed epithelial cell line, suggesting that co-targeting MCM complexes during chemotherapy regimens will provide a selective anti-tumor advantage.

Our work has important implications for the treatment of cancer patients, by potentially increasing the therapeutic efficacy of existing chemotherapy drugs used in PDAC management that are not currently very effective on their own by co-suppressing reserve MCM functions [95]. Colorectal cancer treatment often involves the use of oxaliplatin in chemotherapy, which has been associated with neuropathy that limits the ability to treat patients with this drug [108, 109]. Colorectal and other cancer are often treated with etoposide, but this compound is known to produce toxic cardiac side effects many years after clinical exposure that limit its utility [110, 111]. Our study suggests that

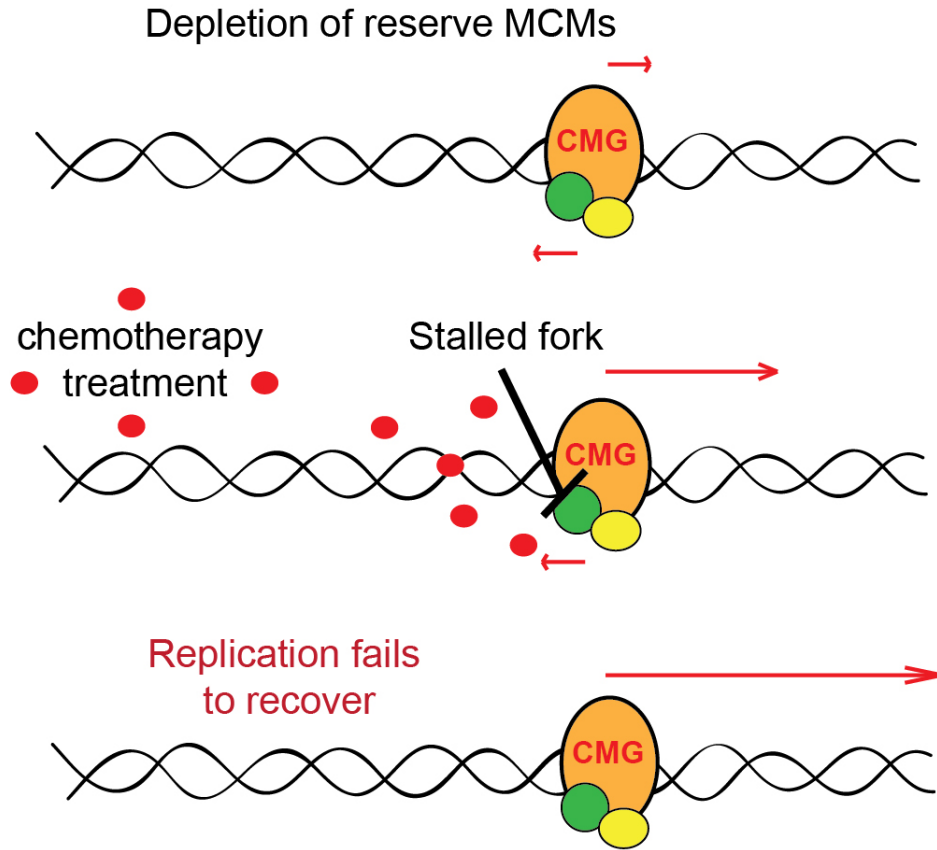


Figure 2.7: Model of how Depletion of Reserve MCM Complex Causes Chemosensitivity. Depletion of MCM protein levels leads to a reduction in the level of reserve MCM complexes loaded onto chromatin. When replication forks stall in S phase due to the replicative stresses provided indirectly by chemotherapeutic agents, the lack of reserve MCM complexes causes fewer CMG helicases to be activated to recover DNA replication, leading to incomplete replication and the induction of apoptosis.

the development and use of anti-MCM drugs in combination with these and similar chemotherapeutic compounds has the potential to increase the therapeutic index of existing clinical compounds. This could allow for the reduced administration of toxic chemotherapy drugs such as oxaliplatin or etoposide that could reduce the toxic side effects associated with these compounds. The clinical utility of anti-MCM drugs is clear,

however, targeting MCMs over sustained periods should be avoided as partial MCM suppression can result in genomic instability and increased DNA damage [15, 45, 117], and mice with sustained depleted MCM complexes on chromatin display increased cancer risk [43]. As such, sustained MCM suppression during chemotherapeutic regimens may be contraindicated for the clinical management of cancer, but shorter durations of MCM co-suppression may overcome these difficulties and allow anti-MCM drug use for cancer treatment. As an alternative approach, chemotherapy treatments alternating with shorter anti-MCM drug treatment may be more effective and less likely to produce DNA damage in non-tumor cells.

CHAPTER THREE:
**C-MYC REGULATION OF CMG HELICASE ASSEMBLY AND ACTIVATION
THROUGH CHROMATIN DECONDENSATION AND CDC45 LOCALIZATION**

Abstract

The oncogene *c-MYC* is essential for cellular proliferation and growth. *c-Myc* is overexpressed in many human cancers, and part of its oncogenic effects is due to a non-transcriptional role in control over the initiation of DNA replication. *Myc* overexpression leads to over-activation of DNA replication origins, causing DNA damage. Activation of replication origins requires the assembly of the replicative Cdc45-MCM-GINS (CMG) helicase that unwinds DNA. When overexpressed, *Myc* binds to intergenic regions of the genome where DNA replication origins are found. We demonstrate here that the mechanism behind how *Myc* promotes CMG helicase assembly and activation is through dramatic chromatin decondensation of higher order chromatin. This depends on the presence of the *Myc*-Box II domain, which is critical for *Myc*-oncogenic transformation. *Myc*-induced chromatin decondensation does not affect MCM assembly, however, it causes recruitment of Cdc45 and GINS to form active replicative helicases. Recruitment of the histone acetyltransferases GCN5 and Tip60 to chromatin via the *Myc*-box II domain is required for chromatin unfolding. Finally, normal

levels of Myc in late-G1 phase also regulate Cdc45 recruitment to the CMG helicase. These findings identify a previously unknown role for Myc in normal cell cycle progression through regulating DNA replication initiation and provide a new mechanistic explanation for Myc driven oncogenic transformation.

Introduction

The proto-oncogene *c-MYC* is essential for cellular proliferation, cell cycle progression and is one of the most commonly amplified genes in cancer [120]. *c-Myc* (Myc) is in a family of basic helix-loop-helix leucine zipper (bHLH-LZ) transcription factors (including N- and L-Myc) that are deregulated in a variety of tumors [66, 121]. Excessive Myc protein levels generate DNA replication stress, damage and genomic instability in only 1-2 cell cycles, likely contributing to tumorigenesis [14, 76]. Myc functions in complexes with other proteins such as Max to regulate transcription [121]. Myc activates and represses a set of direct target genes by binding to DNA elements called consensus E-boxes (CACGTG) in the promoters of these genes [63, 64]. However, the maximum Myc-induced gene expression is less than two-fold, which does not adequately explain all of the potent effects Myc has on cell cycle progression [66, 68, 69]. Myc is commonly studied for its transcriptional roles, but there are a number of unexplained findings regarding Myc that distinguish it from other basic transcription factors and suggest that the mechanistic role of Myc in oncogenesis may extend beyond transcriptional control.

Myc binds to widespread areas of chromatin, and several array projects have shown evidence for Myc binding to over 25,000 genomic loci, which is in stark contrast with other transcription factors [122-124]. Approximately 40% of these sites are greater than 10kb from promoters [122]. More recent studies using genome-wide chromatin immunoprecipitation have shown that the number of Myc binding sites progressively increases from a non-Myc amplified (control) state, through Myc amplified pre-tumor and tumor formation [63]. While two-thirds of Myc binding sites in the control state are in proximity to promoters, the majority of new Myc binding sites in the Myc overexpressed pre-tumor and tumor states are distal, resulting in equal intra- and intergenic binding loci [63]. Overexpressed Myc binds increasingly to non-E-box sites or non-consensus E-boxes (CANNTG) [64, 84, 124, 125]. The mechanisms by which Myc functions to influence oncogenesis through large-scale chromatin binding are unknown. Myc is known to have a non-transcriptional role in the initiation of DNA replication, although the mechanism is poorly understood [14]. The intergenic regions where Myc binds are also the location of replication origins [13].

Initiation of DNA replication begins with the activation of the replicative helicase, which unwinds DNA and marks the G1-S transition [28, 126]. The helicase forms on a precursor protein complex called the pre-Replication Complex (pre-RC) [12]. Pre-RCs are composed of the Origin Recognition Complex (ORC), which binds to future origins of replication and recruits Cdc6 and Cdt1 proteins that load the Mini-Chromosome Maintenance (MCM) 2-7 complex [127-132]. Excess MCM complexes are loaded onto chromatin in a distributed manner throughout intergenic regions, beyond the number required for a normal S phase, and function as reserves in S phase during DNA

replication stress [15, 44]. Myc has been shown to bind to replication origins and is found in complexes with pre-RC proteins [14, 84]. Certain MCM complexes are chosen to become replicative CMG (Cdc45, MCM, and GINS) helicases through the recruitment of the Cdc45 and the GINS (*go-ichi-nii-san*) protein complex [133]. The MCM complexes provide the ATPase activity necessary for the CMG helicase to unwind DNA at the G1-S transition prompting the initiation of DNA replication from replisomes composed of CMG helicases and DNA polymerases [28, 134-136]. This activation minimally requires Cyclin A-Cdk2 and Dbf4-Cdc7 activity [136]. Cdc45 protein levels are extremely low in cells relative to the MCM complex, and its recruitment is rate limiting for helicase formation and activation [17]. In transcriptionally inert embryonic *Xenopus* extracts, maternally-supplied Myc is imported into the nucleus and is required for DNA replication [73]. This suggests that Myc has a non-transcriptional role in DNA replication. Overexpressed Myc causes increased origin activity and DNA damage in both mammalian cells and *Xenopus* extracts, which depends on the presence of Cdc45 and GINS [14, 78]. However, the mechanism by which Myc promotes CMG helicase activation has yet to be demonstrated.

Myc induced oncogenic transformation and replication origin activity is dependent on a highly conserved N-terminal Myc domain called Myc-box II (MBII) [61, 74, 75, 137]. Myc associates with a nuclear cofactor called TRRAP through the MBII domain, which then recruits the histone acetyltransferases (HATs) GCN5 or Tip60 to chromatin to mediate the acetylation of histone tails, resulting in open chromatin and transcriptional activation [85, 87, 88]. Loss of Myc has been shown to produce global nuclear chromatin condensation and histone modifications associated with an inactive

chromatin state [83]. Myc binds to chromatin that is enriched in acetylated and methylated histones H3 and H4, and then promotes further histone H4 acetylation associated with open chromatin [81, 82, 138, 139]. These findings are not typical of classical transcription factors and strongly suggest that Myc is unique and likely functions in a broader chromatin regulatory manner that extends beyond transcriptional control of target genes. However, the effect Myc-induced modification of chromatin has on DNA replication has yet to be determined.

Here we show that the mechanism by which Myc causes initiation of DNA replication involves stimulating dramatic chromatin decondensation, allowing CMG helicases to fully assemble on the pre-loaded chromatin bound MCM complexes through localization of the rate-limiting Cdc45 molecule. The helicases become functional, and result in active replication origins. We further show that chromatin decondensation is dependent on Myc-mediated recruitment of the HATs GCN5 and Tip60 to chromatin via the MBII domain, and requires histone H4 tail modifications to occur. Depletion of endogenous Myc causes a reduction in chromatin-bound Cdc45 protein levels and S phase entry, which can be rescued by Myc overexpression, but is dependent on the MBII domain presence. These findings identify a previously unknown role for Myc in normal cell cycle progression whereby DNA replication initiation is regulated through the assembly and activation of CMG helicases on Myc-mediated open chromatin regions. Our results also provide new mechanistic insight into Myc oncogenic transformation in which overstimulation of DNA replication could result in genomic instability and carcinogenesis.

Materials and Methods

Cell Culture, Synchronizations and Transfections

Mouse keratinocytes (Balb/MK) were maintained in low calcium MEM supplemented with 8% dialyzed FBS (Hyclone) and 4ng/ml EGF (Invitrogen). The retroviral packaging cells lines psi-2 3T3 and PA317 and the BT-549 cell line were maintained in phenol-free DMEM supplemented with 10% FBS (Hyclone). The CHO cell line (clone A03_1) was maintained in DMEM supplemented with 10% FC2 and 0.3 μ M methotrexate.

To create the MK and BT-549 MycER/ Δ MycER cell lines, the psi-2 3T3 cell lines was transfected with pMV7-MycER/ Δ MycER plasmids using polyethylenimine (PEI) and selected with 400 μ g/mL G418 (Invitrogen) for two weeks. Dense cells were then fed fresh media containing no G418 for 24 hours, after which the virus-containing media was removed, filtered, polybrene added, and placed onto PA317 cells at 50% density. After 48 hours, these cells were selected with 400 μ g/mL G418, although little cell death occurred due to high infection rate. After two weeks of selection, dense cells were then fed fresh media containing no G418 for 24 hours, after which the virus-containing media was removed, filtered, polybrene added, and used to infect MK or BT-549 cells at 50% density. MK cells were selected with 50 μ g/mL G418 for two weeks and then maintained in 25 μ g/mL G418 phenol-free media with charcoal-stripped serum. BT-549 cells were selected with 100 μ g/mL G418 for two weeks and then maintained in 50 μ g/mL G418 phenol-free media with charcoal-stripped serum.

MK MycER/ Δ MycER cells were synchronized by EGF deprivation for 3.5 days and stimulated to enter the cell cycle by the re-addition of EGF, with or without MycER/ Δ MycER induction using 2 μ M 4-hydroxytamoxifen (Sigma) at the indicated times. CHO transfections were performed for 24 hrs with *TransIT-LT1* (Mirus) as per manufacturers recommendations. MK siRNA transfections were performed following the manufacturers recommendations using Dharmafect 4 transfection reagent, siRNA concentrations used were 100nM of siLuciferase or siGenome mouse Myc (Dharmacon).

Plasmids

pMV7-MycER/ Δ MycER were provided by M. Eilers [74]. LacI-Myc and LacI- Δ Myc were sub-cloned into the LacI vector from the pMV7-MycER/ Δ MycER plasmids. LacI-VP16 was provided by A. Belmont (University of Illinois). HsCdt1 and HsBRCA1 (6c-w) were expressed using pRcLac [140]. Set8-DBD was generated by proofreading PCR and expressed using pcDNA3-HA-NLS [140]. E. Seto (Moffitt Cancer Center) supplied pIRES GCN5 (WT), pIRES GCN5 (GYG-AYA mutant), which were subsequently subcloned into pcDNA3-HA vector, pcDNA3-Flag Tip60 (WT) and pcDNA3-Flag Tip60 (GQE mutant) [141].

Antibodies

Rabbit polyclonal antibodies unless otherwise stated; anti-Psf2, anti-HBO1, anti-GCN5, anti-Tip60, mouse monoclonal (mAb) anti-ER, mAb anti-Mcm7 (Santa Cruz Biotechnology); mAb anti-actin, mAb anti-Flag (Sigma); anti-Myc (provided by Steve

Hann, Vanderbilt University); rat monoclonal anti-RPA32 (Cell Signaling), mAb anti-Lacl (Millipore); anti-Lacl (Stratagene); mAb anti-HA (Covance); mAb anti-BrdU (Life Technologies); chicken polyclonal anti-Cdc45 [17]; anti-Mcm2 [17]; chicken anti-Mcm4 [17].

Immunoblotting and Immunofluorescence

For immunoblots, equal cell numbers were scraped off plates into cold PBS, lysed and boiled in 1x Laemmli loading dye (for total protein) or were separated into Triton X-100 soluble or resistant (chromatin bound) fractions. Cells were resuspended in buffer A (10mM HEPES pH 7.9, 10mM KCl, 1.5mM MgCl₂, 0.34 M sucrose, 10% glycerol, 1 mM DTT, 5 µg of aprotinin per ml, 5 µg of leupeptin per ml, 0.5 µg of pepstatin A per ml, 0.1 mM phenylmethylsulfonyl fluoride) containing 0.1% Triton X-100 for 5 mins on ice. Nuclei were isolated by low-speed centrifugation (5 min, 1,300 x g 4°C), washed in buffer A without TX-100, and then lysed in buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, and previously described protease inhibitors) for 30 mins on ice. Insoluble chromatin was collected by low-speed centrifugation (5 min, 1,700 x g 4°C) and resuspended in an equal volume of buffer B and 2X Laemmli loading dye. Immunoblotting was conducted using standard procedures [17].

For IF, MK and CHO cells were grown on cover slips. Prior to fixation cells were pre-extracted for 10 mins with cold 0.1% TX100 in PBS-pH 7.4 (5mM MgCl₂, 0.1mM EDTA-pH 8) unless otherwise stated. Cells were then fixed for 15 mins at room temperature using 2% formaldehyde/PBS solution for CHO cells and 4% formaldehyde/PBS solution for MK cells, followed by two 10 min PBS washes. IF

analysis was conducted by permeabilizing cells for 5 mins in 0.2% TX-100/1% normal donkey serum (NDS)/PBS solution at 4°C, followed by two 15 min 1%NDS/PBS blocking-washes. Primary antibody incubation occurred in a moist chamber for 1 hour. If a secondary antibody was used, two 15 min 1%NDS/PBS washes occurred after the primary antibody incubation. For BrdU analysis, cells were treated with 3N HCl for 30 mins prior to primary antibody incubation, followed by three 10 min 1%NDS/PBS washes. After the last antibody incubations, cells were washed twice with PBS solution for 10 mins, incubated with a 0.5µg/mL DAPI/PBS solution for 5 mins to stain the nuclei, then washed twice with PBS solution and mounted onto slides with anti-fade solution where appropriate. Images were obtained using a Zeiss upright fluorescent microscope (CHO cells) or a Leica confocal microscope (MK cells).

DNA Replication and Cell Proliferation Assays

DNA replication was measured by pulsing triplicate wells with 3µCi/mL of [³H] thymidine for 30 minutes and fixing with ascorbic acid followed by scintillation analysis of one-third of the trichloroacetic acid-precipitable material [75]. For BrdU experiments, replicating DNA was labeled with 15µM BrdU incorporation for 30 mins, fixed and analyzed by IF as described above. Cell proliferation (clonogenic) assays with asynchronous MK-MycER/ΔMycER and BT-549 (MycER/ΔMycER) cell lines were conducted in duplicate on 35mm tissue culture plates and treated with the indicated drugs and doses for 48 hrs, then allowed to culture for the remaining 2 weeks. Cells were fixed with an acetic acid/methanol solution, washed with methanol, and stained

with Giemsa for 15 mins to quantify surviving colony numbers. Aphidicolin and gemcitabine drugs were from Sigma.

Statistics

Two-tailed t tests performed throughout, $**P \leq 0.01$, $*P \leq 0.05$.

Results

Myc Elevation Causes an Increase in CMG Helicase Assembly and Activation

Previous studies have indicated that Myc elevation causes over-activation of origin activity in S phase [14, 75]. We re-established a Myc overexpression system by creating the inducible mouse keratinocyte (MK) MycER or mutant Δ MycER (missing the MBII domain) cell lines, showed equal protein expression (Figure 3.1A) and reconfirmed that only MK-MycER cells (Figure 3.1B) demonstrated almost a doubling of DNA replication in early-S phase (15hr and 18hr), indicative of increased origin usage, whereas MK- Δ MycER (Figure 3.1C) did not [75]. Activation of MycER in these cells mimics overexpression conditions for Myc. Note that Δ MycER activation does not appear to confer a dominant-negative effect. Increased DNA replication in the induced MK-MycER cell line was due to more replication foci per cell (Figure 3.1D, quantified in Figure 3.1E) rather than an increased number of cells exiting quiescence and entering S phase.

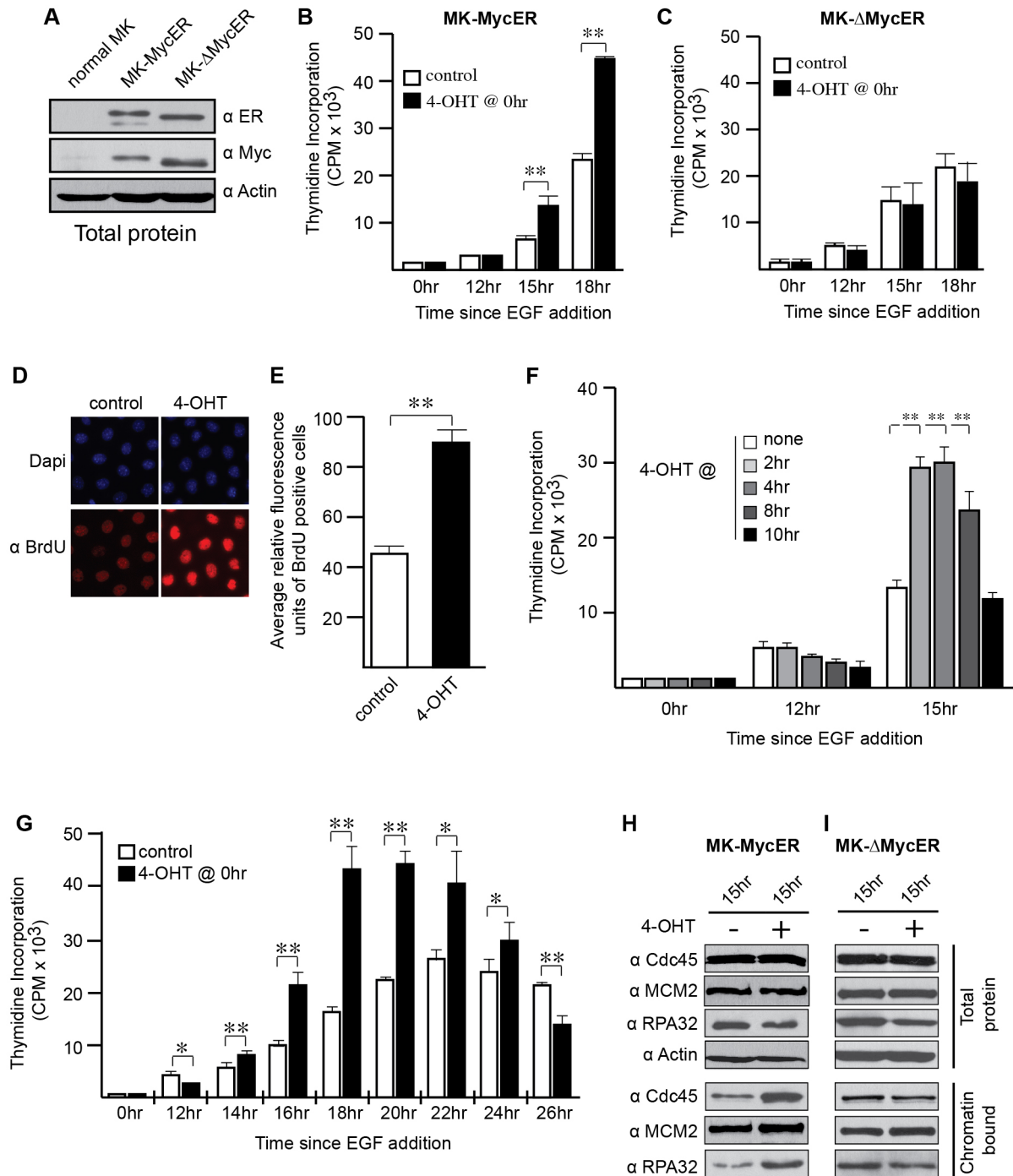


Figure 3.1. Myc Elevation Causes and Increase in CMG Helicase Assembly and Activation. (A) Immunoblot (IB) probed with antibodies to indicated proteins demonstrating the creation and equal protein expression of the MK-MycER and MK-ΔMycER cell lines. EGF-synchronized MK-MycER (B) and MK-ΔMycER (C) cells were treated with 4-OHT at time of EGF re-addition (0hr) and pulsed with ³H-thymidine at indicated times.

Figure 3.1 (continued). (D) Synchronized MK-MycER cells were treated with 4-OHT at 0hr and pulsed with BrdU at 15hrs. Analyzed by confocal microscopy. (E) Graphical representation of the per cell brightness of BrdU positive cells only. (F) Synchronized MK-MycER cells treated with 4-OHT at the indicated times and pulsed with ³H-thymidine at 0, 12 and 15hrs. (G) S phase analysis of Myc induced replication. Synchronized MK-MycER cells treated with 4-OHT at 0hr and pulsed with ³H-thymidine at indicated times. IB analysis of MK-MycER (H) and MK-ΔMycER (I) effects when activated with 4-OHT at 0hr on total protein expression and the chromatin-bound complement of indicated proteins at 15hrs. Values in panels B, C, E, F and G are means of triplicate wells or fields, ±1SD. Experiments conducted in duplicate or triplicate.

To dissect the mechanism by which Myc initiates DNA replication we asked when in G1-phase Myc activation was necessary to elicit origin over-activation. MK-MycER cells were induced at the indicated times throughout early to mid-G1 phase, and origin activation in S phase (15hr) was seen with MycER activation up to 8hrs into G1, but not after 10hrs (Figure 3.1F). In these quiescence-released cells the G1-S transition occurred ~12hrs. The 8hr time period is when the MCM complexes are becoming chromatin-bound, followed shortly after by Cdc45 recruitment to chromatin. Myc has been shown to co-localize with BrdU foci only in early-S phase [14]. We saw that the most dramatic Myc-induced origin activation occurred in early-to-mid S phase (16-20hrs), and diminished as S phase progressed (Figure 3.1G). These results indicate that overexpression of Myc promotes excessive DNA replication during the early half of S phase, a time when euchromatic regions are known to be replicated.

Our results and those of others indicate that Myc-induced origin over-activation is due to an increased number of active CMG helicases [14, 78]. To establish this we analyzed the endogenous total and chromatin-bound proteins of Cdc45, MCM2 and RPA. Although Myc is a transcription factor, MycER activation at the beginning of G1

phase did not cause a change in the total protein levels of the analyzed proteins (Figure 3.1H upper panel). These proteins are functional when bound to DNA, so we assessed their levels chromatin-bound levels in Figure 3.1H, lower panel. MycER induction caused a dramatic increase in the chromatin-bound complement of the Cdc45 protein, a replication-limiting factor for CMG helicase assembly and activation [17]. Only 1/3 of the total Cdc45 protein in cells is chromatin-bound [17], and Myc appears to be promoting the normally soluble complement of Cdc45 to become tightly associated with chromatin. MycER induction also caused an increase in chromatin-bound RPA, an indicator of active CMG helicases. The chromatin-bound Mcm2 protein levels did not change. It has been established that in mammalian cells MCM complexes are loaded in a 2-3-fold excess onto DNA [17], so Myc-induced CMG helicase over-activation would not necessarily require additional MCM complexes to load onto chromatin. No changes in total protein or chromatin-bound proteins levels were seen with Δ MycER activation (Figure 3.1I). We conclude that Myc overexpression in the early to mid G1 phase causes an increase in the number of assembled and activated CMG helicases in S phase, which is dependent on the presence of the MBII domain.

Myc Induced Over-Activation of CMG Helicases Causes Chemosensitization

The excess MCM complexes loaded onto DNA act as tumor suppressors, by enabling additional replication origins to activate when replication forks stall [43]. This would require additional CMG helicases to form and activate on the reserve MCM complexes. Our group and others have shown that reducing MCM complexes on chromatin chemosensitizes cells to replication fork stalling drugs, due to an inability to

recover DNA replication [44, 97]. We reasoned that by creating more CMG helicases than is necessary for a normal S phase, as Myc elevation does, the reserve MCM complexes are being utilized and thus depleted so will not be available to recover DNA replication during drug treatment, which would negatively affect proliferative capacity. The experimental design is outlined in Figure 3.2A, in which activation of MycER or Δ MycER occurred simultaneously with a 2 day drug treatment of either Aphidicolin, a DNA polymerase inhibitor, or Gemcitabine, a pyrimidine analogue that negatively affects nucleotide pool levels. Both of these drugs indirectly stall replication forks, which would then require additional CMG helicases to become active to recover replication. We conducted colony formation assays, and in Figures 3.2B, C, D, F and G we picked a level of MycER/ Δ MycER activation that did not negatively affect cell growth alone, although due to Myc driven over-stimulation of CMGs and consequent deficiency of reserve MCMs would potentially sensitize these cells to fork-stalling drugs. In the MK-MycER cell line, the combination of MycER activation and a low level of Aphidicolin treatment caused a significant loss of proliferative capacity (Figure 3.2B). Representation fields are shown in Figure 3.2C. Under the same conditions, the MK- Δ MycER cell line did not demonstrate a loss of proliferative capacity (Figure 3.2D). A number of clinical studies have shown that Myc amplified tumors respond more favorably to chemotherapy treatment than non-Myc amplified tumors, as measured by parameters such as increased 5 year survival rates or increased remission rates [142-144]. To test our hypothesis in an oncogenic situation, we created the Myc overexpression system in a breast cancer cell line, BT-549 (Figure 3.2E), known to have normal Myc levels and thus allowing us to observe an effect of Myc

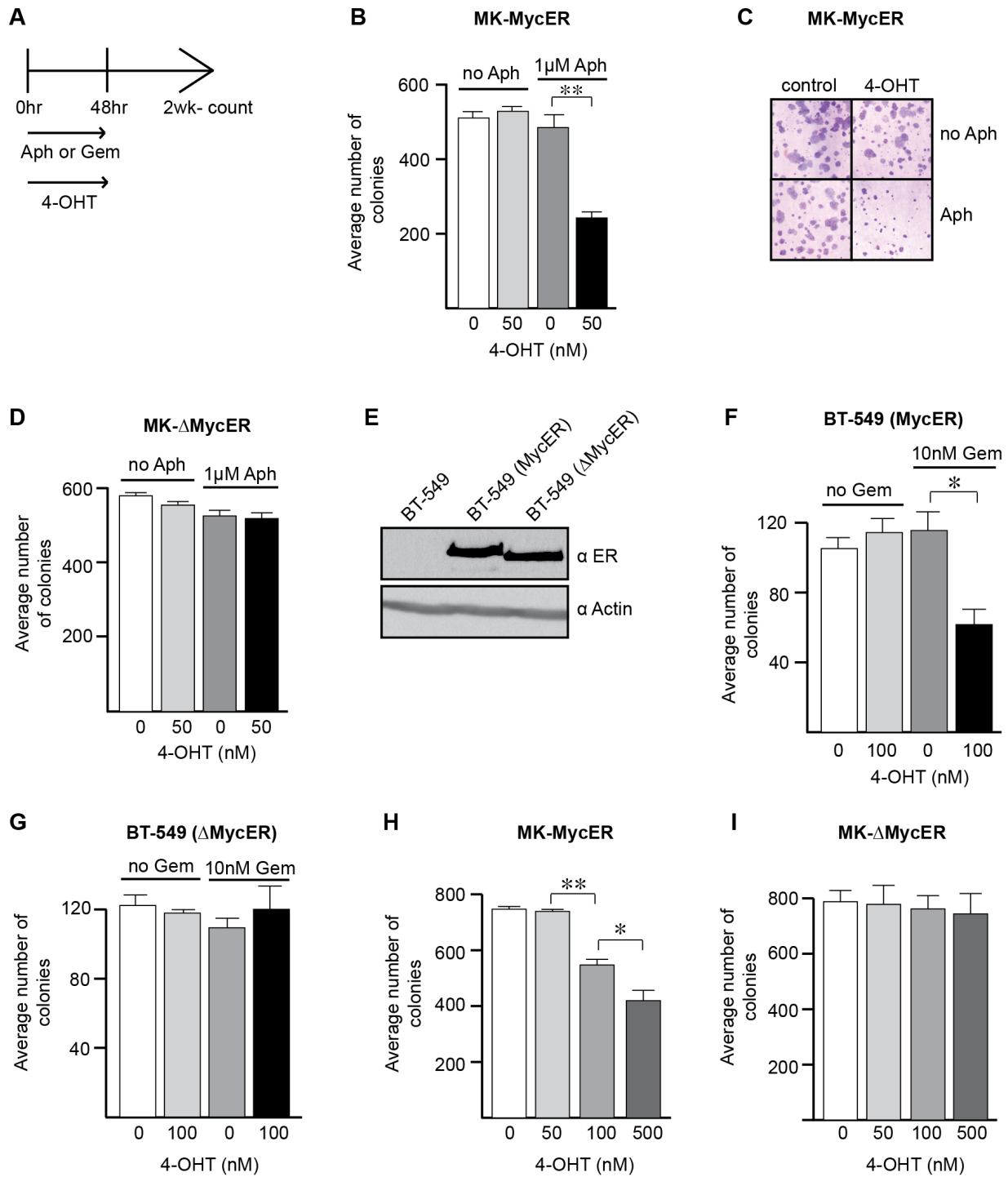


Figure 3.2. Myc Induced Over-Activation of CMG Helicases Causes Chemosensitization.

Figure 3.2 (continued). (A) Diagram showing experimental design for cell proliferation assays. (B) MK-MycER cell proliferation assay, asynchronous cells were plated and then treated with 1 μ M aphidicolin concurrently with 50nM 4-OHT for 48hrs, and then allowed to form colonies before staining and counting. (C) Representative fields from the experiment in (B). (D) MK- Δ MycER cell proliferation assay, as in experiment (B). (E) IB with indicated antibodies demonstrating the creation and equal protein expression of BT-549 (MycER) and BT-549 (Δ MycER) cell lines. Cell proliferation assay for BT-549 (MycER) (F) and BT-549 (Δ MycER) (G) using 100nM 4-OHT concurrently with 10nM Gemcitabine. (H) MK-MycER cell proliferation assay showing significant reduction in proliferative capacity as 4-OHT concentration increases. (I) MK- Δ MycER cell proliferation assay showing no change in proliferative capacity as 4-OHT concentration increases. All cell proliferation assays are means of duplicate plates, \pm 1SD. Experiments were conducted in duplicate.

overexpression. We tested whether we could chemosensitize this breast cancer cell line to Gemcitabine, a chemotherapy drug frequently used in the clinical management of breast cancer and an indirect fork-blocking agent. As seen in Figure 3.2F, co-activation of MycER in concurrently with Gemcitabine treatment resulted in significant sensitization to the anti-proliferative effects of this chemotherapeutic agent. As with the MK cell line, this effect depended on the presence of the MBII domain, as the BT-549 (Δ MycER) cell line was not chemosensitized (Figure 3.2G). Excessive Myc alone has been reported to cause an increase in DNA damage and activation of an ATM/ATR-Chk1 dependent checkpoint [14]. This is suggestive that a loss of proliferative capacity may follow, so we asked whether increasing MycER induction would cause a reduction in colony growth in our experimental assay. As Figure 3.2H shows, as the induction of MycER increases, colony number significantly decreases, however this was not seen following Δ MycER induction (Figure 3.2I), showing that Myc-mediated loss of proliferative capacity is dependent on the presence of the MBII domain. This indicates that normal non-cancerous cells cannot tolerate sustained high Myc overexpression.

We conclude that elevated Myc expression sensitizes normal and cancerous cells to replicative stresses provided by fork-stalling drugs, consistent with the ability of Myc to promote over-activation of CMGs from the pool of reserve MCMs necessary for recovery from such stresses.

Myc-Targeting Induces Large-Scale Chromatin Unfolding

The association of Myc binding at sites with histone modifications related to open and active chromatin led us to hypothesize that Myc may be promoting the assembly and activation of CMG helicases through chromatin unfolding and increased accessibility to the underlying DNA structure. This would also be consistent with our findings that elevated Myc is required in early to mid G1, prior to CMG helicase assembly, to elicit increased DNA replication. There are currently no technological means to assess higher-order chromatin structural changes at DNA replication origins. However, an innovative chromatin remodeling system (Figure 3.3A) utilizing a CHO-derived cell line (A03_1) has been successfully used to address whether proteins can elicit changes in chromatin state (condensed/closed, compared with decondensed/unfolded/open), with physiologically meaningful outcomes [140, 145, 146]. This system contains an engineered 90 Mb homogenous staining region (HSR), through the stable insertion and amplification of a Lac-operator (LacO)/DHFR vector also containing stretches of random intergenic DNA. The final HSR contains ~1,600 such vectors across 60 amplicons, and each amplicon is composed of ~500-kb of vector sequences adjacent to ~1-Mb of co-amplified genomic DNA [147]. Proteins of interest are fused with a LacI-DNA binding domain (DBD), which allows them to be targeted to

the HSR and visualized using anti-Lacl antibodies. The visual appearance of the HSRs are classified as closed (the usual unperturbed state), small open, large open (see Figure 3.3A and B for examples) or indeterminate, as previously described [140]. This system provides insight into regulation of higher-order chromatin dynamics that cannot be analyzed by any other current technological means.

The chromatin remodeling that occurs in this system is due to physiologically relevant events involved in altering chromatin structure by the targeted proteins. Other transcription factors have been tested in this system, such as BRCA1, E2F1 and VP16, and shown to facilitate chromatin unfolding through histone acetylation [145, 148]. A benefit of assessing Myc in this system is that it recapitulates several known physiological functions associated with Myc. Myc is a transcription factor that normally binds to DNA through its C-terminus to carry out its physiological functions, and when Myc is overexpressed as it often is in cancer, it binds to expansive non-promoter genomic regions. Myc has never been tested in this system before, and to determine whether Myc can promote large-scale chromatin decondensation, Myc and Δ Myc were fused to Lacl and transfected into the CHO (A03_1) cell line. As controls, Lacl-BRCA1 (6c-w), Lacl-VP16 and Lacl-DBD alone were expressed and assessed. Figure 3.3B shows example HSRs from the controls, showing the expected results that Lacl-DBD elicits no changes to the HSRs; they remain a small-condensed dot. Lacl-BRCA1 (6c-w) HSRs are mostly open (quantified in Figure 3.3D), and herein referred to as small HSRs, consistent with previous findings [145]. Lacl-VP16 targeting caused a large chromatin decondensation event as previously reported (Figure 3.3B) [148]. Targeting Myc to the HSRs resulted in a massive chromatin decondensation event (Figure 3.3C),

to the extent that Dapi staining in the HSR region was barely apparent due to the very low density of DNA in the center of the large decondensed 'halos'. These are herein referred to as large-open HSRs, to distinguish them from the open HSRs of VP16 and BRCA1. Quantification of chromatin unfolding capabilities (Figure 3.3D) demonstrates that Myc is more potent at decondensing chromatin than BRCA1 or VP16 [140]. Detailed analysis showed that ~70% of LacI-Myc targeted HSRs became large open, compared with ~15% of LacI- Δ Myc targeted HSRs (Figure 3.3E). The majority of LacI- Δ Myc HSRs (>50%) have failed to open and are classified as closed. LacI-BRCA1 (6c-w) also have ~70% open HSRs, however they are small open and look significantly different to LacI-Myc open HSRs (compare Figure 3.3B and C). The vast majority of LacI-DBD HSRs are closed as expected. LacI-DBD is expressed at higher levels than the other proteins, suggesting that the inability to open HSRs is not due to protein crowding effects [140]. Myc is a relatively weak transcription factor, but it is now the most powerful chromatin unfolding protein we have ever tested in this system.

Myc-Driven Chromatin Unfolding Requires MBII and Max Interactions

We next determined which functional domains in the Myc protein were required for Myc-driven chromatin unfolding. The targeting of Δ Myc (missing MBII domain) resulted in mostly closed HSRs (Figure 3.3E). A series of LacI-Myc mutants were created and compared quantitatively to wildtype Myc for their ability to promote chromatin decondensation. Loss of MBI, MBIIIa, MBIIIb and MBIV (but with the NLS intact) did not affect the ability of Myc to unfold chromatin (Figure 3.3F). However, loss of MBII renders Myc unable to induce chromatin unfolding, resulting in primarily closed

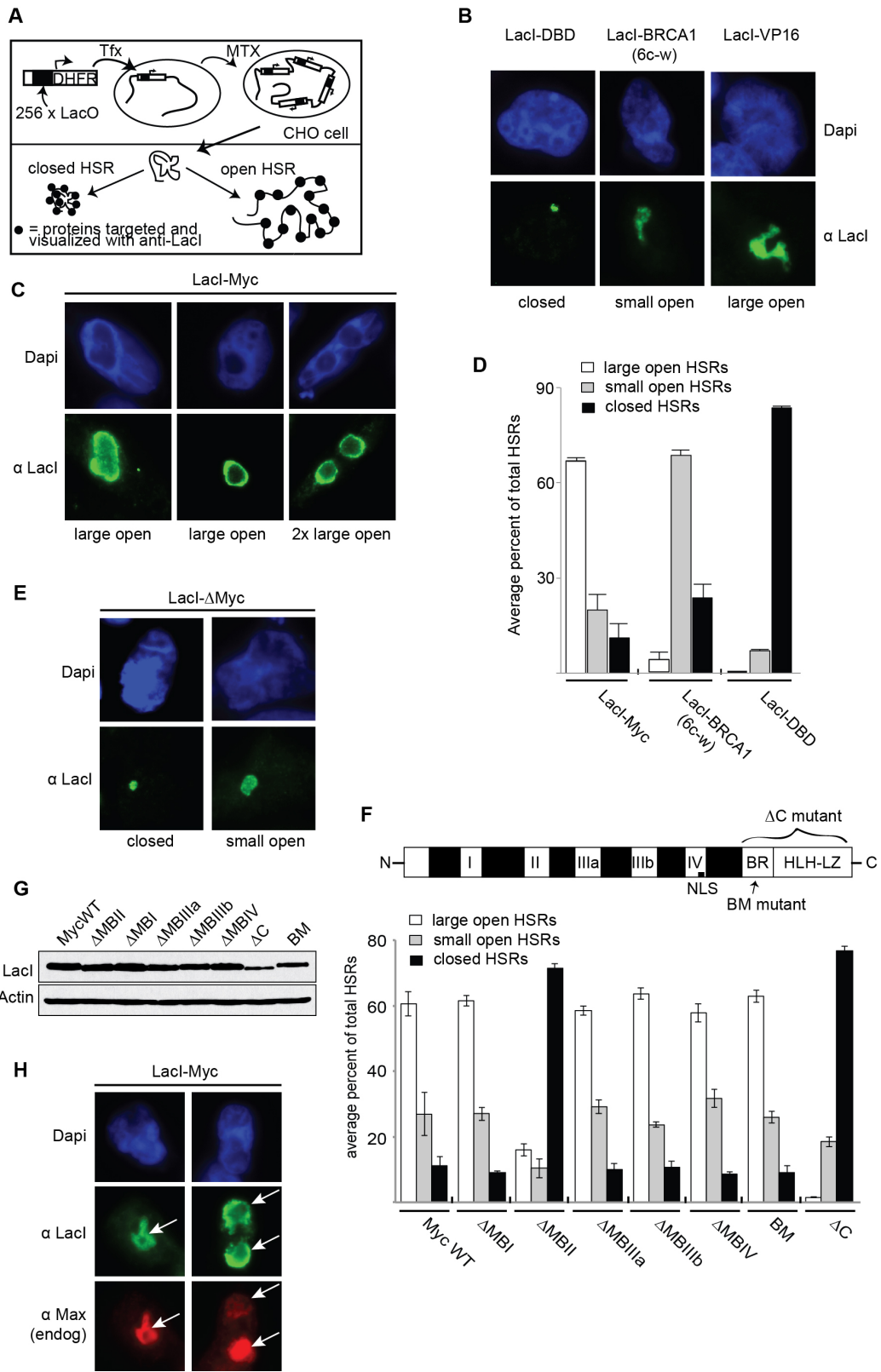


Figure 3.3. Myc-Targeting Induces Large-Scale Chromatin Unfolding.

Figure 3.3 (continued). (A) Schematic to describe the chromatin remodeling system utilized in the CHO (A03_1) cell line used in (B-H). (B) LacI-DBD, LacI-BRCA1 (6c-w) or LacI-VP16 were transiently expressed in CHO cells, followed by Immunofluorescence (IF) with anti-LacI and FITC to detect open or closed HSRs. Nuclei were stained with DAPI. (C) LacI-Myc was expressed and analyzed by IF to detect chromatin decondensation. (D) Graphical representation of effects of the indicated LacI fusion proteins on the ability to decondense chromatin. For each protein, at least 100 transfected cells were scored in triplicate, ± 1 SD. (E) Examples of LacI-Myc Δ MBII targeted HSRs. (F) LacI-Myc deletion (Δ) mutants and a basic region missense mutant (BM) were created and tested for their ability to decondense chromatin. At least 100 transfected cells were scored in triplicate, ± 1 SD. (G) Immunoblot of LacI-Myc mutants from panel (F). (H) Co-localization of endogenous Max with LacI-Myc large open HSRs.

HSRs. Mutation of the basic region site where Myc binds to DNA was not required for chromatin unfolding, but this is expected as we are targeting Myc to DNA through the LacI binding (Figure 3.3F). Deletion of both the basic region and the HLH-LZ Max binding domain (mutant Δ C) rendered Myc unable to decondense the HSRs. All proteins expressed similarly (Figure 3.3G). Endogenous Max is strongly recruited to Myc-unfolded HSRs (Figure 3.3H). These results suggest that Myc-Max interactions are involved in the mechanism underlying chromatin decondensation by Myc. We conclude from these results that targeting Myc to chromosomal regions in vivo causes a dramatic chromatin-unfolding event that is strongly linked to the presence of the MBII domain.

Myc-Induced Chromatin Unfolding Does Not Effect MCM Chromatin Distribution

We next asked whether chromatin decondensation by Myc promoted the assembly of CMG helicases by looking first at the MCM complex. Our group has previously published that chromatin unfolding by Cdt1 facilitates MCM recruitment to open HSRs through HBO1 recruitment [140]. This is a physiologically relevant result as

Cdt1 is involved in the loading of MCM complexes onto chromatin. Using this result as a positive control, we targeted Cdt1 to the HSRs and saw an enrichment of endogenous chromatin-bound Mcm7 to Cdt1-open HSRs (Figure 3.4A, left). We then targeted LacI-Myc, and in contrast to Cdt1, we did not see enrichment of endogenous chromatin-bound Mcm7 to Myc-open HSRs (Figure 3.4A, right). However, Mcm7 is present in open HSRs, but it is not enriched above the general chromatin stain seen throughout the nucleus. We looked for chromatin enrichment of another MCM subunit, Mcm4, in Myc open HSRs, and saw a lack of enrichment for endogenous Mcm4 (Figure 3.4B), but the presence of light Mcm4 staining, as seen with Mcm7. At the boundaries of the large-open HSRs MCM staining is brighter due to the higher density of chromatin. We previously showed in Figure 3.1H that there was a lack of Mcm2 enrichment on chromatin in the induced MK-MycER cell line at 15hrs (early-S phase), so we next asked whether this could also be seen by immunofluorescence. In synchronized MK-MycER cells induced at 0hr with 4-OHT and stained for Mcm2 at 15hrs we saw a *lack* of co-localization of MycER (i.e. the overexpressed Myc) with the tightly-bound chromatin complement of endogenous Mcm2 in 74% of the HSRs examined (Figure 3.4C, quantified in Figure 3.8). Quantification of several hundred cells across multiple fields was classified as high, moderate or low colocalization, and Mcm2 overlap with MycER was moderate 26% of the time. We conclude that targeting Myc to chromosomal regions to elicit chromatin unfolding does not result in the enrichment of the MCM complex. This finding is also consistent with other studies showing that Myc does not regulate MCM loading [14].

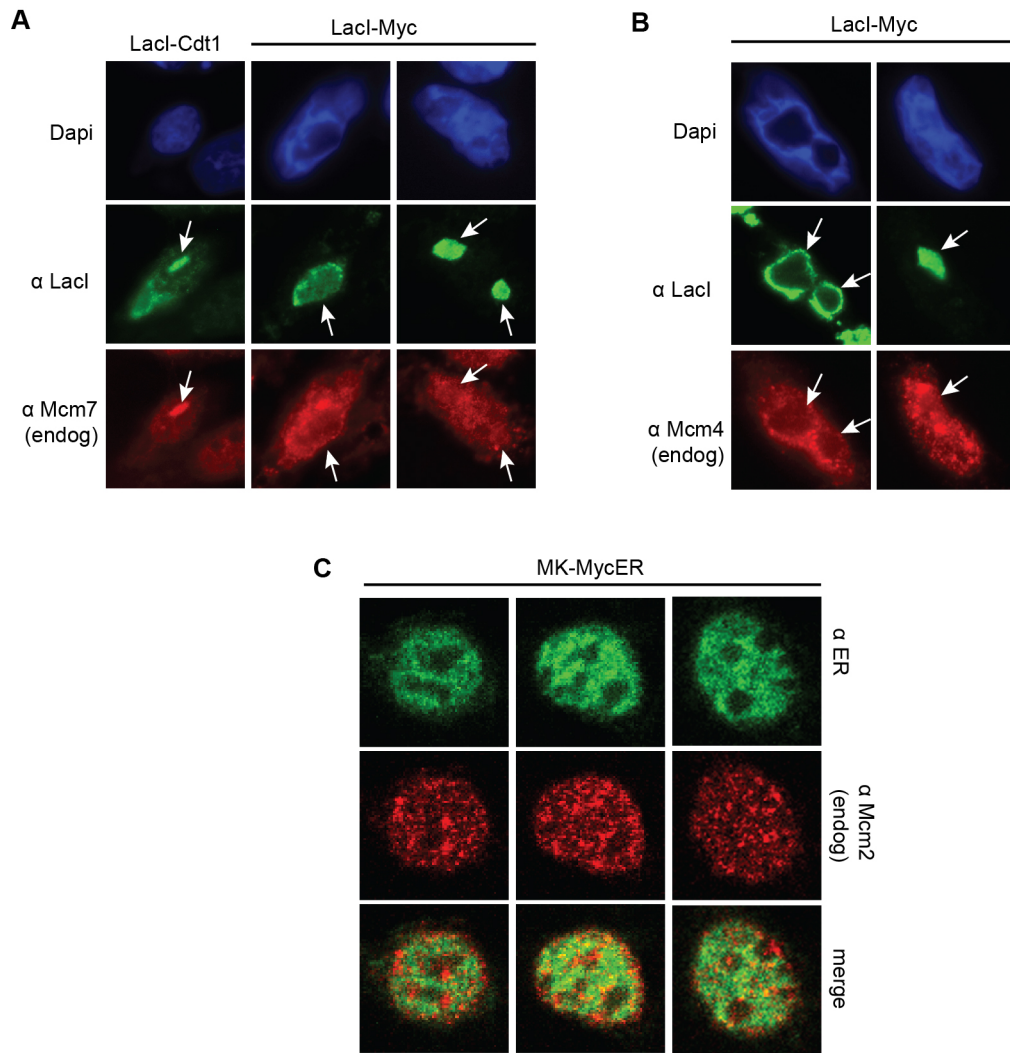


Figure 3.4. Myc Induced Chromatin Unfolding Does Not Effect MCM Chromatin Distribution. In all panels, transfected cells were pre-extracted with a Triton X-100 buffer to remove any proteins not tightly bound to chromatin. (A) Equal concentrations of Lacl-Cdt1 or Lacl-Myc were expressed in CHO cells, followed by IF with anti-Lacl/FITC and anti-Mcm7/Texas Red to detect endogenous (endog) Mcm7. Nuclei were stained with DAPI. White arrows indicate HSR region. Representative pictures of at least 50 transfected cells. (B) Lacl-Myc was expressed, followed by IF with anti-Lacl/FITC and anti-Mcm4/Texas Red to detect endogenous Mcm4. (C) Synchronized MK-MycER cells were treated with 4-OHT at EGF readdition (0hr), pre-extracted and fixed at 15hrs, followed by IF with anti-ER and anti-Mcm2 to detect endogenous Mcm2. Representative images from three fields, with experiment conducted in duplicate.

Chromatin Decondensation by Myc Stimulates CMG Helicase Assembly and Activation

While we have demonstrated a lack of MCM complex enrichment at Myc open chromosomal regions, MCM complexes are loaded onto chromatin in excess and only certain MCM complexes are chosen to become CMG helicases. Myc functions upstream of Cdc45 loading onto CMG helicases, although how and if Myc regulates Cdc45 loading in any direct manner is known. We asked whether chromatin decondensation by Myc promoted the recruitment of Cdc45 and GINS onto the MCM complexes to assemble CMG helicases, and whether these helicase then became active. The normal endogenous Cdc45 staining pattern is punctate and distributed evenly throughout the nucleus (example in Figure 3.5A). When Myc was targeted to HSRs and elicited a successful chromatin-unfolding event, chromatin-bound endogenous Cdc45 was dramatically enriched (Figure 3.5B). The Cdc45 staining has been depleted from the rest of the nucleus and the vast majority of the protein seems to be localized to the 90 Mb region of the decondensed HSR. Cdc45 is extremely rate-limiting for DNA replication and CMG helicase assembly [17], and Myc targeting to the HSR is sequestering the whole Cdc45 supply of the cell to one chromosomal region at the expense of the rest of the replicating DNA in the nucleus. We asked whether the presence of Myc alone at the HSRs caused co-localization of Cdc45 by analyzing the rare closed HSRs targeted by Myc. Cdc45 was not enriched at Myc-targeted closed HSRs (Figure 3.5C). Over 80% of HSRs targeted with Myc had Cdc45 enrichment compared with only 20% for Δ Myc (Figure 3.5D). The vast majority of Myc-targeted,

large-open HSRs were enriched for Cdc45 (>90%), with little to no enrichment of Cdc45 at small open or closed HSRs (Figure 3.5E). This demonstrates that robust chromatin-unfolding event is required to recruit Cdc45 to chromatin. Chromatin-bound Cdc45 is also dramatically co-localized with induced MycER in the synchronized MK cell line at 15hrs, as seen through significant spatial patterning overlap in nearly 100% of the analyzed cells (Figure 3.5F and quantified in Figure 3.8), compared with little to no overlap with Mcm2 in Figure 3.4C.

We asked whether chromatin unfolding elicited by other proteins also caused enrichment of Cdc45 to the HSR region. Two other transcription factors known to elicit chromatin unfolding, VP16 and BRCA1 (6c-w), were analyzed for Cdc45 enrichment. Neither LacI-VP16 opened HSRs (Figure 3.5G) or LacI-BRCA1 open HSRs (Figure 3.5H) caused co-localization with Cdc45. This demonstrates that Myc-specific chromatin decondensation is required for Cdc45 enrichment. Because Myc is a transcription factor, we reasoned that Cdc45 recruitment to unfolded HSRs could be due indirectly to LacI-Myc overexpression causing changes in the expression of an unknown protein that itself promotes Cdc45 binding to HSRs unfolded by any protein capable of causing chromatin decondensation. When a non-targeted HA-tagged Myc was co-expressed with either LacI-VP16 (Figure 3.5I) or LacI-BRCA1 (Figure 3.5J), any transcriptional effects Myc may be promoting did not cause Cdc45 to localize to the open HSRs of these targeted proteins. This demonstrates the requirement for the physical presence of Myc at chromosomal sites to promote Cdc45 enrichment, independent from its role as a transcription factor. We conclude that Myc-mediated chromatin decondensation at Myc-

targeted genomic sites is required for recruitment of the replication-limiting Cdc45 protein to chromatin.

We next asked whether Myc-targeted chromatin unfolding promoted the assembly and activation of functional CMG helicases. We looked to see whether the last member of the CMG helicase, the GINS protein complex, was enriched at Myc-open HSRs. We analyzed GINS by staining for one of its members Psf2, the staining pattern for which was evenly distributed throughout the nucleus (Figure 3.5K). As we found with Cdc45, chromatin-bound endogenous Psf2 was significantly enriched at Myc-open HSRs (Figure 3.5L). Approximately 70% of HSRs targeted with Myc had Psf2 enrichment (Figure 3.5M), and as with Cdc45, the vast majority Myc targeted large open HSRs had Psf2 enrichment (>80%), with only 30% enrichment at small open HSRs and no enrichment at closed HSRs (Figure 3.5N). Chromatin-bound endogenous Psf2 was also seen co-localized with induced MycER in the synchronized MK cell line at 15hrs in 83% of the analyzed cells (Figure 3.5O and quantified in Figure 3.8). We have demonstrated that Myc-induced chromatin decondensation promotes the assembly of CMG helicases, so we next asked whether these helicases become active through looking at RPA co-localization, which binds to single-stranded (and thus unwound) DNA, and BrdU incorporation (incorporated into newly synthesized DNA). The normal staining pattern for RPA is evenly distributed throughout the nucleus (Figure 3.5P).

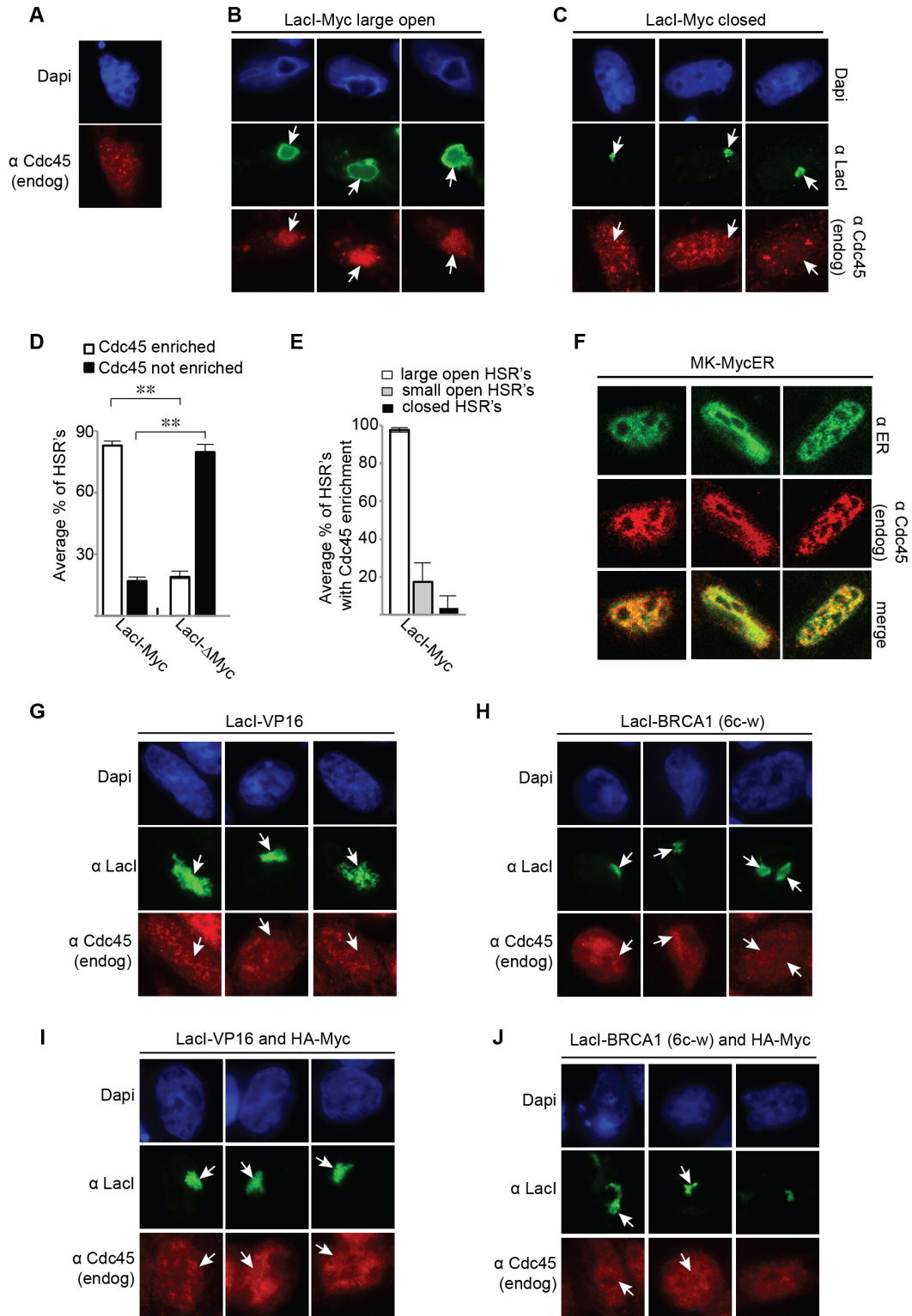


Figure 3.5. Chromatin Decondensation by Myc Stimulates CMG Helicase Assembly and Activation.

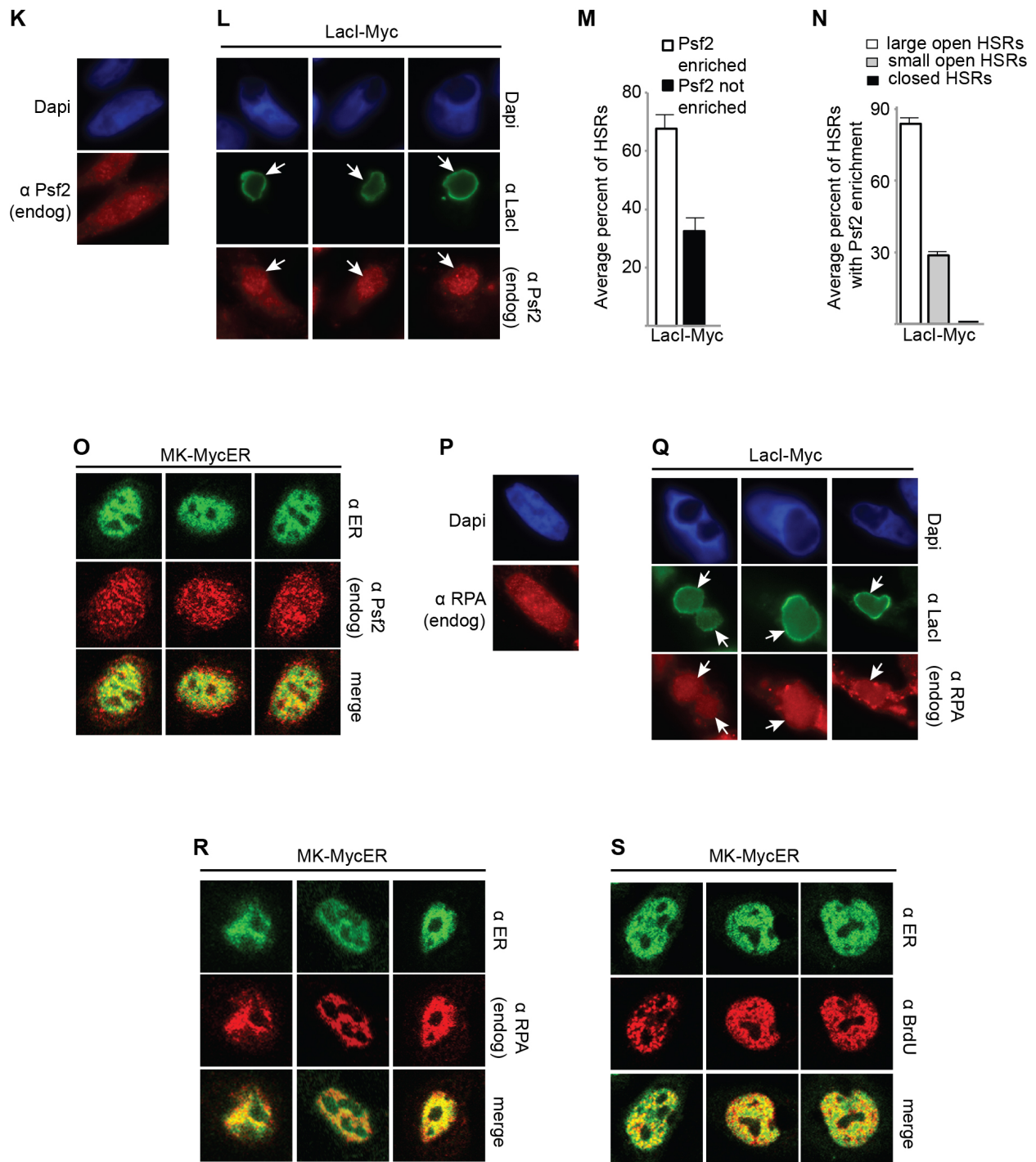


Figure 3.5 (continued). Chromatin Decondensation by Myc Stimulates CMG Helicase Assembly and Activation. CHO cells unless otherwise stated, equal concentration of LacI-proteins expressed. In all panels, transfected cells were pre-extracted with a Triton X-100 buffer to remove any proteins not tightly bound to chromatin. (A) Staining pattern of endogenous Cdc45.

Figure 3.5 (continued). (B) IF of LacI-Myc open and closed HSRs (indicated by arrows) with anti-LacI and anti-Cdc45 to show endogenous protein. (D) Quantification of endogenous Cdc45 recruitment to HSRs targeted by LacI-Myc or LacI- Δ Myc. At least 50 HSRs analyzed in triplicate with \pm 1SD. (E) Cdc45 recruitment in LacI-Myc expressing cells based on HSR size. (F) Synchronized MK-MycER cells induced with 4-OHT at 0hr and analyzed by IF at 15hrs with anti-ER and anti-Cdc45 to detect endogenous Cdc45. Representative images from three fields, with experiment conducted in duplicate. (G) LacI-VP16 open HSRs showing lack of Cdc45 enrichment. (H) LacI-BRCA1 (6c-w) HSRs showing lack of Cdc45 enrichment. (I) LacI-VP16 co-expressed with a non-targeted HA-Myc showing lack of Cdc45 enrichment at open HSRs. (J) LacI-BRCA1 (6c-w) co-expressed with a non-targeted HA-Myc showing lack of Cdc45 enrichment at open HSRs. (K) Staining pattern of endogenous Psf2. (L) Psf2 enrichment to LacI-Myc open HSRs. (M) Quantification of endogenous Psf2 recruitment HSRs targeted by LacI-Myc. (N) Psf2 recruitment in LacI-Myc expressing cells based on HSR size. (O) Synchronized MK-MycER cells induced with 4-OHT at 0hr and analyzed by IF at 15hrs with anti-ER and anti-Psf2 to detect endogenous Psf2. Representative images from three fields, with experiment conducted in duplicate. (P) Staining pattern of endogenous RPA. (Q) RPA enrichment to LacI-Myc open HSRs. (R) Synchronized MK-MycER cells induced with 4-OHT at 0hr and analyzed by IF at 15hrs with anti-ER and anti-RPA32 to detect endogenous RPA. Representative images from three fields, with experiment conducted in duplicate. (S) Synchronized MK-MycER cells induced with 4-OHT at 0hr and pulsed with BrdU at 15hrs, IF with anti-ER and anti-BrdU. Representative images from three fields, with experiment conducted in duplicate.

Chromatin-bound endogenous RPA was enriched at Myc-open HSRs (Figure 3.5Q) and was dramatically co-localized with MycER in 100% of induced MK-MycER cells fixed and analyzed in early-S phase (Figure 3.5R and quantified in Figure 3.8). We could not assess BrdU enrichment at Myc targeted HSRs, as no transfected cells were BrdU positive. This could be due to two reasons. The first is that none of the cells we observed were in S phase and so would not be BrdU positive. However, this is unlikely since over 300 HSRs were examined, whereas for RPA staining (another indicator of S phase) we observed ample enrichment. The second, more plausible explanation is that the LacO-LacI binding affinity is too strong to allow the replication machinery to progress through large chromatin regions and replicate DNA, however, the initial DNA

unwinding can occur as indicated by RPA enrichment. We could however observe BrdU co-localization with MycER in close to 100% of the induced MK-MycER cells assessed in early-S phase (Figure 3.5S and quantified in Figure 3.8), as previously reported [14]. We conclude that chromatin decondensation by specifically by Myc promotes both CMG helicase assembly and activation.

GCN5 and TIP60 Facilitate the Ability of Myc to Decondense Chromatin and Recruit Cdc45

Myc has been shown to bind to genomic sites with histone H3 and H4 methylation and acetylation marks, associated with active and open chromatin, and promote further acetylation of histone H4 tails [82]. Myc recruits the HATs GCN5 and Tip60 to chromatin, and we asked if these potential mediators of Myc functions *in vivo* played a functional role in the mechanism behind Myc-driven chromatin accessibility and CMG formation [86, 88].

We reasoned that histone H4 modifications played a role in Myc-induced chromatin unfolding. We co-expressed Myc with the Set8 histone methylase H4 binding domain (Set8-HBD) (Figure 3.6A) which binds to histone H4 tails and blocks their post-translational modifications, such as acetylation. As seen in Figure 3.6B, co-expression of Set8-HBD significantly reduced the ability of Myc to elicit chromatin decondensation, through an overall shift towards a less-unfolded chromatin state. This suggests that H4 acetylation modifications are likely involved in Myc-driven chromatin unfolding. Myc interacts *in vivo* with the HATs GCN5 and Tip60 through the MBII domain. Myc open HSRs showed enrichment of chromatin-bound endogenous GCN5 and Tip60, but not

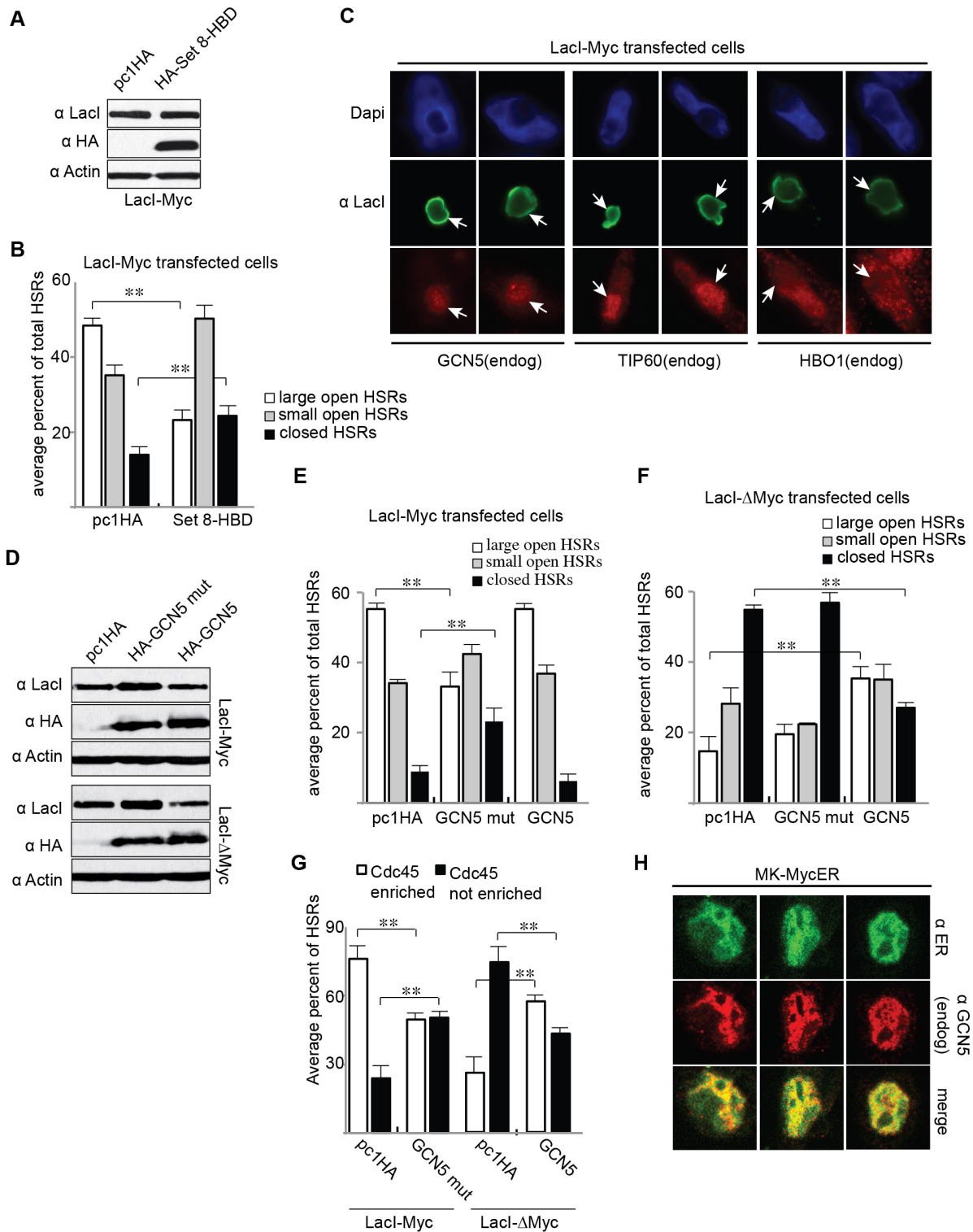


Figure 3.6. GCN5 and TIP60 Facilitate the Ability of Myc to Decondense Chromatin and Recruit Cdc45.

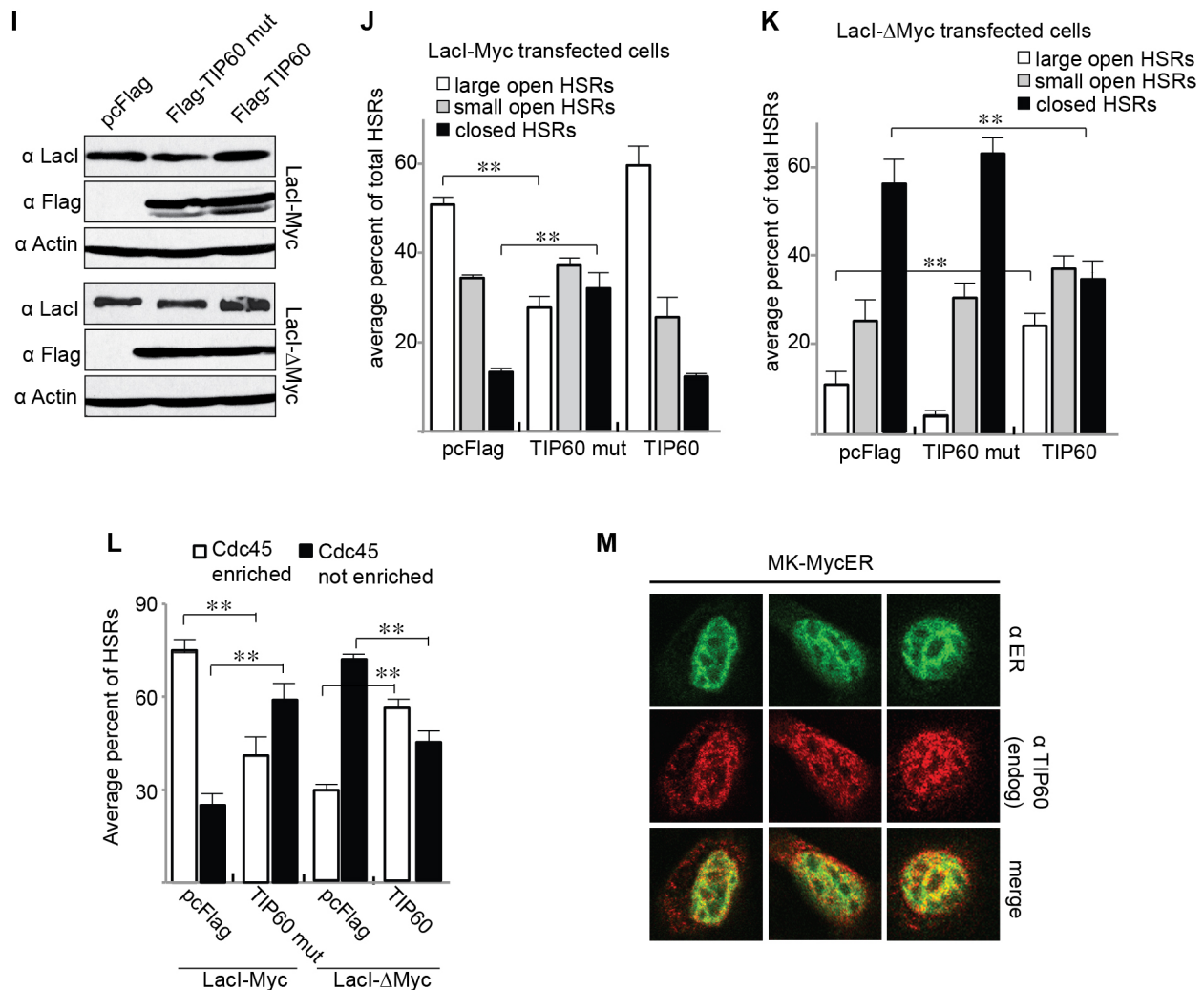


Figure 3.6 (continued). GCN5 and TIP60 Facilitate the Ability of Myc to Decondense Chromatin and Recruit Cdc45. (CHO cells unless otherwise stated) (A) IB of co-transfected Lacl-Myc and Set 8-HBD in a 1:1 ratio. (B) Quantification of the results from (A). (C) Lacl-Myc open HSRs stained with anti-GCN5, anti-TIP60 or anti-HBO1 to detect endogenous proteins. (D) IB of Lacl-Myc or Lacl-ΔMyc cells co-transfected with GCN5 or GCN5 mutant, ratio of 1μg Lacl protein: 2μg GCN5. Quantification of the effects of co-transfection of GCN5 or GCN5 mutant on Lacl-Myc (E) or Lacl-ΔMyc (F) HSR size. At least 100 transfected cells were scored in triplicate, ±1SD. Representative of at least two experiments with similar results. (G) Quantification of endogenous Cdc45 recruitment to HSRs targeted by Lacl-Myc or Lacl-ΔMyc in the presence of GCN5 or mutant. (H) Synchronized MK-MycER cells analyzed by IF at 15hrs with anti-ER and anti-GCN5 to detect endogenous GCN5. (I) IB of Lacl-Myc or Lacl-ΔMyc cells co-transfected with Tip60 or Tip60 mutant, ratio of 1μg Lacl protein: 2μg Tip60. Quantification of the effect of co-transfection of Tip60 or Tip60 mutant on Lacl-Myc (J) or Lacl-ΔMyc (K) HSR size.

Figure 3.6 (continued). (L) Quantification of endogenous Cdc45 recruitment to HSRs targeted by Lacl-Myc or Lacl- Δ Myc in the presence of Tip60 or Tip60 mutant. (M) Synchronized MK-MycER cells analyzed by IF at 15hrs with anti-ER and anti-Tip60 to detect endogenous Tip60.

HBO1, a HAT not associated with Myc-mediated acetylation (Figure 3.6C). This demonstrates specificity for GCN5 and Tip60 recruitment by Myc.

We next asked whether GCN5 and Tip60 were required for Myc-driven chromatin unfolding and Cdc45 recruitment. We co-expressed Lacl-Myc or Lacl- Δ Myc with GCN5 or a catalytically dead mutant GCN5 (Figure 3.6D). Expression of mutant GCN5 with Lacl-Myc produced a significant shift toward condensed HSRs, whereas expression of wild-type GCN5 did not alter the chromatin opening dynamics (Figure 3.6E). The ability of Myc to open chromatin is potent and it might not be possible to make it any better by adding additional GCN5, however, a mutant GCN5 which can interact with the MBII domain may be competing with endogenous GCN5 and preventing successful chromatin decondensation by Myc. Expression of mutant GCN5 with Lacl- Δ Myc did not produce further closed HSRs, however, the expression of wild-type GCN5 facilitated the opening of HSRs, thus overcoming the deficiency of MBII loss (Figure 3.6F). We next asked how these results affected Cdc45 recruitment to HSRs, as a measure of CMG helicase assembly. We found that expression of mutant GCN5 with Lacl-Myc negatively affected the number of Myc-targeted HSRs with Cdc45 recruitment, and wild-type GCN5 facilitated Cdc45 recruitment to Δ Myc-targeted HSRs (Figure 3.6G). Chromatin-bound endogenous GCN5 was also seen co-localized with induced MycER in the synchronized MK cell line at 15hrs in 100% of the analyzed cells (Figure 3.6H and

quantified in Figure 3.8). We next co-expressed LacI-Myc or LacI- Δ Myc with Tip60 or a catalytically dead mutant Tip60 (Figure 3.6I). Expression of mutant Tip60 with LacI-Myc caused a shift toward condensed HSRs, whereas expression of wild-type Tip60 produced no change (Figure 3.6J), as seen with GCN5. Expression of mutant Tip60 with LacI- Δ Myc had no effect, however, the expression of wild-type Tip60 produced an overall shift towards the opening of LacI- Δ Myc targeted HSRs, and thus, like GCN5, overcame the deficiency of MBII loss (Figure 3.6K). Expression of mutant Tip60 with LacI-Myc negatively affected the number of Myc-targeted HSRs with Cdc45 recruitment, and wild-type Tip60 facilitated Cdc45 recruitment to Δ Myc-targeted HSRs (Figure 3.6L), as with GCN5. Chromatin-bound endogenous Tip60 was also seen co-localized (although less dramatically than GCN5) with induced MycER in the synchronized MK cell line at 15hrs in 96% of the analyzed cells (Figure 3.6M and quantified in Figure 3.8). We conclude that the ability of Myc to elicit chromatin decondensation and Cdc45 recruitment is dependent on histone H4 modifications mediated through the recruitment of the HATs GCN5 and Tip60 to chromatin through the MBII domain.

Myc and the MBII Domain are Required in Late G1-Phase for Cdc45 Recruitment to Chromatin

Myc is an immediate-early gene product that is required for the transcriptional induction of many early-G1 gene targets important for cell cycle progression into late-G1. Cdc45 loading onto MCM complexes during CMG helicase assembly occurs in late-G1 in mammalian cells released from quiescence [21]. While we have demonstrated that overexpressed Myc causes Cdc45 recruitment to chromatin, leading to excessive

CMG helicase activation, it is unknown whether normal levels of endogenous Myc, and specifically MBII function, are required in late-G1 at the time that Cdc45 normally loads (~8hrs in MK cells). We wanted to retain the ability of Myc to induce early-G1 gene transcription, and this is likely important for Cdc45 loading and many other late-G1 events, so we designed an experimental strategy (Figure 3.7A) in which synchronized cells (MK-MycER and MK- Δ MycER) were released into G1 and at the same time were transfected with siRNA targeting Myc or Luciferase. We predicted that there would be a delayed loss of Myc protein level so that Myc would be present in early G1 for gene activation, but would be absent by late-G1. Figure 3.7B shows that endogenous Myc was indeed present in early-G1, but became progressively lost by late-G1. Although Δ MycER protein expression was not affected by si-Myc treatment, MycER protein levels were partially suppressed at later intervals (Figure 3.7B, 6hr and 8hr time points). However, even under these conditions the MycER protein level is higher than the endogenous Myc level by late-G1, and is high enough to rescue S phase entry, as seen in Figure 3.7C. To test whether reduction of endogenous Myc affects transcription, we looked at the protein level of monocarboxylate transporter 1 (MCT1), an established Myc target gene [149]. This did not alter with endogenous Myc depletion (Figure 3.7B), indicating that the early-G1 transcriptional effects elicited by Myc were still intact. Delayed loss of endogenous Myc protein resulted in a block to S phase entry (Figure 3.7C), consistent with Myc function being required in late-G1 for entry into S phase [14]. However, activation of MycER (Figure 3.7C left) during si-treatment rescued the ability of the cells to enter S phase, but in contrast, activation of Δ MycER did not (Figure 3.7C

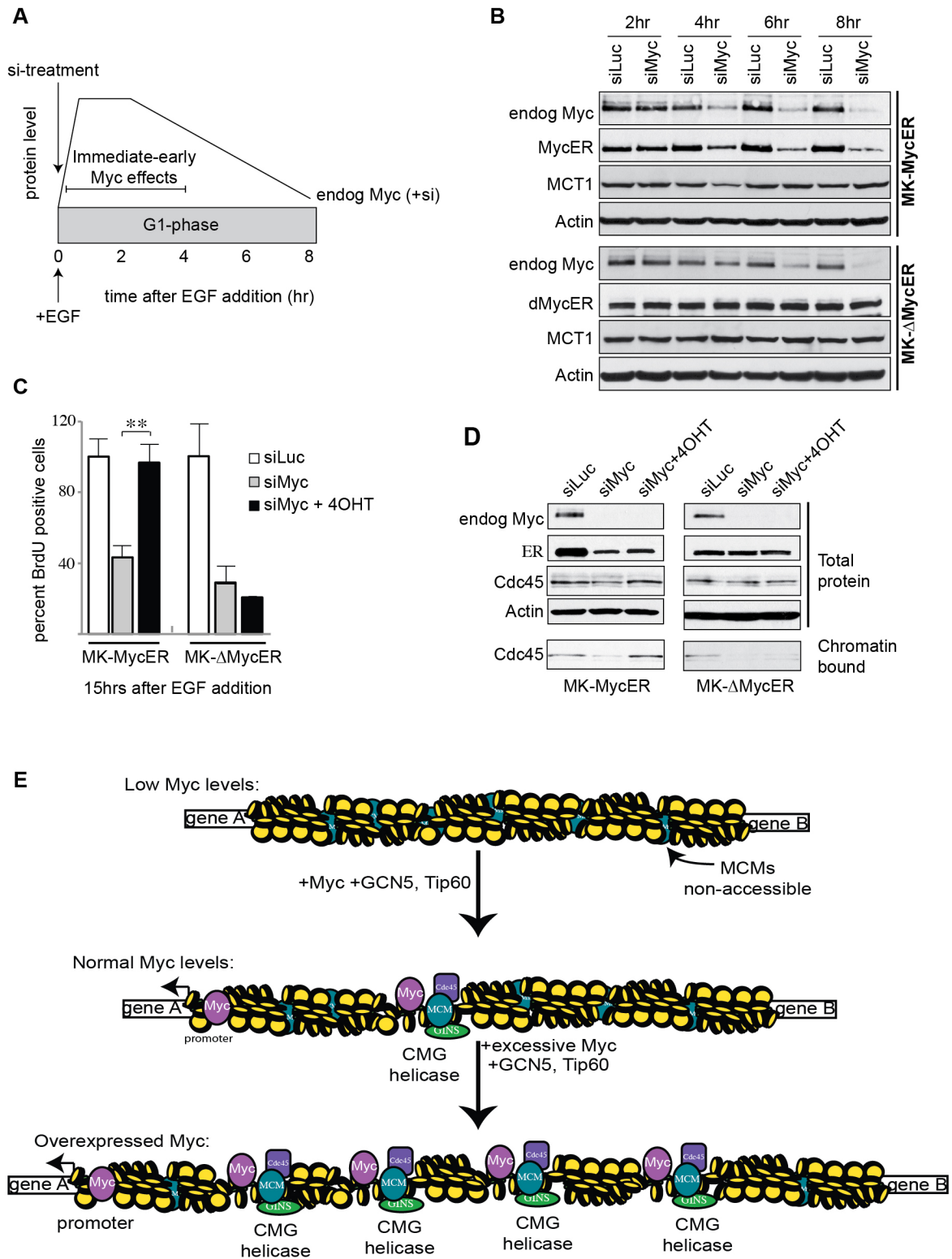


Figure 3.7. Myc and the MBII Domain are Required in Late G1-Phase for Cdc45 Recruitment to Chromatin.

Figure 3.7 (continued). (A) Experimental design for panel (B) showing graphical representation of expected rate of endogenous Myc loss. (B) MK-MycER and MK- Δ MycER were synchronized, and at time of EGF re-addition transfected with siRNA against siLuciferase (control) or siMyc. Immunoblot probed with the indicated antibodies at the indicated time points. (C) MK-MycER and MK- Δ MycER were synchronized, and at time of EGF re-addition transfected with siRNA against siLuciferase or siMyc, \pm 4-OHT activation. Average number of BrdU positive cells at 15hr normalized to percentage of siLuciferase control. Three fields analyzed \pm 1SD. (D) IB analysis total protein expression and chromatin-bound complement of the indicated proteins at 15hrs in synchronized MK-MycER and MK- Δ MycER treated with si-RNA at time of EGF re-addition. (E) Our model of how Myc-induced chromatin unfolding is required for CMG helicase assembly and activation.

right). We next asked whether loss of endogenous Myc by late-G1 affected the ability of Cdc45 to load onto chromatin. In the absence of MycER activation, suppression of endogenous Myc inhibited Cdc45 loading, although did not affect total Cdc45 protein expression (Figure 3.7D, left). However, activation of MycER rescued Cdc45 loading onto chromatin. This rescue was dependent on the presence of the MBII domain, as activation of Δ MycER did not rescue Cdc45 loading onto chromatin (Figure 3.7D, right). We conclude that there is a late-G1 dependence on endogenous normal Myc levels for Cdc45 chromatin loading in order to assemble CMG helicases, which is dependent upon the presence of the MBII domain. Figure 3.7E shows our model of how Myc stimulates CMG helicase assembly and activation. At low Myc levels, chromatin is condensed and MCM hexamers are inaccessible. At normal Myc levels, Myc is bound to genomic promoter and intergenic regions, and creates chromatin accessibility through GCN5 and Tip60 recruitment allowing access of the MCM complexes to form CMG helicases. At overexpressed Myc levels Myc increasingly binds to intergenic regions, where it creates more CMG helicases.

Protein co-localized	High co-localization	Moderate co-localization	Low co-localization
Mcm2	0% (\pm 0%)	26% (\pm 2%)	74% (\pm 2%)
Cdc45	71% (\pm 2%)	28% (\pm 2%)	1% (\pm 1%)
Psf2	22% (\pm 4%)	61% (\pm 3%)	17% (\pm 3%)
BrdU (early S)	92% (\pm 2%)	7% (\pm 3%)	1% (\pm 2%)
BrdU (mid-late S)	none detected		
RPA(early S)	95% (\pm 5%)	5% (\pm 5%)	0% (\pm 0%)
RPA(mid-late S)	0% (\pm 0%)	62% (\pm 11%)	38% (\pm 11%)
GCN5	95% (\pm 1%)	5% (\pm 1%)	0% (\pm 0%)
TIP60	71% (\pm 5%)	25% (\pm 5%)	4% (\pm 1%)

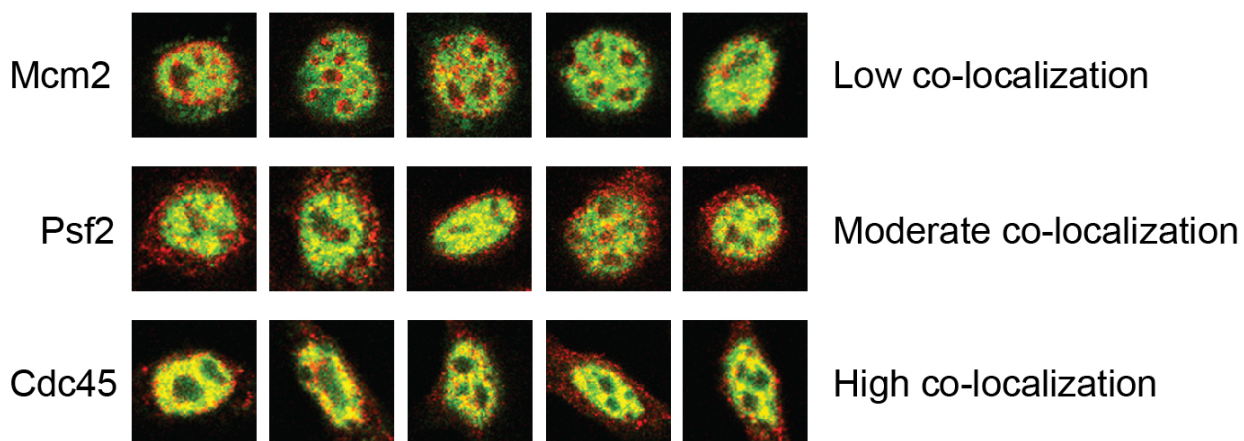


Figure 3.8: Quantifications of the Protein Co-Localizations in Synchronized Myc-Induced MK-MycER cells. Cells were pre-extracted with Triton-X100, analyzed in S-phase at 15hr. Average of three fields \pm 1SD. IF examples of co-localization categories (low, moderate or high co-localization) for Mcm2, Psf2 and Cdc45.

Discussion

Myc is deregulated through amplification or constitutive activation in a large variety of cancers and promotes oncogenesis through its role as a transcriptional regulator of genes involved in cell cycle progression, metabolism, differentiation and apoptosis. Myc deregulation can promote genomic instability, likely contributing to Myc-driven tumorigenesis. Part of the genomic instability incited by Myc is due to a non-transcriptional role in regulating the initiation of DNA replication. Myc is required for S phase initiation, and overexpressed Myc causes excessive DNA replication due to overactivation of CMG helicases [14, 78]. This overactivation produces DNA damage, which could lead to the genomic instability incited by Myc. The mechanisms behind how Myc stimulates excessive DNA replication through replicative helicase activation remains poorly understood, and likely involves both transcriptional and non-transcriptional Myc-driven events. The oncogenic effects of Myc on the replication machinery likely involve upregulation of genes necessary for helicase assembly and activation, such as cyclin-cdk complexes, proteins involved in the loading of helicase subunits, or the helicase subunits themselves [67, 80]. In this study we have provided evidence for a novel mechanism for Myc mediated control over DNA replication initiation. Myc creates higher order chromatin accessibility, which promotes CMG helicase assembly and activation. The ability of Myc to regulate chromatin structure could bridge the transcriptional and non-transcriptional roles for Myc in promoting oncogenesis and normal cell cycle progression.

Myc binds to a large number of genomic sites, both at normal and overexpressed levels. While many of these sites are in the expected promoter regions of Myc-target genes, a significant proportion are at large distances from transcriptional start sites in intergenic regions, and this proportion increases when Myc is overexpressed [63, 64, 122]. Intergenic regions are where the start sites for DNA replication occur, and could explain in part Myc's presence there [13]. Myc targeting to genomic sites correlates with pre-existing and subsequent chromatin modifications to these regions [139]. However, the molecular and regulatory nature of these Myc-associated chromatin modifications is not clear. To investigate how Myc functions to influence CMG helicase activation, and whether chromatin modifications were involved in this mechanism, we utilized an innovative engineered system that allows us to recapitulate many of the characteristics of Myc, such as overexpression and intergenic DNA binding.

We discovered that for a relatively weak transcription factor, Myc is incredibly robust at inducing large-scale chromatin decondensation at targeted genomic sites. This chromatin modification required the highly conserved MBII domain of Myc, and involves Max interactions. Two HATs that bind to Myc through the MBII domain and are known physiological mediators of Myc-regulated processes, GCN5 and Tip60, are required for Myc-mediated chromatin unfolding. To understand Myc's effects on DNA replication we looked at the recruitment of the CMG helicase members, and found that Myc-driven chromatin decondensation stimulates the massive recruitment of GINS, and Cdc45, which is extremely rate-limiting for mammalian helicase assembly and activation (see Figure 3.9). Myc does not influence the distribution of MCM hexamers, but promotes the formation of CMG helicases on the pre-loaded MCMs, which leads to helicase activation

at Myc targeted genomic regions. This Myc-driven helicase assembly and activation is dependent on the chromatin decondensation induced specifically by Myc being targeted to the region. Our results correlate with other studies showing that Myc overexpression leads to a higher number of active helicases per genomic region, increasing the replication origin density and activation that creates DNA damage [78]. We have demonstrated that normal levels of Myc also regulate Cdc45 recruitment during the cell cycle, indicating that the elevated helicase activation following Myc deregulation is a derivative of normal Myc function towards Cdc45 loading and this has become over stimulated.

The wide distribution of Myc binding sites throughout the genome coupled with Myc's ability to cause potent chromatin unfolding over great distances provides an intriguing model that explains the genomic influences of Myc (Figure 3.7E). Chromatin unfolding by Myc stimulates CMG helicase activity in intergenic regions at a distance by causing the chromatin accessibility that allows helicase assembly on the intergenic MCM hexamers in the vicinity of Myc interactions. Myc interactions in the vicinity of promoters will likely be associated with chromatin decondensation that stimulates additional protein interactions that lead to transcriptional activation of Myc-target genes. Overexpressed Myc would be able to expand its influences over genomic regions, promoting a larger amount of chromatin decondensation that may alter transcriptional activation or repression of additional genes or chromosomal processes, which may not normally be under direct Myc control in an unperturbed state.

The ability of overexpressed Myc to induce chromatin accessibility and stimulate excessive CMG helicase activation has important tumorigenic implications. An excess

of MCM hexamers are loaded onto chromatin in mammalian cells than are needed for a normal S phase initiation and completion. The excess MCM complexes act as backups/reserves that become new CMG helicase under conditions of replicative stress, which stall replication forks [97]. This allows replication to be completed and maintains genomic stability. The reserve MCM complexes are also necessary for a normal, unperturbed S phase to complete without DNA damage occurring [43]. The reserve MCMs become activated when replication forks stall due to normal S phase stresses such as challenging topological issues or heterochromatin. Overexpression of Myc causes the premature usage of these reserve complexes, converting them into active CMG helicases to create smaller inter-origin distances [78]. However, this effectively reduces the reserve complement, creating a deficiency in the ability of cells to recover DNA replication following replicative stresses such as normal S phase conditions, or from chemotherapy treatment, which indirectly stalls replication forks (Figure 3.9). A Myc-driven lack of reserve MCM complexes could be an event that occurs early in Myc-overexpressed tumorigenesis, causing the accumulation of undetected DNA damage that drives the oncogenic phenotype. Overexpressed Myc at any stage of tumorigenesis would exacerbate genomic instability and promote additional chromosomal changes arising from the unresolved DNA damage.

Myc-driven reduction in reserve MCM complexes could explain two paradoxical findings regarding Myc overexpression in human cancers. Tumors with high Myc expression are associated with poor survival rates and an aggressive phenotype [150], and yet such cancers also respond better to chemotherapy regimens involving drugs that cause replication forks to stall [142, 143, 151]. We have presented evidence that

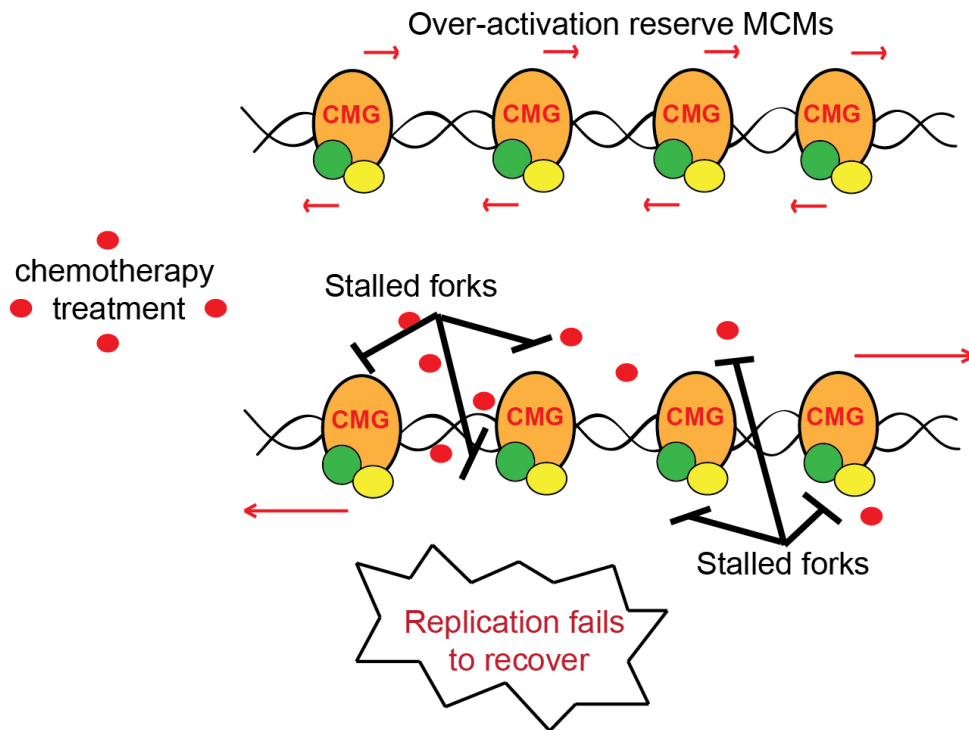


Figure 3.9: Myc Stimulation of CMG Helicase Assembly and Activation Causes a Reduction in the Reserve MCM Complexes. Overexpressed Myc levels cause an over-usage of MCM complexes, converting them into active CMG helicases. When chemotherapy treatments cause replication forks to stall in S phase, there are no reserve MCM complexes to function as new origins, and thus DNA replication fails to recover, promoting DNA damage, which could lead to cell death or genomic instability.

shows acutely elevated Myc sensitizes tumor cells to replicative inhibitors such as gemcitabine. As explained above, the loss of reserve MCMs due to overexpressed Myc promotes DNA damage and genomic instability that are hallmarks of tumor progression,

providing a reason for Myc-driven tumor aggressiveness and poor prognosis. Also the same lack of reserve MCMs due to Myc overexpression means tumor cells are deficient in recovering from the replicative stress of chemotherapy drugs, providing an explanation for the better response to clinical intervention of Myc overexpressed tumors.

CHAPTER FOUR: CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

We hypothesized that the efficacy of the clinically relevant drugs gemcitabine and 5-FU against pancreatic cancer cells, and oxaliplatin and etoposide against colorectal cells, could be increased through co-suppression of the reserve MCM complexes. Using siRNA targeting of MCM subunits, depletion of the reserve MCM complexes caused chemosensitization of multiple human tumor cell types to several chemotherapeutic drugs used in the clinical management of human cancer. This was due in part to an inability of such cells to recover DNA replication following drug treatment, which lead to an increase in apoptosis. Depletion of reserve MCM complexes did not sensitize a non-transformed epithelial cell line to the same doses of gemcitabine that a PDAC cell line was sensitized to. This argues for the development and use of anti-MCM drugs in combination with chemotherapeutic compounds, which has the potential to increase the therapeutic index of existing clinical compounds whilst minimizing side effects on normal cells.

The oncogene *c-MYC* also leads to the mismanagement of CMG helicases in part due to a non-transcriptional role in overactivating replication origins and causing

DNA damage. We sought to elucidate the mechanism by which Myc causes overactivation of CMG helicases. We have identified a previously unknown role for Myc in normal cell cycle progression whereby Myc targeting to genomic sites induces decondensation of higher-order chromatin structure that is required for the assembly and activation of CMG helicases at reserve, accessible MCM hexamers, and overexpression of Myc creates excessive helicase activation due to further Myc-driven unfolding. Our results also provide new mechanistic insight into Myc oncogenic transformation in which overstimulation of DNA replication could result in genomic instability and provide an explanation for Myc driven oncogenic transformation.

Future Directions

Genomic Locations of CMG Helicase Proteins

A group published the genomic mapping sites of Cdc6 and Mcm7 [152]. This group presented unpublished data at a Cold Spring Harbor meeting stating that they followed up this study by classifying the Cdc6 and Mcm7 pre-RC sites into firing origins and dormant origins. They found good correlation between firing pre-RCs and c-Myc binding, and that firing origins have open chromatin structure (Sugimoto and Fujita 2015, unpublished data). This data supports my project and poses other questions, such as what is the correlation of c-Myc binding sites with the genomic binding sites of other CMG helicase proteins, especially Cdc45. If Cdc45 binding sites and c-Myc binding sites overlap or are in a similar vicinity, this would support our work in chapter 3 showing co-localization of Cdc45 and Myc using immunofluorescence. Very little is

known about how Cdc45 is loaded onto chromatin, and no experimental assay has linked Myc and Cdc45 as being direct interactors. It would be interesting to elucidate the recruiting and loading factors of Cdc45 onto DNA, and whether any of these factors directly interact with Myc.

Direct Myc Interactions with the CMG Helicase

Myc has been found in complexes with members of the pre-RC proteins, including Cdt1, Cdc6, ORC and MCM hexamers [14]. Co-immunoprecipitation using anti-Myc also revealed interactions between Myc and Mcm3, 5 and 7, and Cdc6 [14]. It is not known whether Cdc45 and the GINS proteins are also in these complexes, as when this study was conducted the full complement of the replicative helicase was unknown. An approach using tandem affinity purification with mass spectral multidimensional protein identification technology (MudPIT) identified 221 c-Myc associated proteins, of which only 17 were previously known interactors. Of particular interest to this project is that Mcm7 was identified as a c-Myc interactor, and the interaction of Myc with Mcm7 depended on the presence of the MBII domain [79]. The C-terminal of Mcm7 was presumed to interact with Myc, as deletion of amino acids 1-432 did not interfere with the association with Myc [79]. Preliminary data we collected also support these findings, as when we expressed ectopic HA-tagged Myc or Δ Myc and Flag-tagged Mcm7 CT (C-Terminus) and performed an anti-HA co-immunoprecipitation, we could detect Mcm7-CT only with wild type Myc, and not Δ Myc missing the MBII domain (Figure 4.1). We also saw that full length endogenous Mcm7 was co-precipitated with both Myc and Δ Myc, suggesting there may be other interaction

sites or interaction partners between these proteins than just the MBII domain (Figure 4.1, bottom).

However, whether Myc makes a direct physical interaction with these proteins is still uncertain, as there may be additional proteins that are forming complexes with Myc

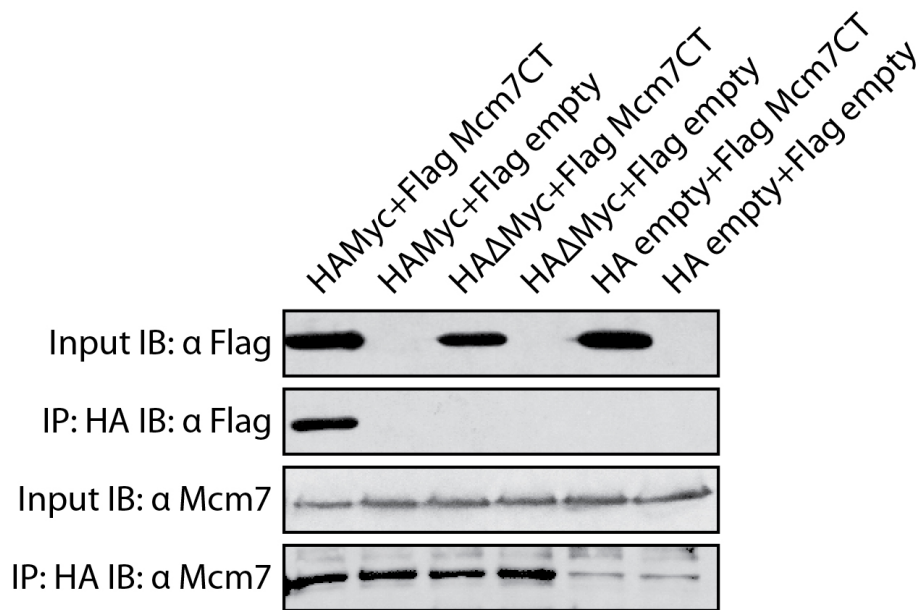


Figure 4.1: Direct Interaction of the MBII Domain of Myc with the C-Terminus of Mcm7. In HEK293T cells (human embryonic kidney cells), equal concentrations of HA-tagged Myc or Δ Myc co-transfected with equal concentrations of Flag-tagged Mcm7-CT (C-terminal 137 amino acids) or empty vector. Immunoprecipitation (IP) with anti-HA antibody, immunoblot (IB) with anti-Flag to detect Mcm7-CT or anti-Mcm7 to detect full length endogenous Mcm7.

and proteins such as Mcm7. To ascertain this in future studies, it would be of interest to purify bacterially expressed Myc, the mutant Δ Myc, and the individual CMG helicase

proteins, incubate them together, and determine whether they co-immunoprecipitate in the absence of any other proteins that may be acting as an intermediate binding partner. Our group previously used this approach to successfully demonstrate that the N-terminal of Rb physically interacts with the C-terminal of Mcm7 [40]. This finding is intriguing, as both Myc and Rb are binding to the same region of Mcm7, yet elicit opposite effects on cell proliferation and DNA replication. Future studies could utilize the cell-free *Xenopus* extracts to determine whether the Myc-Mcm7-CT interaction is necessary for DNA replication to occur. Bacterially purified Mcm7-CT could be titrated into the Myc-rich *Xenopus* extracts, and the DNA replication rate measured via ³²-P incorporation. If the Myc-Mcm7 interaction is necessary for replication to occur, increasing the amounts of the competitive binder Mcm7-CT will cause replication rates to decrease. It has been demonstrated that adding Myc into this system causes a dose-related increase in DNA replication [14]. Pre-incubation of Myc with Mcm7-CT may prevent this increase. Collectively, these future studies would strengthen the position that Myc is directly involved in the CMG helicase.

The MCMs and Cancer

The expression levels of the MCM complex have to be carefully regulated and balanced to prevent genomic instability, a hallmark of cancer often caused by replicative stress [153]. Recent high-throughput sequencing studies have found that in 10% of lung squamous cell adenocarcinomas at least one *MCM* gene is amplified [154]. Overexpression of Mcm7 in mouse epidermal tissue predisposed the animals to develop squamous cell carcinomas quicker and more frequently when exposed to

carcinogens than their normal littermates [155]. Reduction of Mcm2 levels in mice causes lymphomas and thymomas [156]. Point mutations in *MCM* genes are relatively common in tumors, in a study of 178 tumor genomes 12% of lung squamous cell carcinomas were found to contain at least one point mutation [154]. Single nucleotide polymorphisms in *MCM* genes are commonly observed in normal human genomes, at least some of which when recapitulated in budding yeast cause genomic instability, suggesting these may generate genomic instability in susceptible individuals [157]. There is a list of *MCM* cancer alleles mutations in the cBioPortal (<http://www.cbioportal.org>), many of which are found in the conserved ATPase motifs, which may generate MCM complexes that are biochemically defective and lead to reduced DNA replication [153].

From the indicated studies, it appears that both overexpression of the MCMs, consistent with an oncogenic role, and underexpression of the MCMs, consistent with a tumor suppressive role, are linked to cancer development [153]. As we showed in Chapter 2, underexpression of MCMs likely leads to reduced DNA replication. Areas of non-replicated DNA could lead to DNA damage and genomic instability which drive tumorigenesis. Overexpression of MCMs could be causing inappropriate protein-protein interactions that may be titrating out blockers of proliferation, such as overexpression of Mcm7 binding to and blocking the ability of Rb to inhibit the CMG helicase [42]. Or overexpression could affect the stoichiometric balance of the Mcm subunits, leading to nonproductive complexes at the expense of active complexes [153]. In our work from Chapter 3, we argued that overactivation of MCM complexes is causing an increase in origin activity, which could leading to cancer formation due to decreased levels of

reserve MCM complexes. Overexpression of Mcm subunits at the genetic level could also lead to this fate. In all these overexpression/over usage scenarios, the end result is either direct or indirect cellular proliferation, which drives tumorigenesis [153].

Current CMG Helicase Drugs

To date only a few inhibitors have been developed that target the initiation of DNA replication, and the development of drugs to target the MCM complex offers an intriguing avenue for novel cancer treatments [153]. At least three different classes of small molecule helicase inhibitors could be developed for use as chemotherapeutic agents. The first class is enzymatic inhibitors that inhibit the MCM complexes ATPase activity, either through blocking initiation of the unwinding of DNA, or the elongation of the replication fork [153]. The drug heliquinomycin has been shown to inhibit the unwinding properties of the Mcm467 sub-complex, although this may be through an indirect mechanism through interactions with single-stranded that blocking the unwinding of DNA [158]. The fluoroquinolone ciprofloxacin inhibits MCM helicase activity *in vitro*, but whether this is direct, indirect or specific is not known [153]. The second class of inhibitors would be compounds that disrupted physical or genetic interactions between CMG helicase subunits and other proteins necessary for its activation [153]. The third class of inhibitors would be molecules that modulate *mcm* gene expression levels, to either block cell proliferation or return it to normal levels [153]. Trichostatin A, a HDAC inhibitor, reduces *MCM2* gene expression and causes apoptosis in colon cancer cells [159]. Widdrol causes downregulation of *MCM* levels

indirectly due to the compound causing double-stranded DNA breaks, which activates an ATM/ATR checkpoint leading to *MCM* level reduction and growth inhibition [160]. The mentioned drugs are indirect inhibitors of the MCM complex. The development of specific helicase inhibitors is still in its early days. Since we have shown in Chapter 2, and others have shown, that reducing MCM protein levels sensitizes cells to other replication inhibitors, such specific anti-MCM inhibitors could increase the efficacy of existing chemotherapeutics against cancerous cells.

CMG Helicase Purification and Drug Development

Most of the mechanistic work to date on the MCM complex has been performed in yeast or *Drosophila* MCM complexes, due to the ability to purify the CMG helicases from these organisms in large quantities [153]. No high throughput biochemical screens have been conducted on the mammalian MCM complex or CMG helicase due to the fact that these have only recently been purified in small quantities, and large quantities are needed to perform drug screens of inhibiting compounds [153]. There is a strong evolutionary conservation between the *MCM* genes that makes it likely that information from lower eukaryotes may also apply to human DNA replication [153].

In Chapter 3 we established that Myc is causing assembly and activation of CMG helicases on open chromatin. Another way to test whether Myc is causing an increase in the number of CMG helicases formed is to purify the CMG helicase in the presence or absence of Myc. Each member of the human CMG helicase is created through a viral vector that is used to infect SF9-insect cells, which then create large quantities of each individual protein. One or two of the proteins can be tagged for purification, for example,

in our laboratory we tag Cdc45 to allow for purification of fully formed helicases. If a Myc virus is also used to infect the insect cells along with the other 11 viruses, we could establish if more CMG helicases are purified in the presence of Myc compared to the absence of Myc by using the Cdc45 tag to compare the amount of Cdc45 not in complexes to that in complexes with helicase subunits. A mutant Δ Myc virus could also be made to establish whether this does not cause more helicases to form, as we suspect from our findings in Chapter 3. Caveats to this experimental approach are that other Myc cofactors are probably necessary for Myc to assemble helicases, and the insect homologues of these proteins may not be similar enough to the human proteins to help assemble human helicases.

To test whether Myc has a direct effect on CMG helicase activation, once the 11 member complex is purified, human Myc purified protein can be added to the helicase activity assay to assess whether it helps the with the unwinding of the oligonucleotide substrate. As only purified helicase proteins and Myc would be present in the reaction, this would show whether Myc directly influences helicase activity, and the mutant Δ Myc could be tested to see if it has no effect or is a negative regulator of helicase activity. Although these might be interesting avenues to explore, our work in Chapter 3 eludes to Myc having a more indirect role in helicase assembly and activation through chromatin modifications, which these assays are not well equipped to test.

Our results from Chapter 2 indicate the need to develop specific anti-helicase/MCM complex drugs for use in combination chemotherapy regimens. Due to the hexameric organization of the MCM complex, it might prove difficult for cells to develop drug resistance if multiple ATPase sites are targeted simultaneously, allowing

for increased efficacy of an anti-MCM drug cocktail [153]. Our group recently published a study showing that exon 7 in the N-terminus of Rb directly inhibits DNA replication through inhibition of fully formed CMG helicases. By using smaller peptides of exon 7 we hope to find an inhibitor of the helicase that mimics Rb's functions and can be used in drug development to create an anti-helicase compound for use in cancer patients. We are also purifying the human CMG helicase complex to run large-scale drug screening to identify novel inhibitors of its function. As we saw in Chapter 2, inhibition of the reserve MCM complexes could preferentially sensitize cancer cells to existing chemotherapeutic agents whilst minimizing the side effects on normal cells, and would thus be an ideal anti-cancer therapy but able to target multiple tumor types.

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