The Mechanisms and Consequences of DNA Re-replication

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ABSTRACT

DNA replication is a key event during cellular proliferation. In order to maintain genetic stability, cells have evolved different mechanisms to ensure the precise duplication of chromosomes and prevent DNA re-replication. The regulation of DNA replication initiation is critical for preventing re-replication. In mammalian cells, stabilization and activation of the replication initiator Cdt1 leads to DNA re-replication. In this dissertation, I examine the consequences of the re-replication.

The single stranded DNA (ssDNA) initially generated during re-replication activates an ATR/Chk1 mediated pathway and arrests cells in G2/M phase. The checkpoint is essential for the accumulation of re-replicated cells, which can further activate ATM/Chk2, p53, and apoptosis. Our study suggests that cells can have a chance to repair relatively minor DNA damage caused by microscopic re-replication, and only induce apoptosis through the later acquisition of double strand breaks and activation of Chk2 and p53 when re-replication persists. We also identified HDAC6 deacetylase as a new player in re-replication induced checkpoint pathways, presumably by regulating Chk1 protein level and phosphorylation, directly or indirectly.

MLN4924, a new anti-cancer drug, stabilizes Cdt1 and causes re-replication in a variety of human cancer cells. Transient exposure of MLN4924 is sufficient to induce re-replication, which activates checkpoint pathways, apoptosis, and senescence, contributing to the anti-proliferative effect of MLN4924 in cancer therapy. Intriguingly, unlike other

DNA damaging agents used for chemotherapy, p53-negative cells remain susceptible to MLN4924 induced cell death, suggesting an important clinical application.

Although the consequences of re-replication, such as apoptosis and senescence, can be used for killing cancer cells, re-replication itself may lead to gene amplification and tumorigenesis. However, we found no evidence of this deleterious effect in normal breast epithelial cells or breast cancer cells, most likely because re-replication-induced checkpoint, apoptosis, and senescence acted as insurmountable barriers to tumorigenesis.

TABLE OF CONTENTS

I. ABSTRACT

1.0 INTRODUCTION 1

1.1 OVERVIEW OF DNA REPLICATION INITIATION IN EUKARYOTIC CELLS 2

1.1.1 Assembly of Pre-Replicative Complex 3

1.1.2 The Transition to DNA Replication in S Phase 6

1.2 NEGATIVE REGULATION OF THE PRE-REPLICATIVE COMPLEX COMPONENTS 11

1.2.1 Regulation of The Cdt1 Protein 11

1.2.2 Regulation of The Cdc6 Protein 14

1.2.3 Regulation of The Origin Recognition Complex 15

1.3 THE CONSEQUENCES OF PRE-REPLICATIVE COMPLEX DEREGULATION 16

1.3.1 The Consequences of The Cdt1 Protein Deregulation 16

1.3.2 The Consequences of The Cdc6 Protein and The Origin Recognition Complex Deregulation 17

1.3.3 Ways to Induce DNA Re-replication in Eukayotic Cells 17

1.4 THE CONSEQUENCES OF DNA RE-REPLICATION 19

1.4.1 DNA Damage Checkpoint Activation Upon Re-replication 19

1.4.2 The Activation of Apoptosis and Senescence Pathway 22

1.4.3 The Connection Between DNA Re-replication and Gene Amplification 23

1.5 REFERENCES 26

1.6 APPENDIX 46

2.0 ATR PATHWAY IS THE PRIMARY PATHWAY FOR ACTIVATING G2/M CHECKPOINT INDUCTION AFTER RE-REPLICATION 77

2. 1 PAPER..... 78

2.2 REFERENCES 91

2.3 FIGURE LEGENDS 95

2.4 FIGURES 98

3.0 NEDD8-TARGETING DRUG MLN4924 ELICITS DNA RE-REPLICATION BY STABILIZING CDT1 IN S PHASE, TRIGGERING CHECKPOINT ACTIVATION, APOPTOSIS, AND SENESCENCE IN CANCER CELLS..... 104

3.1 PAPER..... 106

3.2 REFERENCES 125

3.3 FIGURES LEGENDS 131

3.4 FIGURES 135

3.5 SUPPLEMENTARY MATERIALS..... 142

4.0 UNPUBLISHED WORK 149

4.1 HDAC6, A NEW PLAYER IN THE G2/M CHECKPOINT PATHWAY INDUCED BY RE-REPLICATION 150

4.1.1 INTRODUCTION 150

4.1.2 RESULTS 151

4.1.3 DISCUSSION 159

4.2 DNA RE-REPLICATION AND GENE AMPLICATION 161

4.2.1 INTRODUCTION 161

4.2.2 RESULTS 163

4.2.3 DISCUSSION 170

4.3 MATERIALS AND METHODS 172

4.4 REFERENCE 176

5.0 CONCLUDING REMARKS 183

5.1 SUMMARY 184

5.2 OUTSTANDING QUESTIONS IN THE FIELD 185

5.2.1 RE-REPLICATION, DNA DAMAGE RESPONSE, AND SENESCENCE PATHWAY 186

5.2.2 RE-REPLICATION AND GENE AMPLIFICATION 187

5.2.3 RE-REPLICATION AND TUMORIGENESIS 188

5.3 FUTURE PLANS 190

5.4 REFERENCE 192

CHAPTER ONE

INTRODUCTION

CHAPTER ONE: INTRODUCTION

It is vitally important for cells to maintain genome integrity and genetic stability during the cell cycle. Failures to do so can result in disastrous consequences, causing various birth defects, many developmental abnormalities, cancer and a range of genetic diseases (1). The regulation of DNA replication initiation is one of the important mechanisms to guarantee the precise duplication of chromosomes. Recent research in the field has shed light on how replication initiation proteins are negatively regulated to prevent re-initiation and re-replication within the same cell cycle (2). The deregulation can lead to DNA re-replication in different systems. Furthermore, it is suggested that the deregulation can increase DNA breaks, chromosomal fusions and aneuploidy (2-4). However, it is not clear whether these consequences are directly associated with DNA re-replication.

1.1 OVERVIEW OF DNA REPLICATION INITIATION IN EUKARYOTIC CELLS

The initiation of DNA replication in eukaryotic cells is a three-step process. The first two steps include the recognition of replication origins and the recruitment of the

presumptive DNA helicase MCM2-7 (minichromosome maintenance 2-7) complex to form the pre-RC (pre-replicative complex). The third step is the loading of additional proteins to form pre-IC (pre-initiation complex), and the activation of MCM2-7 helicase, both requiring CDK (cyclin-dependent kinase) and Cdc7/DDK (Dbf4-dependent kinase) activities.

1.1.1 Assembly of pre-Replicative complex

In eukaryotic cells, DNA replication is initiated at many different areas called replication origins. The assembly of pre-RC consists of two-steps: the recognition of replication origins by the ORC (origin recognition complex) and the recruitment of downstream factors, such as Cdc6, Cdt1 and Mcm2-7, during late M and G1 phase.

In budding yeast *Saccharomyces cerevisiae*, an origin, also named as ARS for autonomously replicating sequence, is composed of a highly conserved 11-bp ACS (ARS consensus sequence) and several poorly conserved B domains (5-7). These origins are repeatedly used in an efficient way in successive cell cycles. They are recognized and bound by the initiator protein, a six-subunit complex called ORC (8).

The sequence that ORC (initiator protein) binds is significantly different between various eukaryotic organisms. In fission yeast *Schizosaccharomyces pombe*, an origin is a 1kb long ORC-binding DNA sequence containing A-T rich element (9, 10). No consensus sequence element has been identified in the origins. Moreover, they exhibit less efficiency and internal redundancy compared to *S. cerevisiae* origins (11).

The situation is less clear and more complicated in metazoan cells. Until recently, there were only about 20 well-defined replication origins identified so far, such as origin II/9A in the fly *Sciara coprophila*, chorion DNA replication origins in *Drosophila* follicle cells, the human lamin B2 origin or the Chinese hamster DHFR (dihydrofolate reductase) origins (12-15). Among these, only the lamin B2 origin acts as a single specific initiation site (16). The other origins like c-myc, rDNA, or DHFR origins, contain broad initiation zones of up to >55kb sequence and containing multiple inefficient initiation sites (17-21). Although deletion of specific sequences in those sites can abolish origin activity *in vitro*, no consensus sequences have been found (6, 14, 22). Therefore, there is no clear evidence that ORC binds to any essential *cis*-acting DNA elements for origin activity.

Recent advances in technology such as DNA microarrays and deep sequencing have made it feasible to map replication origins in a genome-wide scale with high resolution. These studies, consistent with earlier results, have suggested that DNA replication in metazoans initiates at specific regions, but the specificity may not be determined simply by sequence, but by other factors such as transcription regulatory factor and DNA or chromatin structure (23-26).

Despite the lack of sequence dependence in higher eukaryotes, the ORC proteins were found highly conserved in all eukaryotes studied, from *S. cerevisiae*, *Xenopus laevis* to *Mus musculus*, *Homo sapiens* (27-32). So, what are the functions of ORC proteins? As an initiator protein, ORC can recognize origins and bind to DNA (33). The ORC-DNA interaction is sequence- and ATP- dependent in yeast (8). ORC1-5 are all related to the super-family of AAA^+ ATPases (ATPases Associated with various cellular Activities)

(34). Of the five proteins, only the ATP-binding activity of ORC1 is required for DNA binding though not all of them have ATP binding motifs (32). However, little sequence specificity is found to affect ORC loading onto DNA in metazoan (35). Instead, DNA topology was shown to affect the loading (36). Moreover, ATP seems not to influence the binding process. Despite these reports, the mechanism for DNA binding by ORC and origin selection is still vague in metazoan.

Studies in various organisms have shown that chromatin association of ORC is required for Cdc6 and Cdt1 loading (32, 37-39). In *S. cerevisiae*, upon the loading of Cdc6 protein, which is also an AAA^+ ATPase, the ORC1 ATPase is activated and the ORC-Cdc6-DNA changes its conformation to increase the association (32, 40). The loading of Cdt1 and MCM2-7 proteins is dependent on Cdc6 ATPase (41). A Cdt1-MCM heptamer is proposed to be loaded on ORC-Cdc6-DNA origin (42). In *S. cerevisiae* Cdt1 directly interacts with ORC6 to facilitate MCM2-7 chromatin association (which is salt sensitive) (43). After initial MCM2-7 binding, Cdt1 and Cdc6 dissociate from origins upon ATP hydrolysis of Cdc6, and MCM2-7 helicase gets stably loaded at DNA (which is salt resistant) (Fig. 1) (41). The loading of MCM2-7 complex is then repeated and there are about 10-20 MCM2-7 complexes loaded per origin in both yeast and animal cells (44-46). The excessive loading of MCM2-7 complexes may protect cells from replication stress (47). It has been shown recently that although MCM2-7 forms a single hexamer in solution, once loaded on DNA it can form stable, head-to-head double hexamers with globular shape (2). With DNA passing through the central channel, MCM2-7 can slide along DNA passively. Although MCM2-7 recruitment is only dependent on ORC, Cdc6

and Cdt1 in yeast, higher eukaryotes may utilize additional proteins for the loading of MCM2-7. For example, Mcm9, a metazoan specific MCM2-7 homologue, was shown to aid Cdt1 in loading MCM2-7 in *Xenopus* (48). In human cells, the histone acetyl-transferase Hbo1 has a similar function (49). However, *in vitro* data also suggests that both of the proteins are not required factors, but may only facilitate the loading. Overall, ORC serves as a foundation to recruit Cdc6, Cdt1 and subsequently Mcm2-7 to form the pre-replicative complex and to initiate DNA replication (Fig. 1) (32, 33, 38, 50, 51).

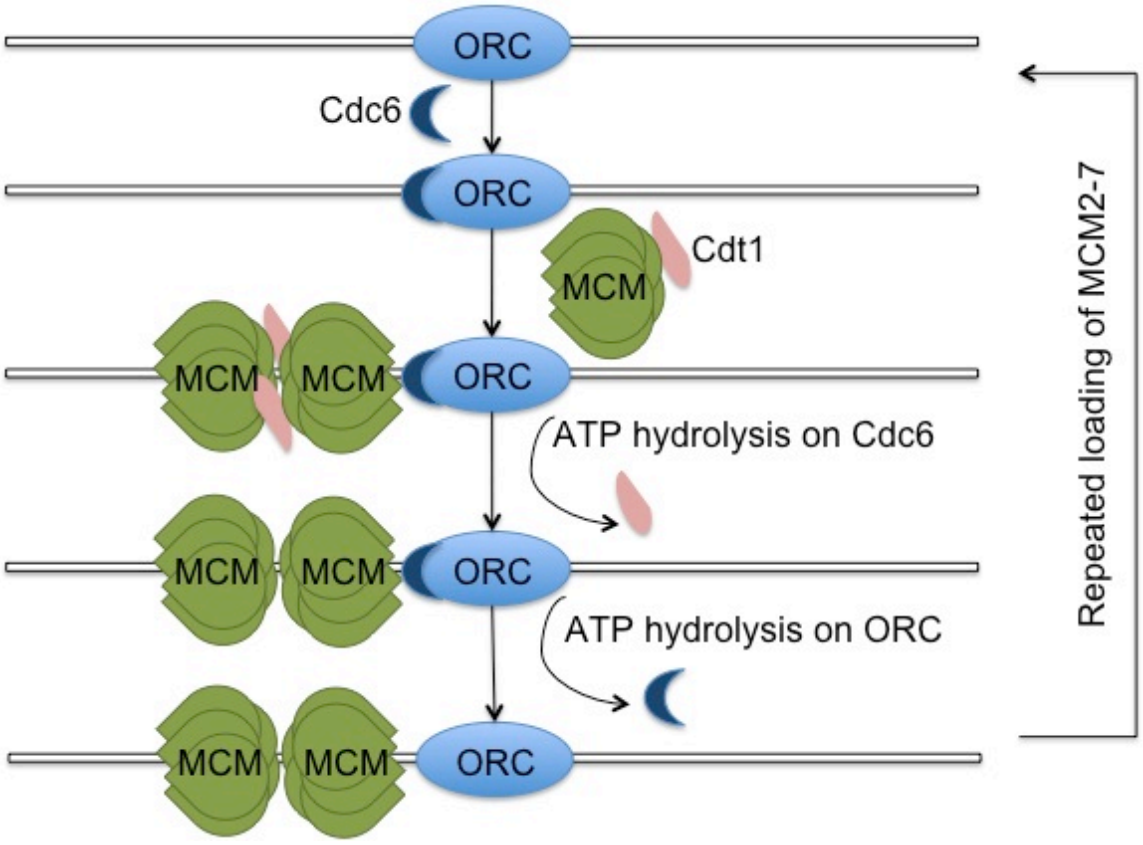

Figure 1. The assembly of pre-RC. ORC (origin recognition complex) binds to potential origin sequences in M or G1 phase. Cdc6 is subsequently recruited by ORC, which further recruit Cdt1 and MCM (separately or in complex). ATPase activities of Cdc6 and ORC are required for

the dissociations of Cdt1 and Cdc6, respectively, which stabilize MCM chromatin binding and complete the pre-RC assembly. The MCM complexes can be repeated loaded.

1.1.2 The transition to DNA replication in S phase

Pre-RC bound origins are potential sites for DNA replication initiation. A variety of proteins or protein complexes must bind to the origins to form pre-IC, activate MCM2-7 complex and initiate DNA replication. The progression to this step requires S phase CDK/DDK activities.

It has been shown that intact MCM2-7 complexes lack DNA helicase activity *in vitro* (52-54). However, recent research has found that Mcm2/5 interface might act like an ATP-dependent "gate". After "gate" closure under certain reaction conditions, MCM2-7 helicase activity can be reconstituted *in vitro* (55). As discussed previously, excess MCM2-7 hexamers are loaded on origins in G1 phase. The complex is not active until Cdc45 and GINS (Go-Ichi-Ni-San) binding (44, 56-60). It is possible that the association of Cdc45 and GINS helps to stabilize Mcm2/5 interface "closure" structure and turns on MCM2-7 helicase activity. Other proteins involved in the activation process include MCM10, Sld3, Sld2, Dpb11 and And-1. Several important replication initiation proteins or protein complexes will be further discussed below.

- **Mcm10**

  Mcm10, also named DNA43, was first identified in two independent screens. DNA43 was identified in a screen for mitotic DNA synthesis errors,

whereas Mcm10 was identified in the mini-chromosome maintenance screen that isolated the members of MCM2-7 complex (61, 62).

Mcm10 mutants were shown to interact with MCM2-7 complex, Cdc45 and subunits of DNA polymerase ε and δ (63, 64). Work in *S. pombe* and *Xenopus* egg extracts has shown that Mcm10 is loaded onto origins in an MCM2-7 dependent manner and the loading itself is necessary for Cdc45 binding (65, 66). Mcm10 is also required for the recruitment of DNA polymerase α primase to chromatin (67). In *S. cerevisiae*, Mcm10 can similarly be recruited by MCM2-7 before Cdc45, and more intriguingly, it can bind to origins after Cdc45 in a complex with DNA pol α primase (68). Furthermore, the degradation of yeast Mcm10 leads to instability of DNA pol α though this is not the case with human Mcm10 (68, 69). The functions of Mcm10 in *Xenopus* egg extracts and human cells will be further discussed below.

Taken together, Mcm10 plays an important role in DNA replication. However, many details related to the mechanism still remain unclear.

- **And-1/Ctf4**

Ctf4 was first identified in *S. cerevisiae* in a chromosome transmission fidelity screen (70). It was shown to interact with DNA pol α although the gene is not essential for viability (71, 72). Mcl1 is Ctf4 homolog in *S. pombe*, and it is essential for *S. pombe* viability and genome integrity (73). Similar to Ctf4, Mcl1 can also physically interact with DNA pol α. However, the exact role of Ctf4/Mcl1 was not clear until the recent work on And-1.

And-1 is the human homolog of Ctf4. It was identified more than ten years ago. But little has been known about its function until recently (69, 74). And-1/Ctf4/Mcl1 was shown to be a new replication initiation factor that can link helicase with DNA polymerase. And-1 is first loaded onto chromatin in a Mcm10 dependent manner. The Mcm10-And-1 interaction is further required for the recruitment of DNA pol α subunit p180 and replication initiation. It was mentioned earlier that Mcm10 degradation led to DNA pol α instability in *S. cerevisiae*. In human cells, And-1 is used instead of Mcm10 to maintain DNA pol α stability. Taken together, And-1 serves as a critical factor downstream of Mcm10 and plays an important role in replication initiation.

- **Cdc45**

Cdc45 was originally identified in a screen for cold-sensitive mutants that affect the cell cycle (75). As mentioned earlier, chromatin loading of Cdc45 requires Mcm10 loading (65, 66). Cdc45 can also be loaded in G1 phase but the stable binding between MCM2-7 and Cdc45 only happens in S phase (76-78). Once loaded, Cdc45 can move with replication fork and is required for both DNA replication initiation and elongation (56, 79).

The function of Cdc45 might be separated into two parts. The first function is to form a CMG (Cdc45-MCM2-7-GINS) complex to support the helicase activity (80). Cdc45 can interact with MCM2-7 complex and probably serve as a helicase cofactor (59, 81). The second function is to recruit DNA Pol α onto chromatin. Cdc45 was shown to interact with DNA pol α in vitro (82, 83). It

was shown that chromatin loading of DNA pol α depended on Cdc45 loading in *Xenopus* egg extracts (82). In addition, Cdc45 is also needed for RPA (single strand binding protein complex) loading. Indeed, Cdc45 and RPA are loaded onto chromatin in an inter-dependent manner (76). The co-existence of Cdc45 and RPA on chromatin may further support helicase activity and help origin unwinding (59, 84, 85).

Both functions of Cdc45 may be regulated by another protein called Sld3. Sld3 is similarly required for replication initiation and forms a complex with Cdc45. Sld3 mutant inhibits Cdc45 and Mcm2 interaction in *S. cerevisiae* (78). In *S. pombe*, Sld3 is suggested to play a role in Cdc45 chromatin loading (86). There is also evidence that the loading of RPA is prevented in Sld3 mutant (78). In summary, Cdc45 and Sld3 may work together on replication origins (87).

- **GINS**

GINS is a replication initiation protein complex identified by two independent groups, using two distinctive approaches. One group performed a screen for multicopy suppressors of Sld5 mutant and co-immunoprecipitations to find potential Sld5 interaction proteins. Psf1 (Partner of Sld Five 1), Psf2 and Psf3 were thus isolated. Together with Sld5, the complex was subsequently named GINS, short for Japanese 5, 1, 2 and 3 (Go, Ichi, Nii, San). They also purified a similar complex from *Xenopus* egg extracts (88). The other group performed a completely different screen for essential replication proteins using an inducible

degron system (89). They identified Cdc105, Cdc101 and Cdc102, which turned out to be Sld5, Psf1 and Psf2 in GINS complex.

Both of the studies have shown that GINS has critical functions in replication. GINS associates with chromatin in S phase and moves with the replication fork (89). The chromatin loading of GINS is co-dependent on Cdc45, Dpb11 and Sld3. Indeed, the recruitments of any these proteins are dependent on the chromatin association of the others (88, 90). Although it is clear that GINS plays an essential role during DNA replication, no individual enzyme activity has been reported. GINS appears to function as part of the CMG complex and to facilitate MCM2-7 helicase activity (80).

- **Summary**

The transition from pre-RC to pre-IC, in other words, the sequential recruitments of pre-IC components, such as Cdc45, to chromatin, depend on CDK and Cdc7/DDK activity. In G1 phase, when CDK and DDK activity is low, the loading of pre-IC component is inhibited. When CDK and DDK are activated in S phase, multiple substrates are phosphorylated including the MCM2-7 complex (32). Phosphorylation of MCM2-7 complex is required for stable binding of CDC45 in S phase of *S. cerevisiae* (76, 91). Sld2 (possible vertebrate ortholog RecQ4) and Sld3 are phosphorylated by CDK and the phosphorylated proteins bind Dpb11 (human ortholog TOPBP1) (92-96), allowing the loading of GINS and DNA pol ε (97). After the stable formation of CMG complex with a variety of

factors (skipped for simplicity), active MCM2-7 helicase initiates DNA replication.

1.2 THE NEGATIVE REGULATION OF THE PRE-RC COMPONENTS

As discussed above, Cdc6 and Cdt1 are required for MCM2-7 loading on origins. It is equally important to inactivate or remove the two "helicase loaders" once cells enter S phase. Recent research in the field has shed light on how these pre-RC components are regulated to prevent replication re-initiation in a CDK dependent and independent manner.

1.2.1 Regulation of the Cdt1 protein

The mechanisms to inhibit Cdt1 are rather different in various organisms.

Before the introduction of regulation pathways in different experimental systems, it is necessary to understand two distinct E3 complexes, Cul4-Ddb1^{Cdt2} and SCF^{Skp2}. The two complexes can degrade Cdt1 under different conditions (Fig. 2). Cul4-Ddb1^{Cdt2} complex degrades Cdt1 during S phase in a replication-dependent manner or after DNA damage. SCF^{Skp2} complex can target Cdt1 for degradation after the Cdk phosphorylation in S phase. Both the complexes belong to the CRL (cullin-RING ligase) family of ubiquitin E3 ligases. CRL family is the largest E3 super-family in eukaryotes (98). The SCF complex is the prototype of CRL and consists of the cullin Cul1 protein (which serves as a scaffold), the RING finger protein (bound to Cul1 at its C-terminus), the

adaptor Skp1 (bound to Cul1 at its N-terminus), and a F-box protein (which binds to Skp1 and serves as a substrate recognition subunit). Cul4-Ddb1^{Cdt2} complex contains Cul4 as a scaffold, Rbx1 as the RING finger protein, Ddb1 as an adaptor, and Cdt2 as an SRS (substrate recognition subunit).

In yeast, Cdt1 is mainly regulated in a Cdk-dependent manner. Cdt1 is excluded from the nucleus during S phase in *S. cerevisiae* (99). However, in *S. pombe*, Cdt1 is degraded by Cul4-Ddb1^{Cdt2} complex in S phase and after DNA damage (100). In *Xenopus* egg extract, Cul4-Ddb1^{Cdt2} pathway is the dominant pathway for Cdt1 degradation, whereas SCF^{Skp2} has only a small role, if any, in the degradation (101, 102). In *Caenorhabditis. elegans*, Cul4 or Ddb1 inactivation during S phase can similarly stabilize Cdt1 (103, 104). In contrast, Skpt1, the *C. elegans* Skp2 homolog, is not required for Cdt1 degradation (103). Only in human cells, both the E3 ligases seem to be important for Cdt1 protein level regulation. After entering S phase, Cdt1 binds to Cyclin A through a Cy (cyclin binding) motif and is subsequently phosphorylated on T29. The phosphorylated Cdt1 interacts with Skp2 and is targeted for degradation by SCF^{Skp2} E3 ligase during S/M transition (105). As a redundant pathway for S phase Cdt1 destruction, Cul4-Ddb1^{Cdt2} can degrade Cdt1 in a replication-dependent manner. Cdt1 interacts with chromatin bound PCNA (proliferating cell nuclear antigen) through its PCNA interaction motif (PIP box). Subsequently, Cdt1 is recognized by Cul4-Ddb1^{Cdt2} complex and targeted for the destruction (101, 106-110). This Cul4-Ddb1^{Cdt2} mediated pathway can also be activated upon DNA damage (Fig. 2).

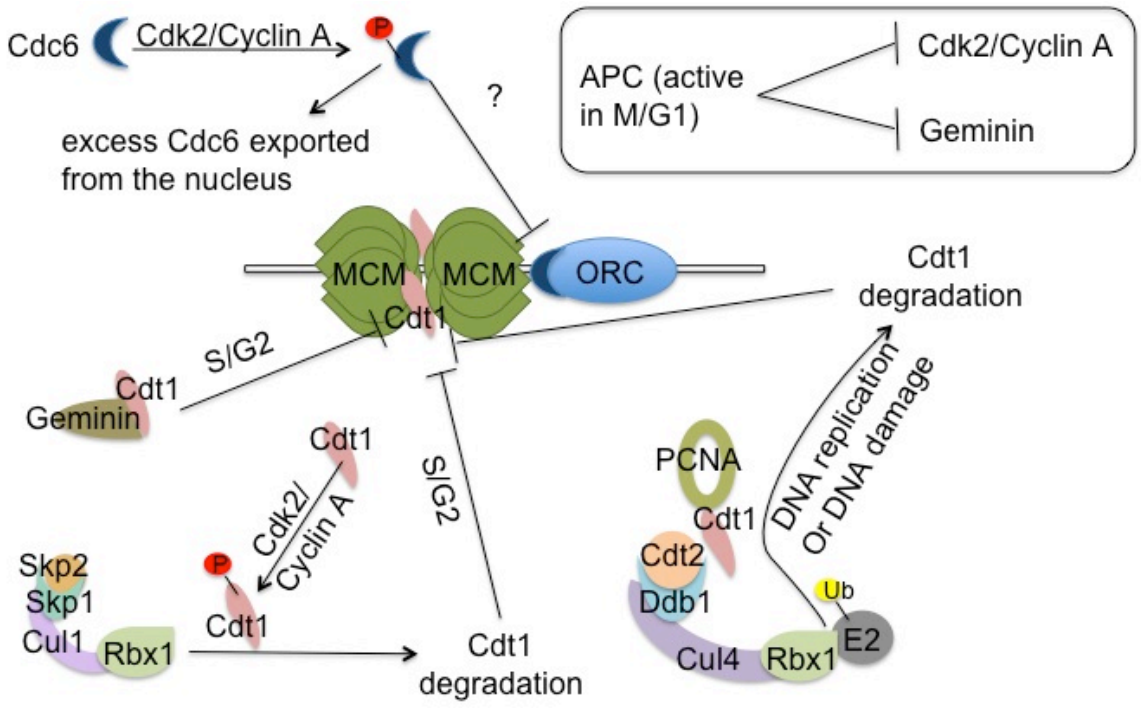

Figure 2. The negative regulation of pre-RC components to prevent DNA re-replication in higher eukaryotic cells. Cdt1 is down regulated in both protein activity and abundance to prevent re-replication in metazoan. Cdt1 is phosphorylated in a Cdk-dependent manner and degraded by SCF^{Skp2} ubiqutin E3 ligase in S/G2 phase. Cdt1 can be degraded in a replication-dependent manner by Cul4-$Ddb1^{Cdt1}$ E3 ligase. This degradation pathway is also used after DNA damage. In addition, Geminin inhibits Cdt1 activity in S/G2 phase. Cdt6 is phosphorylated and inhibited by S-Cdk as well. The mechanism of this inhibition is still unknown. Moreover, both S-Cdk and Geminin proteins are regulated by APC, which is inactivated in M and early G1 phase. Therefore, S-Cdk and Geminin can be accumulated at G1/S transition and inhibit pre-RC components in S and G2 phase.

Another mechanism to inhibit Cdt1 is by interaction with Geminin (Fig. 2). Geminin is conserved in almost all metazoan, from *C. elegans* to humans. It is an APC (anaphase promoting complex) substrate and accumulates in late G1 as APC is inactivated. Geminin then binds to Cdt1 and inhibits MCM2-7 loading till late M phase (111-113).

1.2.2 Regulation of the Cdc6 protein

Cdc6 inhibition is a major mechanism to prevent DNA re-replication in yeast, especially *S. pombe*, whereas its role in mammalian cells remains uncertain.

In *S. pombe*, Cdc18 (*S. pombe* Cdc6 homolog) is phosphorylated by cyclin dependent kinase in S phase (the S-Cdk) (33). The modified protein is then degraded. In *S. cerevisiae*, Cdc6 is similarly regulated although several non-redundant pathways are employed to block origin re-initiation (99, 114). In higher eukaryotes, especially mammalian cells, Cdc6 can also be phosphorylated by S-Cdk, but the role of phosphorylation is still controversial (115).

In mammalian cells, Cdc6 is phosphorylated during S phase. Both *in vitro* and *in vivo* results have shown that S-Cdk can phosphorylate Cdc6 near the N-terminal NLSs (nuclear localization signals) to block the NLSs activity so that the bulk of the Cdc6 is exported out of the nucleus into the cytoplasm (116, 117). However, multiple groups have suggested that only soluble endogenous or ectopically expressed Cdc6 can be exported from nucleus in human cells so that the chromatin-bound Cdc6 still perseveres in the nucleus throughout S phase (118, 119). Taken together, Cdc6 does not seem to be a major target of negative regulation of pre-RC in mammalian cells.

Cells have developed different ubiquitin ligases to degrade Cdc6. Upon S-Cdk modification, Cdc18 is degraded via the $SCF^{Pop1/Pop2}$ ubiquitin ligase in *S. pombe* (120, 121). During early G1 phase, Cdc6/Cdc18 can be degraded by APC^{Cdh1} in both yeast and human cells and in late G1 by SCF^{Cdc4} following phosphorylation by S-Cdk (122, 123). It is not clear though whether this disruption plays a role in preventing re-initiation. Indeed,

Cdc6 reaccumulates at a high level during S, G2 and M phase in mammalian cells, but this Cdc6 could be mostly cytoplasmic (122). APC-mediated Cdc6 degradation can also be activated by ionizing radiation after p53 or p21 inhibition of Cdk2 (124). In addition, UV or DNA alkylations can target Cdc6 to Huwe1 (also known as Mule/UreB1/ARFBP1/Lasu1/HectH9) ubiquitin E3 ligase for degradation (125).

1.2.3 Regulation of ORC and MCM2-7 complex

Like Cdc6, ORC is also a Cdk substrate (114, 126). In *S. cerevisiae*, ORC2/6 are phosphorylated in G1/S transition and play a role in inhibiting origin re-firing (114). Similarly, ORC2 phosphorylation may serve to limit re-initiation in *S. pombe*, although Cdc18 is the main target of pre-RC inhibition (127). In higher eukaryotes, the chromatin binding of ORC1, the largest ORC subunit, is regulated to control initiation. For example, in human cells, ORC1 has been shown to get degraded in S phase through an SCF^{Skp2} mediated pathway (128). It has also been reported that the chromatin binding of ORC1 is reduced in S and G2 phase (129). However, other studies have suggested that both ORC1 level and chromatin association remain the same (130-132). The reason of the difference is unclear. Moreover, the mechanism and the consequence of ORC regulation in mammalian cells still remain undefined.

MCM proteins are also regulated by Cdk-dependent modification. Cdk can phosphorylate Mcm2 and Mcm4 in vivo and in vitro (133-137). In *S. cerevisiae*, MCM2-7 proteins are normally exported from nucleus in S phase and this fails to occur after Cdk inactivation. This nuclear export of Mcm2-7 in S phase is one of the mechanisms to

inhibit re-replication (138). In *Xenopus*, Mcm4 is phosphorylated by Cdk and then loses the chromatin loading capacity (133, 135, 137, 139). Mouse Mcm4/6/7 seems to lose its helicase activity after Cdk phosphorylation (136). Despite this, there is no evidence to suggest that MCM2-7 is directly down regulated in metazoan. In addition, Cdt1 stabilization/activation seems to be sufficient to induce re-replication, suggesting the presence of a functional MCM2-7 complex. In summary, although further research is necessary to evaluate the importance of Cdk-dependent MCM phosphorylations, it is quite possible that as with Cdc6, there is enough functional MCM2-7 proteins still located in the nucleus during S and G2 phase.

In summary, although ORC and MCM2-7 are regulated at G1-S transition by S-Cdk activity, the significance of these regulations remains to be determined.

1.3 THE CONSEQUENCES OF PRE-RC DEREGULATION

1.3.1 The consequences of the Cdt1 protein deregulation

The deregulation of Cdt1 in different organisms gives different phenotypes. In yeast, Cdk regulates various pre-RC components to inhibit re-licensing. No re-replication can be observed after deregulation of Cdt1 alone. However, in higher eukaryotes, Cdt1 seems to be a major focus to prevent re-initiation. In *C. elegans* or Hela cells, failure to degrade Cdt1 induces re-replication (104, 106). In *Drosophila* and some human cells, failure to inhibit Cdt1 by Geminin induces re-replication (115). In MCF10A cells, it

seems necessary to increase both Cdt1 protein level and activity to observe re-replication (140).

1.3.2 The consequences of Cdc6, ORC and MCM2-7 complex deregulation

In *S. pombe*, Cdc18 seems to be a main substrate of Cdk. Indeed, the overexpression of Cdc18 itself leads to massive re-replication, which can be further increased by Cdt1 overexpression (51, 141). However, no re-replication can be detected after Cdt1 overexpression. In metazoan, no severe consequences are observed after Cdc6 deregulation alone.

ORC and MCM2-7 may play a role to inhibit re-licensing. However, no significant re-replication is detected in metazoan after separate deregulation of their proteins.

1.3.3 Ways to induce DNA re-replication in eukaryotic cells

As discussed above, Cdc18 overexpression can induce re-replication in *S. pombe* (141). However, in *S. cerevisiae*, the mechanisms preventing re-replication are not redundant. All Cdk-dependent pathways for inhibiting the pre-RC must be inactivated to induce massive re-replication. Fractional re-replication can be detected when Orc2/6 phospho-mutants are combined with constitutively active Cdc6, Cdt1 and MCM proteins (114).

In metazoans, the repression of Cdt1 is more important for inhibiting re-initiation. For example, overexpression of non-degradable Cdt1 itself is sufficient to cause DNA re-

replication in *Xenopus* (101). In *Drosophila* and certain human cell lines like HCT116, the loss of Geminin, which can in turn activate Cdt1, induces DNA re-replication (115, 142). In addition, Cdt1 overexpression alone, or combined with Cdc6, can cause re-replication in certain $p53^{-/-}$ human cells (143).

Although Cdt1 deregulation seems to be the main mechanism in mammalian cells to induce re-replication, Cdk activity is still used to repress re-initiation in some of them. In *C. elegans*, Cul4 depletion can lead to DNA over replication (104). Cdt1 accumulation was considered as the mechanism of re-replication in this scenario. However, it was shown recently that the loss of Cul4 protein resulted in increase of CKI-1 (Cdk Inhibitor 1) level, which in turn negatively regulated Cdk and inhibited Cdc6 export from the nucleus (144). Indeed, co-expression of non-degradable Cdt1 with non-exportable Cdc6 can cause re-replication in *C. elegans*. Moreover, the depletion of Emi1, an APC inhibitor, has been shown to induce re-replication in Hela and MCF10A cells (140). After Emi1 depletion, APC is activated and can degrade Geminin, CyclinA and CyclinB1, which can in turn cause re-replication through Cdt1 activation and Cdk inhibition.

Recently, MLN4924, a potential cancer drug, has been reported to induce extensive DNA re-replication in human colon cancer cells (145). MLN4924 is a NEDD8 pathway inhibitor. NEDD8 is a small ubiquitin-like protein. The modification of NEDD8 (called neddylation) on CRL ligases, which include SCF^{Skp2} and Cul4-$Ddb1^{Cdt2}$, is necessary for their ubiquitin ligase activities (146). After MLN4924 treatment, neddylation of Cullins is inhibited and CRL mediated ubiquitin-ligase activities decreased. This in turn increases protein abundance of substrate of CRL, such as Cdt1.

However, before my work it was not verified whether MLN4924 induces re-replication through stabilizing Cdt1. I address this question in chapter three.

1.4 THE CONSEQUENCES OF DNA RE-REPLICATION

It is critical to consider the consequences of DNA re-replicaiton in order to understand the significance of negative pre-RC regulation and inhibition of origin re-firing. What kind of DNA structures is generated during the process? Which signaling pathways are activated by those structures? What are the functions of those pathways? Is it possible to repress them? What is the ultimate fate of re-replicating cells? If they can survive, what will happen next? In a word, what is the biological significance of DNA re-replication?

1.4.1 DNA damage checkpoint activation upon re-replication

In *S. cerevisiae*, fragmented chromosomes can be detected by pulsed field gel electrophoresis after DNA re-replication. Moreover, it has been shown that a Rad-9 dependent DNA damage checkpoint pathway is activated upon re-replication, indicating the appearance of DNA breaks (147). In mammalian cells, re-replication can be induced by the loss of Geminin, Cdt2 or Ddb1. Using Comet assay or γ-H2AX staining, the presence of both DSBs (double-stranded DNA breaks) and ssDNA (single-stranded DNA) can be detected (106, 142, 143, 148-150). In cells depleted of Geminin, a p53-independent G2/M checkpoint is activated with ATM or ATR/BRCA1 mediated Chk1

and Chk2 phosphorylation leading to Cdc2 inhibition, whereas Cdt1 overexpression activates a p53-dependent ATM/ATR pathway (142, 143, 148).

The next question is, which signals activate the checkpoint pathways. If they are abnormal DNA structures as stated above, how does re-replication generate those DNA structures such as excess ssDNA? Hypothetically, there are several different mechanisms to produce ssDNA in re-replicating cells. The first mechanism is that the increase of re-initiation sites on DNA can directly generate more ssDNA. The second is that, the increased MCM2-7 helicase activity is uncoupled from DNA polymerase activity and creates excess ssDNA. Indeed, ssDNA is induced in Xenopus after the addition of aphidicolin, a DNA pol α inhibitor (151). It is possible that the unusual loading of MCM2-7 leads to enhanced helicase activity that cannot be coupled with DNA polymerase. Then the uncoupling creates more ssDNA. Last but not least, re-replication can lead to fork collapse and generate DSBs, which can be processed by Mre11 nuclease to form ssDNA (152). When cells undergo re-replication, multiple initiation events may happen on the same locus. In this scenario, more than one replication fork move in the same direction. Both folks will collapse when they encounter each other, creating DSBs. In addition, DSBs can possibly be generated by the second fork attemping to replicate over single-strand stretches in replicating DNA left behind by the first fork (153). More work is clearly required to verify which of the above structures reach a threshold to activate the checkpoint pathways. There are other potential chromosome structures that can possibly signal checkpoint pathways. For example, cohesin ring structure (which is formed by the cohesin complex surrounding the two sister chromatids) is present from S

phase to anaphase (154). DNA re-replication may have a physical force on the ring, which can activate checkpoint pathways.

Another critical question is, what will the checkpoint activation lead to? Can the re-replicating cells be rescued from abnormal DNA structure and genome instability, or is cell death inevitable? Interestingly, multiple reports have shown controversial results. Accumulation of re-replicated cells after Geminin depletion is suppressed by checkpoint inactivation (142, 148, 149). Indeed, after depletion of Geminin, ATM/Chk2 and ATR/BRCA1 are activated. ATR/Brca1 can further activate FA (fanconi anemia) pathway, which is important for both G2/M checkpoint and homologous DNA repair. After depletion of checkpoint proteins like ATR and FANCA, cells are driven into mitosis and go through mitotic catastrophe before entering apoptosis, suggesting that the G2/M checkpoint functions to arrest the cell cycle to repair DNA damage and protect cells from mitotic catastrophe. Because the cells are prevented from entering mitosis, checkpoint activation is essential to allow the persistence of cells undergoing re-replication. However, the checkpoint can also be detrimental for re-replication. For example, the presence of wild type p53 leads to a decrease in the number of cells with re-replication probably due to p53 mediated apoptosis (143). Moreover, in *Xenopus*, addition of recombinant Cdt1 can induce DNA re-replication and checkpoint pathway. Here, inactivation of the checkpoint increases re-replication, implying that an intra-S phase checkpoint prevents DNA synthesis of re-replication (155). Consistent with this, two color FACS (flow cytometry analysis) showed that cells with most amount of DNA by PI (propidium iodide) staining were no longer incorporating BrdU (bromodeoxy-

uridine), most likely because the checkpoint pathway eventually suppress DNA sythesis (142). Overall, it is suggested that (a) different checkpoint pathway is activated by Geminin depletion or Cdt1 overexpression and (b) that checkpoint pathways can both increase and decrease extent of re-replication depending on the cellular context. Clearly, further work is necessary to elucidate this issue.

1.4.2 The activation of apoptosis and senescence pathways

Cdt1 overexpression induced DNA re-replication can induce a p53-dependent checkpoint pathway. The activation of p53 can further lead to apoptosis (143). In Geminin-depleted cells, apoptosis can also happen if the G2/M arrest is overridden (142, 149).

It has been indicated that there are at least two barriers to tumorigenesis that inhibit cellular proliferation and tumor progression (156, 157). The two barriers are apoptosis and senescence. Recent research has shown that DNA re-replication induced by expression of an activated oncogene can lead to cellular senescence (158). The work was done with oncogenic Ras (H-RasV12) expression in normal human fibroblasts. FISH (fluorescence *in situ* hybridization) experiments showed that certain replication origins are amplified more than once in those cells. Moreover, DNA combing assay has detected increased active replicons. This DNA hyper-replication can activate ATR/ATM mediated DDR (DNA damage response) pathways that will lead to cellular senescence. Indeed, DDR activation is required for the senescence and its inactivation allows cellular proliferation and contributes to cell transformation (158). It is not clear though whether

re-replication induced by pre-RC deregulation in cancer cells can similarly cause cellular senescence. I address this question in chapter three.

Taken together, re-replication can lead to cell cycle arrest, apoptosis and possibly senescence. These pathways can all inhibit cellular proliferation and may contribute to preventing tumorigenesis (Fig. 3).

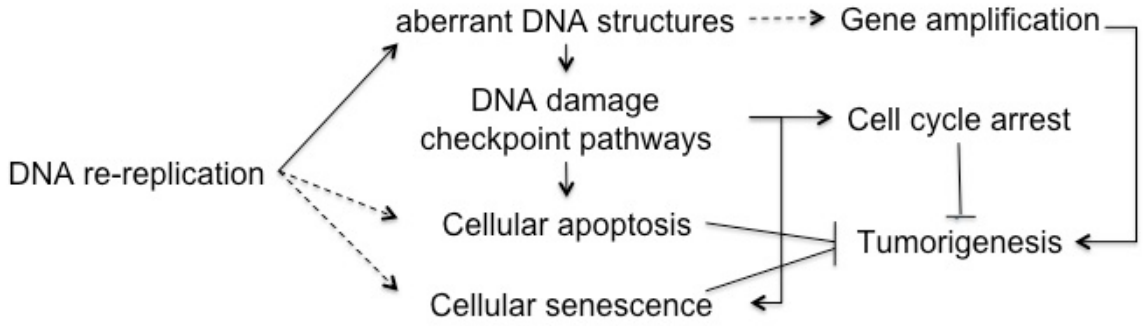

Figure 3. The consequences of DNA re-replication. Dashed lines indicate hypothetical pathways. For details, see text.

1.4.3 The connection between DNA re-replication and gene amplificaiton

Gene amplification is a copy number increase of certain chromosome region (159). It can stimulate abnormal cell growth and lead to tumorigenesis (160). In mammalian cells, amplified genes can exist in a variety of forms including extrachromosomal copies called double minutes, tandem arrays within a chromosome with cytologically visible HSR (homogeneously staining region), and distributed insertions across the genome. The mechanisms of gene amplification are not fully understood. It has been suggested that amplification is initiated by a DSB in cells deficient in DNA damage checkpoints (161-165). The models of how a DSB leads to

gene amplification are various. One of them is a BFB (breakage-fusion-bridge) cycle (166). In this process, as shown in Fig. 4, the broken end of a chromosome can fuse through NHEJ (non-homologous end-joining) mechanism, and form a dicentric chromosome. Breakage occurs at anaphase, and an asymmetric break can generate an inverted duplication of DNA sequences. The process will be repeated in subsequent cell cycles until the ends are stabilized by telomere addition. The BFB cycles can eventually create multiple copies of the chromosome region near the break. If cells bearing amplified DNA have a selection advantage, the cells can be overgrown in the population (167).

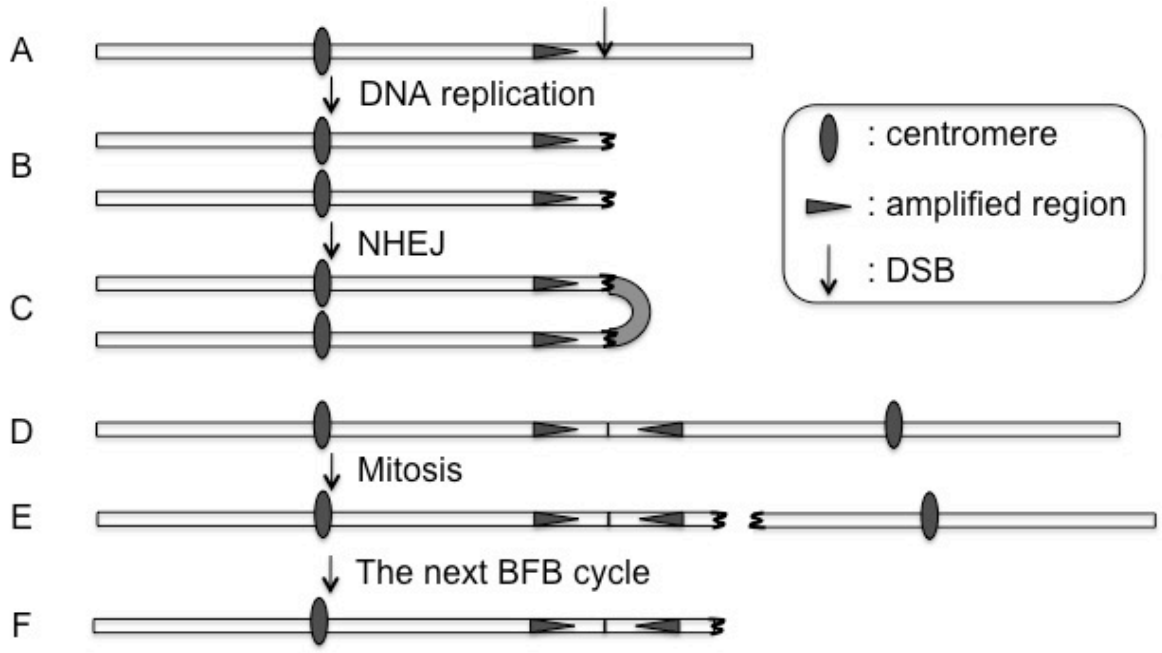

Figure 4. The process of breakage-fusion-bridge (BFB). After replication, the broken ends of the sister chromatids (B) that come from DNA with a DSB (A) can be fused together (C) and form a dicentromeric chromosome (D). An asymmetric breakage at anaphase can give rise to

an inverted duplication of DNA sequence (E). The broken end of the resulting chromosome (F) will lead to the next BFB cycles in the subsequent cell divisions.

Recent studies using new techniques such as the array-CGH (array comparative genomic hybridization) have found gene amplification is contained in most solid tumors (168-171). In addition, it has been shown that gene amplification contributes significantly to tumorigenesis (172, 173). However, normal mammalian cells lack the ability to easily amplify genes in culture, although CNVs (copy number variations), duplications of chromosomal regions can be detected in the genomes of individuals and cancers (174-176). This suggests that copy number increase may occur in the germ-line and be fixed during evolution or during the progression of a cancer *in vivo*, whereas gene amplification is inhibited in normal cultured cells. The existence of inhibition mechanisms is further supported by the observation that hybrids between tumor and normal cells lose the amplification ability (177).

DNA re-replication has been believed to create genomic alterations and threaten genome instability. It is also considered to be one of the models for copy number increase during gene amplification (178, 179). However, this theory has never been experimentally tested. As discussed above, both DSBs and ssDNA are generated in re-replicating cells. Hypothetically, DSBs can further be the substrates for gene amplification in cells that are able to enter cell cycle with the damaged DNA (Fig. 3) (161-165). Therefore, it is worth testing whether re-replication can induce gene amplification in this scenario. This is something I test in chapter four.

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1.6 APPENDIX

MECHANISMS TO CONTROL REREPLICATION AND IMPLICATIONS FOR CANCER

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Attributions:

Mechanisms and consequences of re-replication, re-replication and tumorigenesis, outstanding questions in the field, and conclusion were written by S.S.Hook. Introduction, ways to induce rereplication, mechanisms to prevent re-replication were written by J.J.Lin.

ABSTRACT

Recent advances in the replication field have highlighted how the replication initiator proteins are negatively regulated by inhibitor proteins and ubiquitin-mediated degradation in mammalian cells to prevent rereplication. When these regulatory pathways go awry, uncontrolled rereplication ensues and a G2/M checkpoint is evoked to prevent cellular death. Many components of the checkpoints activated by rereplicaton are important for cancer prevention by facilitating DNA damage repair processes. The pathways that prevent rereplication themselves have also recently been implicated in

preventing tumorigenesis. Studies from patient tumors, genetically altered mice, and mammalian cell culture suggest that deregulation of replication licensing proteins results in an increase in aneuploidy, chromosomal fusions, and DNA breaks. These studies provide a framework to address how regulators of replication function to maintain genomic stability.

INTRODUCTION

Duplication of chromosomal DNA is a key event in the cell cycle. In eukaryotes, DNA replication initiates at areas known as replication origins, which are recognized by a six-subunit ATPase complex called the origin recognition complex (ORC) [1]. This complex binds DNA in late mitosis or early G1 synergistically with a second AAA+-ATPase, Cdc6 [1,2]. The replication licensing factor, Cdt1 then recruits the MCM2-7 complex, the likely replicative DNA helicase, to the replication origins in a manner dependent on concerted ATP hydrolysis by Cdc6 and ORC subunits [3] to form the prereplicative complex (pre-RC) at the licensed origin. Subsequent replication initiation depends on activation of the helicase activity of MCM2-7 directly or indirectly by cdk2 (cyclin dependent kinase 2) and DDK (Dbf4 and Drf1- dependent kinase) [1] (Fig. 1 middle). It is important that replication initiation happens once and only once per cell cycle. To ensure this, cells inactivate the processes for pre-RC formation once S phase is initiated to prevent re-licensing and re-initiation. In doing so, rereplication within the same cell cycle is prevented. After mitosis, the pre-RC machinery is de-repressed so that origins can be licensed again for the next cell cycle.

In this review, we will focus on the newest advances in our understanding of the mechanisms and consequences of rereplication, ways mammalian cells try to prevent rereplication, and how rereplication is related to tumor formation.

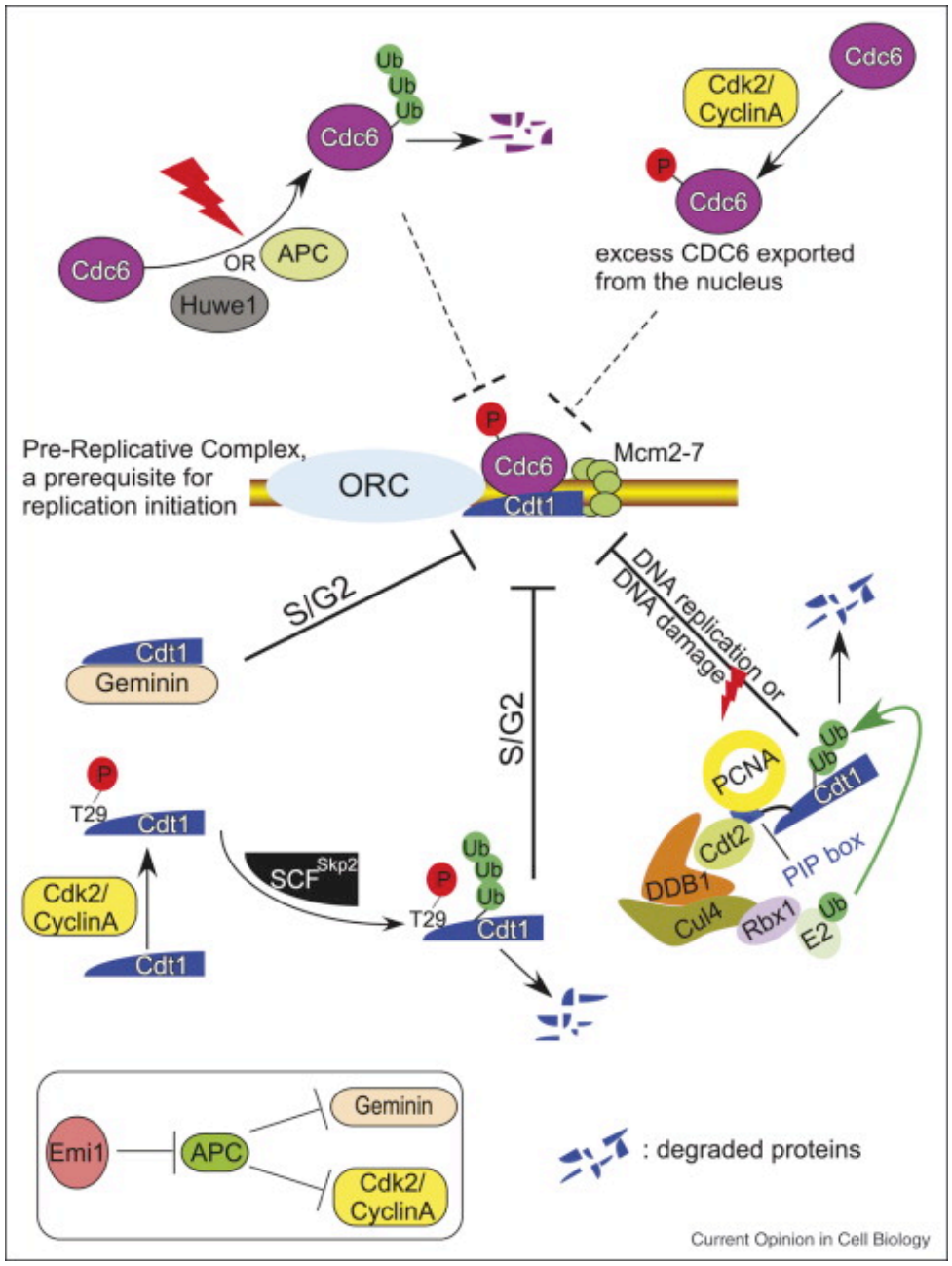

Fig. 1. Mechanisms to prevent rereplication in mammalian cells. In the center is the

pre-RC which is a pre-requisite for replication initiation. The pathways highlighted with black lines are known to inhibit pre-RC formation and by doing so have been demonstrated to prevent rereplication. The pathways denoted with dashed lines also inhibit pre-RC formation but have not yet been shown to prevent rereplication. The red lightening bolt denotes pathways activated by DNA damage. The boxed section shows Emi1 which has been shown to prevent rereplication by stabilizing geminin and cyclin A.

WAYS TO INDUCE REREPLICATION

In various experimental organisms, the array of mechanisms to inhibit rereplication is somewhat different, and hence disruption of the regulatory pathways to induce rereplication also varies. In yeast, cdk2-dependent suppression of individual pre-RC components is the main mechanism to prevent rereplication [4]. However, in higher eukaryotes, including mammals, other cdk-independent pathways have been reported to suppress rereplication. In mammals, Cdt1 is the major target that is repressed once cells enter S phase for the prevention of re-licensing, though Cdc6 and ORC are also regulated [[5] and [6]]. Overexpression of Cdt1 itself or with Cdc6 causes rereplication in p53−/− human cancer cells [7]. This is in contrast to the yeasts, where the overexpression of Cdc18 (the ortholog of Cdc6) but not Cdt1 is sufficient to override the control of DNA replication in fission yeast, whereas simultaneous deregulation of the four pre-RC components are required to induce immense rereplication in budding yeast [[5] and [8]].

To repress Cdt1 after the onset of S phase, mammalian cells have developed multiple mechanisms, which include association with the inhibitor, Geminin, and degradation by the ubiquitin proteasome system by two distinct E3 ligases — SCF^{Skp2} (which is cdk dependent) and $Cul4–DDB1^{Cdt2}$ (Fig. 1) [[4] and [6]]. Depletion of the Cdt1

inhibitor, geminin, leads to DNA rereplication in Drosophila and certain human cancer cell lines [5]. However, in other human cell lines like HeLa or MCF10A, in Xenopus egg extracts, and in Caenorhabditis elegans loss of geminin does not induce rereplication. By contrast, Cdt2 depletion at least in HeLa cells, stabilizes Cdt1 levels in G2 phase and induces robust rereplication [9•]. Similarly, Cul4 depletion in C. elegans [10] and a non-degradable Cdt1 overexpression in Xenopus [11•] lead to rereplication. Thus, geminin and Cdt1 degradation mechanisms down regulate Cdt1 activity to differing extents in different experimental systems.

Although the primary mechanism to induce rereplication in mammalian cells might be through regulating Cdt1, some mammalian cells like their yeast counterparts, continue to repress rereplication through the activity of cdk2/cyclinA. Recently, the depletion of Emi1, an inhibitor of anaphase-promoting complex/cyclosome (APC/C) activity during S and G2 phases, has been reported to cause rereplication in HeLa and a nonmalignant breast epithelial cell line MCF10A [12]. APC activation leads to the degradation of geminin, cyclin A, and cyclin B1 which in turn induces rereplication through Cdt1 activation and inactivation of cdk2/cyclinA and cdk1/cyclinB1 [[12] and [13]].

MECHANISMS AND CONSEQUENCES OF REREPLICATION

Regardless of the exact mechanism of inducing rereplication, the manner by which it happens has not been well studied. So far, many researchers rely on detecting rereplication using fluorescence-activated cell sorting (FACS) analysis of cells. In order

for cells to display rereplication by FACS analysis, the majority of the cells need to contain significant amounts of rereplicated DNA. This fact led researchers to turn to either comparative genomic hybridization (CGH) analysis, pulse gel electrophoresis, heavy/heavy DNA labeling, or fluorescence in situ hybridization (FISH) analysis to measure rereplication on a smaller, more precise scale.

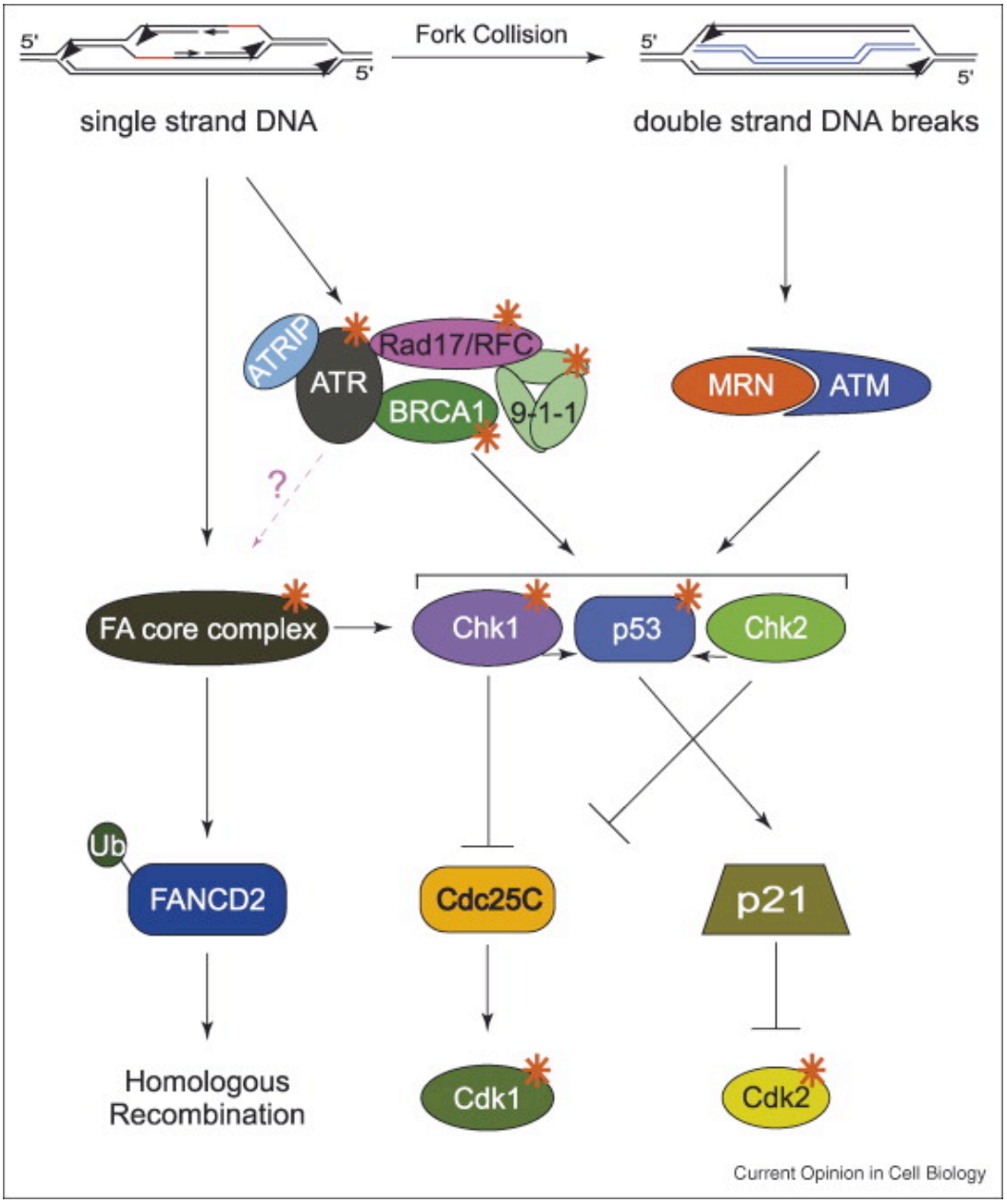

Fig. 2. Checkpoints activated upon rereplication. Multiple rounds of replication could give rise to replication origins within replication bubbles which would activate ATR-dependent signaling pathways. Replication fork collision could generate DNA double strand breaks and activate ATM-dependent pathways. Orange stars denote proteins essential for checkpoint activation in response to rereplication. The pink dashed line denotes a hypothetical pathway. The 9–1–1 complex is composed of Rad9–Rad1–Hus1. The MRN complex is made up of Mre11–Rad50–Nbs1.

Many have postulated that rereplication occurs primarily though ad hoc re-initiation at sites previously replicated and not because of a co-ordinated single round of rereplication, because FACS profiles show the appearance of cells where the DNA content is increased beyond 4N in a broad range of ploidy and not as a discrete peak at 8N (for review see [14]). The ad hoc re-initiation is induced by either overexpression of licensing factors, such as Cdt1 and Cdc6, or by depletion of inhibitors of licensing such as geminin and Emi1. Overexpression of Cdt1 with Cdc6 causes rereplication preferentially at early firing origins at only 2–4 h after an initial firing in S phase [7]. Recent studies have illustrated that even if only a fraction of the genome is rereplicated, the same origin can fire many times, and latent origins begin firing [[15], [16] and [17••]]. Although re-licencing might be a major contributor to rereplication, it seems in S. cerevisiae that pre-RC formation alone is not sufficient to induce rereplication [15]. Perhaps this observation correlates with an older study using minichromosomes that showed that rereplication can be blocked during elongation [18]. The mechanism of this latter observation and whether this phenomenon exists in mammalian cells is unclear.

One of the major consequences of rereplication is the activation of DNA damage checkpoints [8]. Rereplication activates the serine/threonine kinases, ATM and Rad3-

related (ATR) and ataxia-telangiectasia mutated (ATM). These kinases phosphorylate and activate substrate kinases, chk1 and chk2 to evoke a G2/M cell cycle block (Fig. 2). Chk1 and chk2 in turn phosphorylate and inactivate cdc25C, the major phosphatase responsible for activating mitotic cdk1/cyclinB. If rereplication is induced while members of these checkpoint pathways are knocked-down by siRNA techniques, cells die because of their failure to arrest before mitosis. Viability and accumulation of cells with extensively rereplicated DNA is restored by an artificial G2 block [19]. The approach of co-depleting geminin with potential mediators of the G2/M checkpoint has been useful to establish that proteins such as Fanconi Anemia (FA) proteins, BRCA1, p53, etc., are important to establish the checkpoint in response to rereplication. Recent elegant work by Julian Blow's group shows that uncontrolled rereplication is necessary for checkpoint activation and that a single co-ordinated round of rereplication in G2/M is not sufficient to elicit a checkpoint response. Additionally, they show that rereplication results in head-to-tail fork collision and generation of small double stranded fragments of rereplicated DNA [17]. Thus, the G2/M checkpoint is activated by rereplication to halt the cell cycle and prevent cells from mitotic catastrophe.

MECHANISMS TO PREVENT RE-REPLICATION

There are several mechamisms in play to prevent rereplication because global rereplication can be deleterious to a cell's survival. So far in mammalian cells, the majority of these mechanisms impinge on the regulation of the replication initiation proteins, Cdt1 and Cdc6.

As mentioned above, Cdt1 is negatively regulated both by geminin and by proteolytic degradation. After cells initiate DNA replication in late G1, APC is inactivated and as a consequence its substrates such as geminin accumulate. Geminin binds to Cdt1 and inhibits its activity until late M phase [5], when APC gets activated and degrades geminin. To remove excess Cdt1 and prevent origin re-firing, cells have developed pathways for Cdt1 degradation. After the G1/S transition, Cdt1 associates with cyclin A through a cyclin binding motif (Cy motif) and gets phosphorylated by cdk2 on T29. Phospho-Cdt1 binds to Skp2 and is targeted for destruction via the SCF^{Skp2} E3 ubiquitin ligase during the S to M cell cycle transition [6] (Fig. 1). Surprisingly however, Cdt1 mutants incapable of being phosphorylated by cdk2/cyclinA and of binding to Skp2 are still degraded in S phase [6]. This conundrum was not resolved until recently when a new mechanism for Cdt1 degradation depending on the Cul4–$DDB1^{Cdt2}$ ubiquitin ligase was characterized [[9•], [11•], [20], [21], [22], [23•], [24] and [25•]]. During DNA replication or after DNA damage (like UV irradiation), Cdt1 interacts with chromatin-bound proliferating cell nuclear antigen (PCNA) through its conserved N-terminal PCNA interaction motif (PIP box) (Fig. 1). The interaction is required for the subsequent recognition of Cdt1 by Cul4–$DDB1^{Cdt2}$, which ubiquitinates and promotes the degradation of Cdt1. Cdt2/L2DTL was discovered to be essential for the proteolysis probably through functioning as a substrate receptor interposed between the Cdt1 substrate and the Cul4–DDB1 enzymatic complex. Thus, in mammals there are two redundant pathways for degradation of Cdt1 once cells enter S phase.

Although there is considerable evidence in yeast that ORC and especially Cdc6

are important to prevent rereplication, their role in mammalian cells is not clear [5]. Some evidence in mammalian cells suggested these proteins were negatively regulated after replication initiation, but elimination of this regulation was not sufficient to induce DNA rereplication [[4] and [5]]. Overexpression of Cdc6, however, may have important implications for tumor formation by a mechanism independent of replication control (see below).

REREPLICATION AND TUMORIGENESIS

Clearly, rereplication elicits a DNA damage response and many of the signaling proteins involved in these responses have been mutated or deleted in a variety of types of cancers [[26] and [27]]. In this section, however, we will focus on the current knowledge concerning the players important in regulating rereplication and their potential involvement in tumor formation.

In many types of mammalian cells, Cdt1 overexpression alone can cause rereplication which is augmented by Cdc6 overexpression. Interestingly, upregulation of these two proteins is also seen in several models of tumorigenesis. For example, elevated levels of Cdt1 and/or Cdc6 are seen in tumors and in tumor-derived cell lines [[28], [29••], [30] and [31]] and NIH3T3 cells overexpressing Cdt1 cause tumors when injected into immune-compromised mice [28]. In addition, transgenic mice overexpressing Cdt1 in T cells develop thymic lymphoblastic lymphomas when p53 is deleted [32]. Although it is possible that overexpression of Cdt1 and/or Cdc6 in human tumors is a side effect of increased proliferative capacity, no correlation was noted between expression levels of

either Cdt1 [31] or Cdc6 [29••] and the proliferation marker, Ki-67.

A major mechanism by which tumors arise is genomic instability. Not only does overexpression of Cdt1 cause tumors, recent data suggest that Cdt1 may be doing so by inducing genomic instability. Over 50% of all human cancers contain mutations in the tumor suppressor gene p53. Deletion of p53 may synergize with overexpression of Cdt1 and Cdc6 to cause genomic instability in non-small cell carcinomas [31]. In addition, thymocytes from Cdt1 transgenic mice (also null for p53) had enhanced aneuploidy compared to p53−/− control thymocytes. Although some of the thymocytes from transgenic mice contained the usual 40 chromosomes, the vast majority contained between 49 and 58 [32]. Genomic instability stimulated by Cdt1 overexpression, however, is not completely dependent on p53 since in normal human fibroblasts where p53 is wild type, Cdt1 overexpression still results in a large percentage of cells with aneuploidy [30]. Likewise, tumor cells from mice transplanted with Cdt1 overexpressing cells [28] also show aneuploidy as well as end-to-end chromosome fusions, chromosome gaps and breaks [32]. One could imagine that downregulation of negative regulators of Cdt1, such as the Cul4–DDB1^{Cdt2} ubiquitin ligase complex would also cause genomic instability because Cdt1 overexpression results in genomic instability. Indeed, DDB1 siRNA causes a significant increase in chromosomal breaks [33]. In fact, it may be the balance of the replication initiator proteins, Cdt1 and Cdc6 relative to the levels of the replication inhibitor, geminin, that is crucial during tumorigenesis. For example, a subset of patients with mantle cell lymphomas had a ‘deregulated licensing signature,’ that is high Cdt1 and Cdc6 with low geminin levels. These patients had an increase in

chromosomal alterations by CGH analysis and ultimately a shorter median overall survival [34].

Although increased levels of Cdt1 and Cdc6 facilitate re-initiation of replication origins, recent papers suggest that some of their ability to promote genomic instability may not be dependent on their replicative functions. In quiescent cells, Cdt1 overexpression in either rat or human fibroblasts results in chromosomal gains or losses without any detectable rereplication [30]. In addition, increased Cdt1 induced by DDB1 siRNA, induced chromosomal breaks in regions that did not correlate to known fragile sites that are often broken during replication stress [33]. The most intriguing argument for replication initiators inducing genomic instability by a novel mechanism comes from the observation that Cdc6 can be recruited to the INK4/ARF tumor suppressor locus, recruit histone deacetylases, and stimulate heterochromatinization [29••]. This observation might explain why tumors that contain high levels of Cdc6 often also repress the three tumor suppressors, $p15^{INK4b}$, ARF, and $p16^{INK4a}$, which are all transcribed from this locus [29••].

Elevated levels of Cdc6 in tumors suggest that Cdc6 degradation might be an important barrier to tumor formation. Cells use at least two different ubiquitin ligases to degrade Cdc6. It was known that during early G1, Cdc6 can be degraded by APC^{Cdh1} [35]. Recently, this work has been extended to show that cdk2 promotes pre-RC formation by phosphorylating ser54 of Cdc6 which prevents degradation by APC [[36•] and [37]]. In addition, ionizing radiation activates APC-mediated degradation of Cdc6 through p53 inhibition of cdk2, possibly through transcriptional induction of the cdk inhibitor, p21 [37]. DNA damage stimulates Cdc6 degradation by a second mechanism

which is p53, APC, and cell-cycle-independent. HUWE1 (also known as Mule/UreB1/ARFBP1/Lasu1/HectH9), a HECT domain ubiquitin E3 ligase, associates with chromatin-bound Cdc6 and facilitates degradation after cells are exposed to UV or DNA alkylation [38] (Fig. 1). The use of different E3 ligases in response to differing forms of DNA damage may have to do with whether ATR (UV or alkylation) or ATM (ionizing radiation) is the primary signal transducer. In addition, to ubiquitin-mediated proteolysis, Cdc6 is also cleaved by caspase-3 in early apoptosis after DNA damage [39] or cancer drug therapy [40]. Despite so many mechanisms by which to reduce Cdc6 levels, it is unclear whether elevated Cdc6 in tumors is the cause or effect of increased cell proliferation.

OUTSTANDING QUESTIONS IN THE FIELD

Cells employ several mechanisms as barriers to tumorigenesis, including but not limited to cell cycle checkpoints and oncogene-induced senescence. Two exciting papers have recently suggested that DNA damage checkpoint pathways are required for oncogenes to induce senescence as an adaptive cellular mechanism to slow the progression of preneoplastic lesions to neoplasias [[41•] and [42•]]. These groups show that oncogenes induce repeated origin firing, asymmetric fork progression, pre-mature fork termination, and double strand DNA breaks. As a result of this replication stress, the ATR/ATM checkpoint pathways are activated. Inactivation of the checkpoints by ATM shRNA, chk2 shRNA, or p53 shRNA (a substrate for both ATM and chk2), abrogated oncogene-mediated senescence [[41•] and [42•]] and promoted transformation and tumor

growth [42•]. The model from these studies is that oncogenes induce replication stress and cells respond with DNA damage checkpoint activation and subsequent senescence. Interestingly, overexpression of both the oncogenes used in these studies, mos and ras, caused increases in Cdc6 protein levels, an effect which would facilitate rereplication or suppress the INK4/ARF tumor suppressor locus. One could hypothesize that since rereplication also activates DNA damage checkpoints, rereplication might also induce senescence.

The implication from these two studies is that after the initial checkpoint activation, some cells are able to 'escape' senescence and it is these cells that go on to form the malignant neoplasias that are so deleterious for an organism. The question then arises, 'How do cells escape senescence?' Perhaps it is through additional gene alterations. Over 25 years ago, it was proposed that replication stress (or possibly rereplication) is a mechanism for gene amplification [43]). Although this idea is an attractive one, currently, there is no solid data for this idea. In theory, origin re-firing in a short space of time could result in the second fork catching up the initial origin and the generation of a double strand break [17]. If this break is repaired by homologous recombination, as has been postulated as the means to repair replication induced breaks [44], one could imagine that all three copies of the replicated region might be spared [17]. Of course this is only theoretical as it is not clear how the cell would try to repair DNA damage caused by the collision of replication forks. Many genes known to be important for cancer prevention and implicated in the cell's response to rereplication are also required for repair of DNA breaks. The MRN complex which is important for chk2

activation during rereplication [58] is crucial for double strand break repair to keep the DNA ends in close proximity to one another. BRCA1, a protein required for the G2/M checkpoint pathway activated by rereplication, also functions as a part of the homologous recombination machinery (Fig. 2). Studies are just being published that suggest that defects in components of the G2/M checkpoint activated in response to rereplication give rise to gene amplification in breast cancer [[45] and [46]]. Future research should shed light on the long-standing question of whether rereplication indeed results in gene amplification.

Somewhat surprisingly, there have not been mutations or alterations identified in the core components of the pre-RC in human cancers. Although loss of function alterations would be incompatible with cellular survival, the same cannot be said for gain of function mutations that could lead to rereplication. High Cdt1 levels correlate with tumorigenesis but mutations that increase Cdt1 activity have not been identified. One could hypothesize that the levels of geminin, an inhibitor, might be low in cancers. There have been reports, however, that show that in 49% of small cell lung carcinomas, geminin levels are dramatically changed, but counterintuitively, only 12% of the tumors have decreased geminin whereas 37% have increased geminin [31]. It is unclear whether these differences in geminin levels are seen on a per cell basis or are merely an indirect consequence of an altered cell cycle profile. Additionally, it could be that geminin levels are high in low-grade tumors and as the grade increases, geminin levels become low. So far, no comprehensive studies have been done that correlate the expression of components of the replication machinery with tumor grade.

Although many groups have been studying how geminin is degraded to facilitate rereplication, another possibility is that geminin is regulated through changes in subcellular localization. It has been shown that at the end of mitosis avian geminin can be exported from the nucleus to the cytoplasm to facilitate MCM loading [47]. It remains possible that during tumorigenesis, geminin is mislocalized and cannot function to restrain replication origin firing.

To date, there is no indication that elevated levels of the MCM complex components promote rereplication. However, several studies indicate that the MCM complex might be a target of negative regulation during rereplication. First, MCM subunits 2, 3, and 7 are phosphorylated by ATR in response to replication stress [48]. Second, rereplication can be blocked during elongation [18] and the MCM complex is required for elongation [8]. In budding yeast, the MCM subunits are also subject to nuclear export in response to cdk activity to prevent pre-RC formation [8]. Thus, whether excess MCM activity will affect rereplication and whether this might impact tumorigenesis remains to be elucidated.

Finally, an outstanding question is how a cell distinguishes between developmentally regulated, beneficial endoreduplication and aberrant rereplication? Two types of mammalian cells, megakarocytes and trophoblastic cells are able to undergo endoreduplication (also called endomitosis) or duplication of the entire genome successive times without transversing the G2/M transition. Trophoblast cells, essential for placental development and the barrier between material and fetal tissues, undergo concomitant differentiation, endoreduplication to reach 512–1024N DNA content, as well

as invasion into the endometrium [49]. Megakaryocytes on the contrary, go through several rounds of endoreplication (to 128N) as a transition from a megakaryocyte progenitor to a terminally differentiated megakaryocyte capable of forming platelets [50].

Until recently, it was unclear whether replication proteins themselves were key regulators of endoreduplication. Laskey's group has shown that in geminin knock-out mice, cells commit to the trophoblastic lineage and begin undergoing premature endoreduplication at the eight-cell stage [51•]. In addition, they found that normally geminin is degraded during trophoblast endoreduplication, indicating that a similar mechanism prevents both rereplication and endoreduplication [51•]. In megakaryoblastic cell lines that are able to undergo differentiation-induced endoreduplication, geminin levels go down [[52] and [53]]. Cyclin E overexpression stabilizes Cdc6 levels (probably through cdk2) and Cdc6 overexpression in the absence of megakaryocyte differentiation can cause cells to endoreduplicate [52]. It is tempting to speculate that Emi1 depletion which causes degradation of cyclin B1 [13] might also facilitate endoreduplication because inhibition of cdk1/cyclinB1 activity promotes endoreduplication in both trophoblastic cells and megakaryocytes [[49] and [50]]. From these limited studies, it appears that the mechanisms to limit rereplication might also act to inhibit endoreduplication. The key to whether a cell endoreduplicates would not only depend on pathways that prevent rereplication but would also depend on the concerted context of the differentiation program that is initiated, the signal transduction pathways activated, the gene expression changes, and the extracellular signaling environment.

CONCLUSIONS

The balance between levels of the initiation factors, Cdt1 and Cdc6, and their negative regulators is crucial in determining whether cells will rereplicate their DNA. High Cdt1 and/or high Cdc6 promote rereplication as seen in various tumor models, and correlate with genomic instability. By contrast, depletion of the Cdt1 inhibitor, geminin induces rereplication though a role in tumor formation is not well established. The major recent advance in understanding how cells prevent rereplication has been in elucidating the pathways that regulate the degradation of Cdt1 and Cdc6. Cdt1 is targeted for destruction by SCF^{Skp2} and $Cul4–DDB1^{Cdt2}$, whereas Cdc6 is regulated by HUWE1 and APC. Now that techniques to visualize replication foci in live cells [54] and crystallographic and proteometric analysis of the ligase complexes [[9•], [55], [56] and [57]] are being published, our arsenal with which to study rereplication is rapidly increasing. The recent expanse in knowledge of how the replication initiation pathways are controlled has revealed that cells use diverse, distinct and precise mechanisms to prevent rereplication. By employing such mechanisms, cells control tumorigenesis by increasing the fidelity with which genomes are passed to daughter cells.

REFERENCES AND RECOMMENDED READING

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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CHAPTER TWO

ATR PATHWAY IS THE PRIMARY PATHWAY FOR ACTIVATING G2/M CHECKPOINT INDUCTION AFTER RE-REPLICATION

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SUMMARY

DNA replication is tightly controlled to ensure accurate chromosome duplication and segregation in each cell cycle. Inactivation of Geminin, an inhibitor of origin licensing, leads to re-replication in human tumor cells within the same cell cycle, and triggers a G2/M checkpoint. We find that the primary pathway to signal that re-replication has been detected is the ATR kinase and the Rad9/Rad1/Hus1 (9-1-1) clamp complex together with Rad17/RFC clamp loader. ATM kinase and the Mre11/Rad50/Nbs1 complex do not appear to play significant roles in the checkpoint. Chk1 activation occurs at early stages, whereas Chk2 activation occurs much later. Overall we conclude that ATR/Chk1 pathway is activated at an early time point after the loss of Geminin and contributes to checkpoint arrest essential for the accumulation of re-replicated cells, whereas activation of the ATM/Chk2 pathway is a by-product of DNA re-replication at a later period.

INTRODUCTION

Duplication of chromosomal DNA is a key event in the cell cycle. Cells have developed multiple mechanisms to ensure accurate duplication of genetic materials. In eukaryotes, DNA replication initiates at areas known as replication origins, which are recognized by a six-subunit complex called origin recognition complex (ORC) (1-3).

Cdc6 and Cdt1 are then independently recruited at ORC associated origins either in the late mitosis or early G1 phase (1,3,4). The MCM2-7 complex is subsequently recruited by Cdc6 and Cdt1 to the replication origins to initiate DNA replication (5,6).

Geminin, an inhibitor of DNA replication initiation (7-9), can physically interact with and inhibit the activity of Cdt1, ensuring firing of origins once per cell cycle. Geminin depletion by small interfering RNA (siRNA)[1] can induce DNA re-replication and activate G2/M checkpoint in both human and *Drosophila* cells (10-12). It was shown previously that Geminin depletion caused the activation of DNA damage protein kinases ATR (Ataxia Telangiectasia and Rad-3-related), Chk1 and Chk2, and induced G2/M cell cycle arrest in human colon cancer cells (12-14). Abrogation of this checkpoint by ATR depletion leads to apoptosis (12,13). Thus the accumulation of re-replicated cells after Geminin depletion was critically dependent on G2/M checkpoint activation.

Since re-replication is expected to lead to gene amplification, we consider it important to understand the re-replication induced checkpoint pathway. Although not much is known about the sensor proteins involved in the re-replication induced checkpoint pathway, a lot is known about the DNA damage pathways. Studies in yeast and mammals have implied that there are several important proteins working as DNA damage sensors in checkpoint pathways.

Rad9, Rad1, Hus1 and Rad17 proteins are required for the checkpoint activation (15-17). Rad9, Rad1 and Hus1 form a heterotrimeric protein complex 9-1-1 and

structurally resembles a proliferating cell nuclear antigen (PCNA)-like sliding clamp in both yeast and human cells (18-20). However, Rad17 is a checkpoint protein which shares homology with replication factor C 1 (RFC1) in structure and associates with four small RFC subunits (RFC2-5) to form a complex related to the PCNA clamp loader (16,19,21). Rad17 binds to chromatin before DNA damage and recruits Rad9-Rad1-Hus1 complex in response to DNA damage, probably acting as a clamp loader to load the 9-1-1 complex onto the damage sites. Once the 9-1-1 complex is bound to chromatin, it facilitates the phosphorylation of the substrates (like Chk1 kinase) by ATR (22). In cells lacking ATR, the Chk1 phosphorylation is blocked (23). Furthermore, in cells with reduced Rad17 or lacking Hus1, this phosphorylation is also inhibited (22,24).

The Rad17/RFC and 9-1-1 complexes are involved in ATR/Chk1 pathway, whereas Mre11/Rad50/Nbs1 (MRN) complex are implicated to play an important role in ATM/Chk2 pathway (16,25). MRN complex was suggested to work in both DNA damage checkpoint and repair pathways (26,27). Upon the induction of double-stranded breaks (DSBs), MRN complex gets recruited to the proximity of DNA damage sites independent of ATM (28) and is involved in the initial process of DSBs due to Mre11 nuclease activity (29). Furthermore, MRN complex is suggested to enhance ATM accumulation at damage sites and facilitate ATM activation (16,30-32), which is in turn necessary for the activation Chk2 kinase (33-35). Additionally, in vitro studies using either *Xenopus* extracts or purified human proteins have shown that ATM activation

requires MRN complex for its DNA-tethering and ATM-binding ability (36,37). Collectively, MRN complex is suggested to function as a DNA damage sensor and amplifier for the ATM/Chk2 signaling pathway.

Re-replication produces both single-stranded DNA and double-stranded DNA breaks (10,13), and both ATR/Chk1 and ATM/Chk2 pathways are activated. To measure the relative importance of the two pathways we therefore decided to check whether the cofactors for ATR or ATM activation in the DNA damage pathways are equally important for the checkpoint activation due to re-replication [Fig. 1].

EXPERIMENTAL PROCEDURES

Cell lines and drugs – Human colorectal cancer cell line HCT116 ($p53^{+/+}$) was grown in 10% fetal bovine serum and 1% penicillin-streptomycin in McCoy's 5A modified medium (Cellgro). The concentration of daunorubicin (Sigma) used was 0.05uM.

SiRNA – Short interfering (siRNA) oligonucleotides (Invitrogen) were made to following target sequences (sense): geminin (GEM), UGCCAACUCUGGAAUCAAA (12); Rad9 (Rad9), GUCUUUCCUGUCUGUCUUC; Rad17 (Rad17), CAGACUGGGUUGACCCAUC; Mre11 (Mre11), ACAGGAGAAGAGAUCAACU; ATM (ATM), GCGCCUGAUUCGAGAUCCU; and control oligonucleotide (GL2),

AACGUACGCGGAAUACUUCGA. Transfections were performed with 100nM siRNA oligonucleotide duplexes with Lipofectamine RNAiMAX (Invitrogen) to 1×10^6 HCT116 cells per 6㎝ dish according to the instructions of the manufacturer.

Antibodies and immunoblotting – Rabbit anti-geminin was raised as described earlier (9). Rabbit anti-Rad9, rabbit anti-Rad17, rabbit anti-ATM, mouse anti-CDC2, mouse anti-FANCD2 (Santa Cruz Biotechnology); rabbit anti-Mre11 (Novus Biologicals); rabbit anti-phospho-Chk1 (Ser317), rabbit anti-phospho-Chk2 (Thr68), rabbit anti-phospho-CDC2 (Tyr15) (Cell Signaling Technology); mouse anti-actin, mouse anti-Chk1, and mouse anti-Chk2 (Sigma) were used for western blotting. Cells were lysed in lysis buffer containing 0.1% NP40, 50mM Tris-HCl 7.4, 150mM NaCl, 5mM EDTA, 50mM NaF, 1mM Na_3VO_4 and protease inhibitor cocktail (Sigma). Equal amounts of cell lysates were resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then proteins were transferred and blotted with indicated antibodies.

FACS analysis – Cells were collected by trypsinization and fixed with 70% ethanol overnight in -80℃. Cells were centrifuged and stained with 500μl propidium iodide solution (0.05% NP40, 50 ng of propidium iodide per ml, and 10 μg of RNaseA per ml) after fixation. The labeled cells were analyzed on a Becton Dickinson flow cytometer with Cellquest Pro software.

RESULTS

Rad9 and Rad17 protein are required for G2/M checkpoint activation after Geminin depletion

ATR was previously shown by our lab to be crucial in arresting cells in response to re-replication (13). Since 9-1-1 (Rad9-Rad1-Hus1) and Rad17/RFC complexes are known to be important for regulating ATR activity, we chose to address whether one representative member of each complex is involved in the G2/M checkpoint induced by Geminin depletion.

RNA interference was performed to silence the expression of either Rad9 or Rad17 protein alone or together with Geminin in HCT116 cells [Fig. 2A]. As shown in Fig. 2C, Rad9 or Rad17 protein levels were significantly decreased in cells transfected with Rad9 or Rad17 siRNA but not with the control siRNA (GL2) or Gem siRNA. In addition, Geminin protein levels were extensively reduced only in cells transfected with Gem siRNA.

Previous results have shown that co-depletion of checkpoint protein (like ATR) and Geminin abrogates checkpoint activation and in turn decreases the accumulation of re-replicated cells because the cells enter mitosis with re-replicated chromosomes and undergo apoptosis (12,13). So an easy way to verify the importance of a protein in the checkpoint pathway is to assay the percentage of cells accumulating with >4N DNA

content by FACS analysis. Fig. 2B and Table 1 show that knockdown of Rad9 or Rad17 suppressed the accumulation of re-replicated cells after depletion of Geminin. Depletion of Rad9 or Rad17 alone does not have any effect on re-replication. Therefore both the proteins are likely important in the G2/M checkpoint pathway. Consistent with this, biochemical analysis showed the activation of checkpoint proteins was decreased in cells where Rad9 or Rad17 proteins were knocked down together with Geminin. Geminin depletion causes Chk1, Chk2 phosphorylation and Cdc2 phosphorylation on Tyr15 [Fig. 2C, lane 2 and 6]. In addition, the Fanconi anemia core complex gets activated after Geminin depletion and monoubiquitinates FANCD2, activating DNA repair pathways (12-14,38). In the absence of Rad9 or Rad17 proteins, phosphorylation of Chk1, inhibitory phosphorylation on Cdc2 and monoubiquitination of FANCD2 were decreased after Geminin depletion [Fig. 2C, lane 4 and 8]. Therefore both Rad9 and Rad17 proteins are independently required for the checkpoint activation after re-replication.

Mre11 and ATM are not required for checkpoint pathway and overreplication in Geminin depleted cells

It was shown previously that both single-stranded DNA and double-stranded DNA breaks were generated during DNA re-replication (13). Since double-stranded DNA breaks activate ATM (26), we wanted to test whether ATM is required for the checkpoint activation and accumulation of re-replicated cells. Furthermore, MRN

complex is implicated to be the DNA damage sensor for detecting dsDNA breaks. We decided to choose Mre11 protein as a representative member of the complex to test the requirement of the complex for the checkpoint activation upon Geminin depletion.

In Fig. 3B, either Mre11 or ATM protein levels were notably reduced in cells treated with the corresponding siRNA. Surprisingly, the accumulation of re-replicated cells was not affected by depletion of either Mre11 or ATM together with Geminin protein [Fig. 3A and Table 1]. Consistent with the FACS results, Chk1 activation and Cdc2 phosphorylation on Tyr15 were not influenced by the co-depletion of Mre11 or ATM [Fig. 3B, lane 4 and 8]. Additionally, FANCD2 monoubiquitination was not changed.

Although the percentage of re-replicated cells did not change after co-depletion, Chk2 phosphorylation on Thr68 was significantly decreased [Fig. 3B, lane 4 and 8]. This is consistent with the previous reports that ATM and MRN are required for the activation of Chk2 (33,35,37,39). It also suggests that ATM and Mre11 knockdown have sufficiently decreased the corresponsive protein levels to deregulate their functions.

Together, these data suggests that the ATM/MRN pathway is not involved to induce G2/M arrest and re-replication after Geminin depletion.

Chk1 is required for Chk2 activation after Geminin depletion

To our surprise, Chk2 phosphorylation on Thr68, which is normally a

consequence of ATM activation (16,33-35), was similarly reduced after co-depletion of Geminin and Rad9 or Rad17 [Fig. 2C, lane 4 and 8]. This could be either a direct or an indirect result due to the decrease of ATR activity. To distinguish the two possibilities, we performed siRNA against Chk1, the downstream substrate of ATR, together with Geminin and examined Chk2 activation. If ATM/Chk2 and ATR/Chk1 pathways work in parallel, and the reduction in Chk2 activation is simply due to the crosstalk between the two pathways, Chk1 depletion will not affect Chk2 activation. Otherwise, ATM/Chk2 pathway can probably located downstream of ATR/Chk1 pathway.

In Fig. 4, Chk1 protein level was significantly decreased in cells treated with the SiRNA. After Geminin depletion, Chk2 was activated and phosphorylated on Thr68 [Fig. 4, lane 2]. However, the phosphorylation was reduced without the presence of Chk1. The results suggested Chk1 activation is probably upstream of Chk2 activation. Additionaly, instead of functioning in parallel and overlapping with each other, ATM/Chk2 and ATR/Chk1 pathways might work together after Geminin depletion.

Chk1 is activated earlier in re-replication while Chk2 activation occurs at later stages

As shown above, the Rad9/ATR/Chk1 pathway is required for the accumulation of re-replicated cells but MRN/ATM/Chk2 was not. In addition, Chk1 activation is possibly upstream of Chk2 activation. We wondered whether Chk2 activation was truly a

later event in cells undergoing re-replication.

Around 18 hours after Geminin siRNA transfection, a G2/M cell cycle arrest was observed and there was a little more re-replication in Geminin depleted cells compared to control siRNA (GL2) treated cells [Fig. 5A]. 24 hours after the knockdown, a more obvious G2/M arrest was observed in Geminin knockdown cells and the percentage of re-replicated cells increased further. The phosphorylation of Chk1 protein at these two time points was similar [Fig. 5B]. However, Chk2 protein phosphorylation was not notably seen until 30 hours after the siRNA transfection, when 32.5% of re-replication was detectable in the cells in the population. In summary, Chk1 was activated around the time of G2/M cell cycle arrest and before the immense accumulation of re-replicated cells, while convincing Chk2 activation was observed relatively late, much after the significant accumulation of re-replicated cells.

DISCUSSION

The loss of Geminin in human cancer cells causes a G2/M checkpoint activation and DNA re-replication. In this paper, we demonstrate that Rad9 and Rad17 proteins are both required for the checkpoint activation and accumulation of re-replicated cells. The downstream substrate Chk1 in the same pathway is phosphorylated probably before the huge accumulation of re-replicated cells. Nevertheless neither Mre11 nor ATM is

necessary in this process. The Chk2 protein, normally activated in an ATM-dependent manner (16,33-35), is activated comparatively late in the process.

Geminin is an inhibitor of Cdt1, a pre-RC (pre-replicative complex) component (7-9,40). After Geminin depletion in mammalian cells, an extra round of DNA replication is initiated and cells with greater than G2 DNA content appear. Generally, the activation of ATR and Chk1 is considered as a consequence of the formation of single-stranded DNA, whereas the ATM and Chk2 activation is more observed in cells with double-stranded DNA breaks (41). The generation of single-stranded DNA in the re-replicated cells might therefore account for the induction of the G2/M checkpoint mediated by ATR, Rad9/Rad1/Hus1 and Rad17/RFC. DNA re-replication continues as the cells are arrested in G2/M phase. Upon further re-replication, more forks collapse, which possibly produces double-stranded DNA breaks together with single-stranded DNA. During the process, the MRN complex probably works as a sensor for the double-stranded DNA breaks and an amplifier to activate ATM and in turn Chk2 kinase. Additionally, the Mre11 nuclease might help to process the double-stranded breaks to single-stranded DNA and contribute to the ATR regulated pathway. The ATM/MRN/Chk2 pathway activation is therefore a byproduct of the accumulated re-replication that amplifies the downstream signals (inhibition of Cdc25c and Cdc2) for checkpoint activation, but is not the major determinant for the accumulation of re-replicated cells [Fig. 6].

A primary role of ATR/Chk1 in the G2/M checkpoint activation following re-replication is consistent with previous studies in *Drosophila*, *Xenopus* and human colon cancer cells. In *Drosophila*, Chk2 inactivation does not create an immense change on Geminin deficiency induced re-replication (11). In *Xenopus* egg extracts, depletion of Geminin causes a Chk1 dependent G2 arrest (14). In human cancer cells, co-depletion of Chk2 does not produce a significant effect on the accumulation of re-replicated cells after Geminin silencing as compared to Chk1 co-depletion (12).

Despite the distinctive functions of ATR/Chk1 and ATM/Chk2 in DNA damage checkpoint, there is plenty of evidence showing crosstalk between the two pathways (27), which means that either Chk1 or Chk2 can be the substrates of both ATM and ATR proteins. We speculate that at the early time point after Geminin depletion, the ATR activation signal induced by single-stranded DNA has not reached the threshold to result in noteworthy phosphorylation of Chk2. As cells were arrested by G2/M checkpoint, more DNA re-replication occurs and the ATR activation signal is amplified and joined by the ATM activation signal to eventually activate Chk2. Thus although the ATR and ATM kinases respond to DNA damage and activate both Chk1 and Chk2, our results suggest that the lesions caused initially by re-replication are primarily the generation of ssDNA leading to the preferential activation of ATR and Chk1.

Given the many reports of activation of p53 after over-activity of Cdt1 and Cdc6 (42-44), our results also suggest a hierarchical process of checkpoint activity leading to

the activation of p53. The ATR/Chk1 pathway initially causes the G2/M arrest that allows re-replication to continue. At later stages of re-replication, fork collapse and replication across single-stranded nicks (on a template with unligated Okazaki fragments) creates double stranded DNA breaks that activate ATM/Chk2 and p53. Such a graduated response to re-replication would give the cell a chance to repair minor degrees of transient re-replication during the G2 phase while reserving the use of p53 to induce apoptosis only when there is extensive and prolonged re-replication.

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ABBREVIATION

siRNA, short interfering RNA; ATR, ataxia telangiectasia and Rad-3-related; ATM, ataxia telangiectasia mutated; RFC, replication factor C; MRN, Mre11-Rad50-Nbs1; ssDNA, single-stranded DNA; FACS, fluorescence-activated cell sorter.

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FIGURE LEGENDS

Figure 1. Model of DNA damage checkpoint activation induced by Geminin depletion. Two pathways are possibly used to activate G2/M checkpoint arrest. However, it is unknown yet whether both pathways are used or one of them is preferred.

Figure 2. Rad9 and Rad17 proteins are required for the accumulation of re-replicated cells and G2/M checkpoint activation after Geminin depletion in HCT116 cells. (A) Schematic of transfection protocol. (B) Histogram of cells transfected with indicated siRNA duplex following the protocol described in panel A. The cells were harvested and stained with propidium iodide (PI) for DNA content before flow cytometry analysis. y axis, cell count; x axis, PI fluorescence; the percentage of cells containing greater than 4N DNA is shown. (C) G2/M checkpoint activation is suppressed without Rad9 or Rad17 in Geminin depleted cells. HCT116 cells treated as in Fig. 2A were immunoblotted for the indicated proteins.

Figure 3. Mre11 and ATM proteins are not required for the accumulation of re-rereplicated cells and G2/M checkpoint activation in HCT116 cells. (A) Histogram of cells with indicated siRNA transfection following protocol in Fig. 2A. The percentage of cells with >4N DNA is shown. (B) Checkpoint activation after indicated siRNA transfection. HCT116 cells treated as in panel A were immunoblotted for the indicated proteins.

Figure 4. Chk1 is required for Chk2 activation after Geminin depletion. HCT116 cells were transfected with siRNA of Chk1 and/or Geminin following the protocol in Fig.2A. Cells were harvested at 72 hours after transfection and immunoblotted for the indicated proteins.

Figure 5. Time course of checkpoint kinase activation after Geminin depletion in HCT116 cells. (A) Histogram of cells with indicated siRNA transfection. HCT116 cells were transfected with either control siRNA duplex GL2 or Geminin and harvested at 18, 24 or 30 hours. Cells were stained with PI and analyzed by flow cytometry. The percentage of cells with more than 4N DNA is shown in the panel. (B) Chk1 protein is activated in an early period of DNA rereplication and Chk2 protein is activated later. HCT116 cells treated as shown in panel A were immunoblotted for the indicated proteins.

Figure 6. Model for the checkpoint activation after re-replication by Geminin depletion. The G2/M arrest induced by Geminin depletion and DNA rereplication in HCT116 cells is primarily due to the activation of ATR/Chk1 pathway. Chk2 activation is a late consequence of extensive re-replication.

Table. 1 Percentage of re-replication after indicated siRNA

HCT116 cells were transfected with indicated siRNA duplex following the protocol described in Fig. 2A. The cells were harvested for FACS analysis. Cells with greater than

4N DNA content are regarded as cells undergoing re-replication. Mean ± standard deviation from three experiments are shown.

Checkpoint protein knocked down in columns 4 and 5	GL2 RNAi	GEM RNAi	Checkpoint protein RNAi	Checkpoint protein + GEM RNAi
Rad9 Knockdown	5.57±1.09	52.93±2.51	6.65±0.54	25.46±1.97
Rad17 Knockdown	4.73±1.68	49.85±0.88	7.43±0.73	24.14±4.00
Mre11 Knockdown	3.94±0.31	56.60±1.25	3.44±0.16	55.06±1.59
ATM Knockdown	3.43±0.46	52.38±2.33	6.03±1.15	52.69±3.44

Figure 1. Model of DNA damage checkpoint activation induced by Geminin depletion.

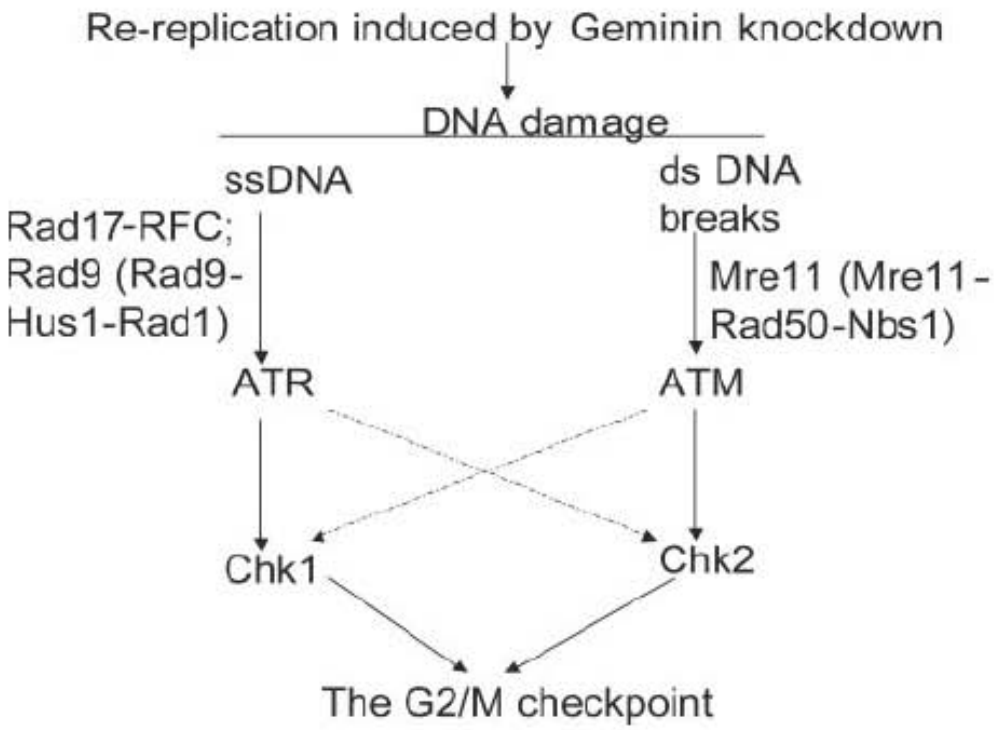
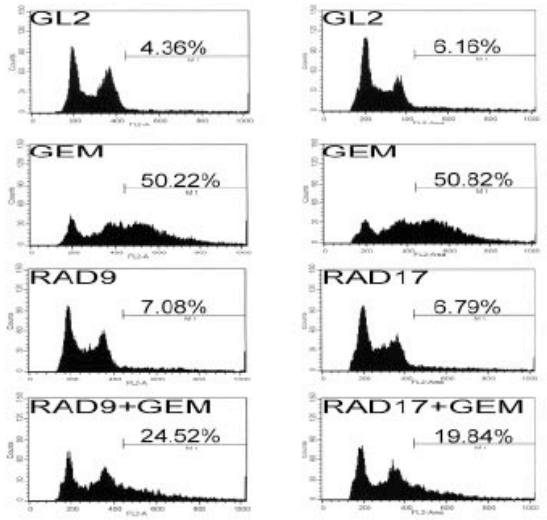


Figure 2. Rad9 and Rad17 proteins are required for the accumulation of re-replicated cells and G2/M checkpoint activation after Geminin depletion in HCT116 cells.

A

DAY\ SiRNA	GL2	GEM	Rad9	Rad9+ GEM
DAY 1	GL2	GL2	Rad9	Rad9
DAY 2	GL2	GEM	GL2	GEM
DAY 3	CHANGE MEDIA			
DAY 4	HARVEST FOR ANALYSIS			

B



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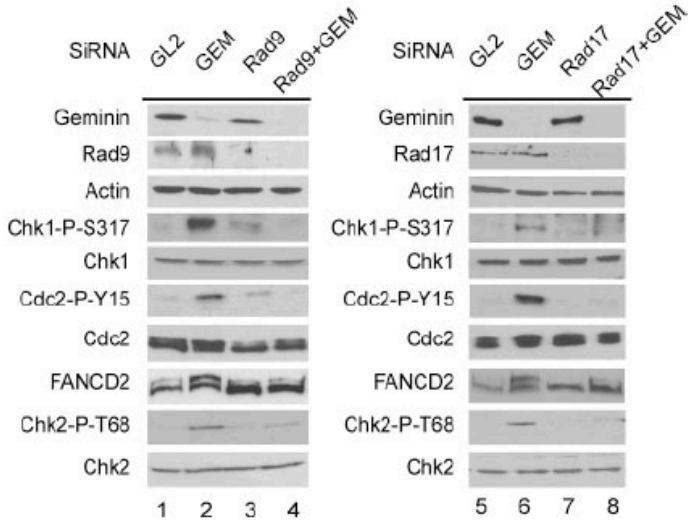


Figure 3. Mre11 and ATM proteins are not required for the accumulation of re-rereplicated cells and G2/M checkpoint activation in HCT116 cells.

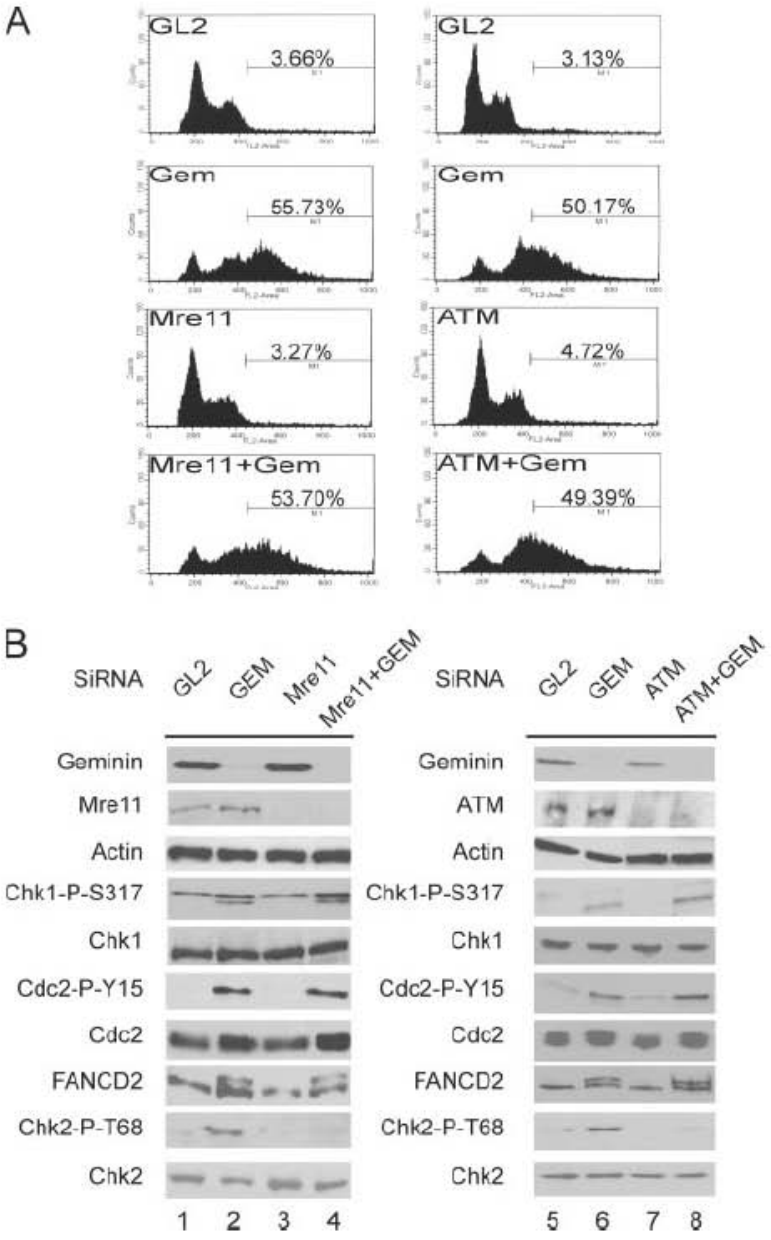


Figure 4. Chk1 is required for Chk2 activation after Geminin depletion.

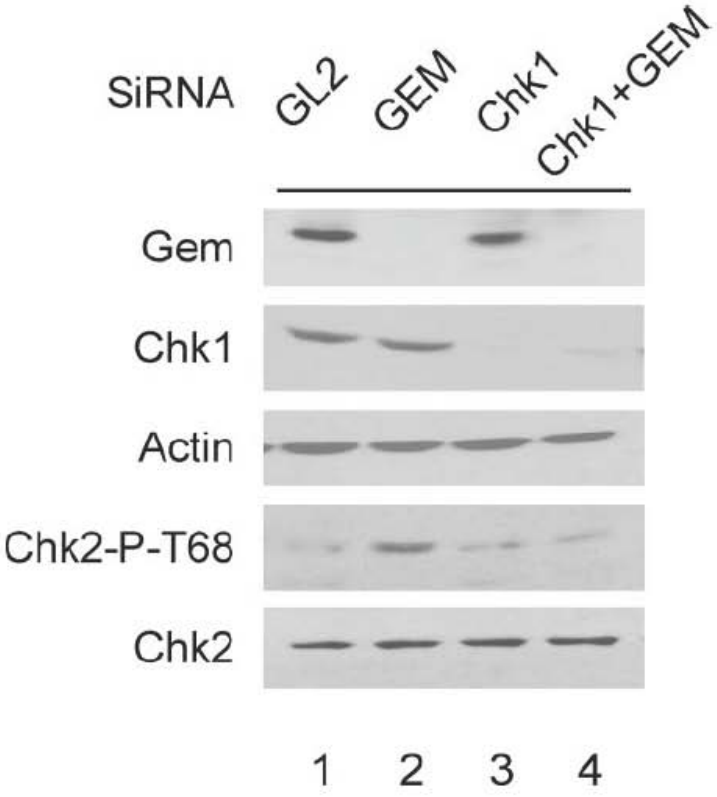


Figure 5. Time course of checkpoint kinase activation after Geminin depletion in HCT116 cells.

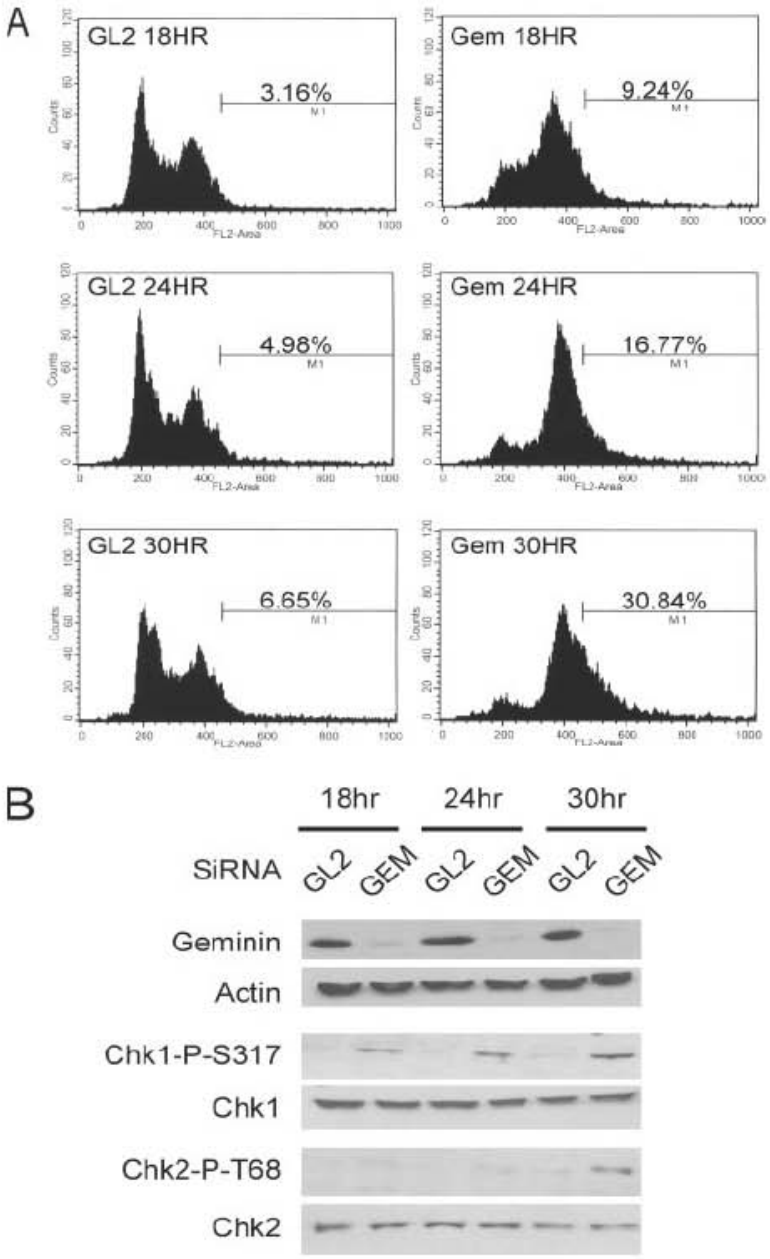
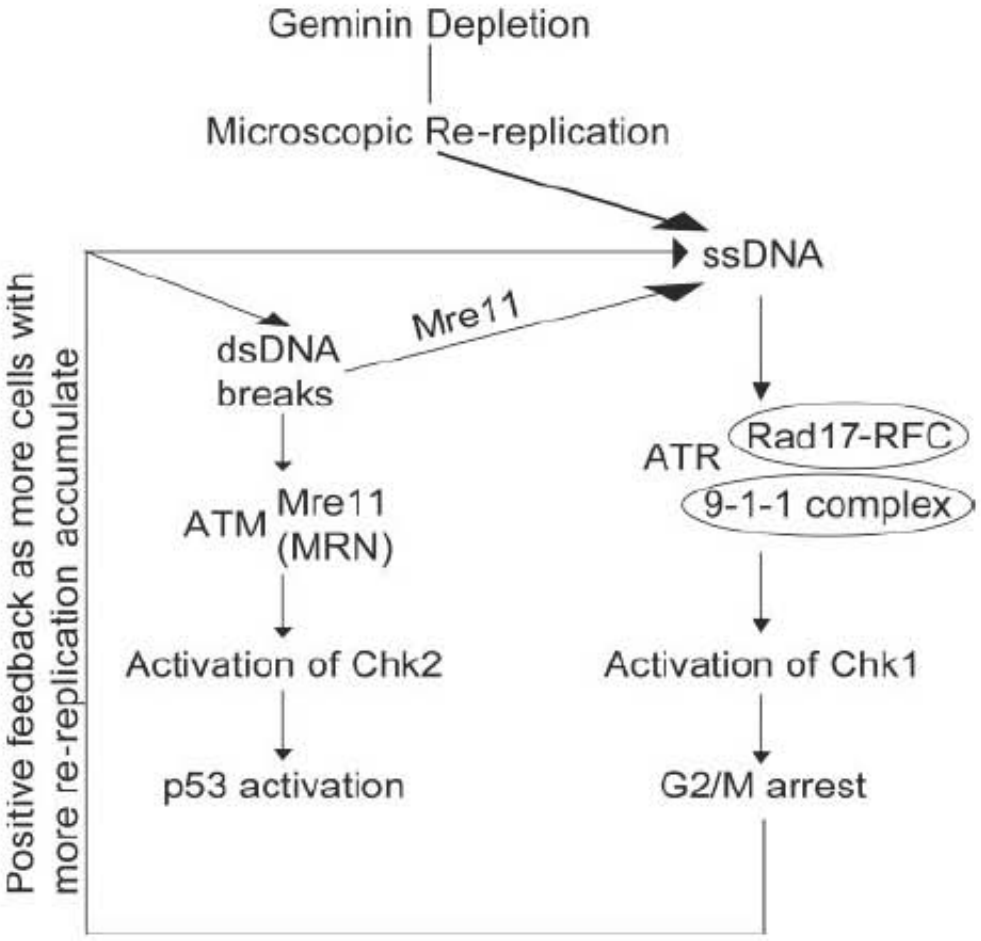


Figure 6. Model for the checkpoint activation after re-replication by Geminin depletion.



CHAPTER THREE

NEDD8 TARGETING DRUG MLN4924 ELICITS DNA RE-REPLICATION BY STABILIZING CDT1 IN S PHASE, TRIGGERING CHECKPOINT ACTIVATION, APOPTOSIS AND SENESCENCE IN CANCER CELLS

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Precis: Findings reveal insights into the mechanism of action of an important new drug in clinical trials, also showing that even transient exposure to p53 mutant cancer cells may be sufficient to produce potent anticancer effects.

Attributions:

The Timelapse Movie analysis (Fig. 6A) was done by U.N.

P53 and p21 western blots (Fig. 6F) were done by M.A.M.

MLN4924 IC50 measurement (Fig. S4C) was done by M.A.M, U.N., and P.G.S.

All other experiments were done by J.J.L.

ABSTRACT

MLN4924 is a first-in-class experimental cancer drug that inhibits the NEDD8-activating enzyme, thereby inhibiting cullin-RING E3 ubiquitin ligases and stabilizing many cullin substrates. The mechanism by which MLN4924 inhibits cancer cell proliferation has not been defined, although it is accompanied by DNA re-replication and attendant DNA damage. Here we show that stabilization of the DNA replication factor Cdt1, a substrate of Cullins 1 and 4, is critical for MLN4924 to trigger DNA re-replication and inhibit cell proliferation. Even only one hour of exposure to MLN4924, which was sufficient to elevate Cdt1 for 4-5 hours, was found to be sufficient to induce DNA re-replication and to activate apoptosis and senescence pathways. Cells in S phase were most susceptible, suggesting that MLN4924 will be most toxic on highly proliferating cancers. Although MLN4924-induced cell senescence appears to be dependent on induction of p53 and its downstream effector $p21^{Waf1}$, we found that $p53^{-/-}$ and $p21^{-/-}$ cells were even more susceptible than wild-type cells to MLN4924. Our results suggested that apoptosis, not senescence, may be more important for the anti-proliferative effect of MLN4924. Further, our findings show that transient exposure to this new investigational drug should be useful for controlling p53-negative cancer cells, which often pose significant clinical challenge.

INTRODUCTION

Duplication of the genetic material is a key event in the cell cycle. In eukaryotes, replication origins are recognized and bound by a six-subunit complex called ORC (Origin Recognizing Complex) (1-3). Cdc6 and Cdt1 are subsequently recruited independently to those sites in late M or early G1 phase (1, 3, 4), followed by the recruitment of MCM2-7 complex to initiate DNA replication (5, 6). It is vitally important that the initiation of replication at replication origins is tightly controlled such that it occurs only once during the cell cycle. Mammalian cells have developed different mechanisms to prevent re-initiation and subsequent re-replication of DNA within the same cell cycle. One such mechanism is the inactivation of Cdt1 during S and G2 phases (7, 8). After replication initiation, Cdt1 is either inhibited by a small protein called Geminin (9, 10) or degraded by two distinct E3 ligases – cdk-dependent SCF^{skp2} and Cul4-$DDB1^{cdt2}$ in S or G2/M phase (8, 11). Deregulation of those pathways by depletion of Geminin, Cul4 or Cdt2 activates (or stabilizes) Cdt1 and consequently induces DNA re-replication in different systems (7, 12-14).

Studies have shown that cullin-RING ligases (CRLs), a subclass of E3 ligases that includes both SCF^{skp2} and $CRL4^{Cdt2}$, are modified by an ubiquitin-like protein NEDD8, which subsequently facilitates their ligase activities (15-18). Thus, through the modulation of this activity, the NEDD8 pathway regulates the abundance of CRL substrates. MLN4924, a potential cancer drug currently in phase I clinical trials, is a small molecule inhibitor of NEDD8 activating enzyme (NAE) (19, 20). MLN4924 treatment in HCT116 human colon cancer-derived cell line inhibits NAE, and therefore the NEDD8 conjugation pathway, resulting in an increase in protein abundance of CRL substrates such as Cdt1 (21). This is accompanied by an increase in the percentage of

cells containing more than 4N DNA, indicating DNA re-replication was occurring. Cells treated with MLN4924 also undergo significant apoptosis contributing to the drug's anti-proliferative activity. Various CRL substrates play critical functions in cellular growth and survival pathways and the question remained as to which substrates are critical for MLN4924 induced re-replication and apoptosis.

In this paper, we examine whether Cdt1 is the key factor for the induction of DNA re-replication in HCT116 cells treated with MLN4924. Among the different approaches for stimulating Cdt1 activation, MLN4924 shares a similarity with that of Cdt2 depletion in inactivating the $CRL4^{cdt2}$ E3 ligase, as opposed to Geminin depletion, which activates Cdt1 by a different pathway. We verified this hypothesis and detected a synergistic effect between MLN4924 treatment and Geminin depletion. Transient exposure of cells to MLN4924 led to DNA re-replication, as well as activation of the apoptosis and senescence pathways. This allowed us to test whether a specific part of the cell cycle was particularly susceptible or resistant to MLN4924. Finally, we compared the sensitivity of wild-type (WT) HCT116 cells and isogenic $p53^{-/-}$ or $p21^{-/-}$ HCT116 cells to MLN4924, and discovered that WT HCT116 cells were less susceptible to MLN4924 induced cell death. The results indicate that p53-deficient cancer cells may be more sensitive to MLN4924, emphasizing the therapeutic opportunity with this class of investigational drugs.

MATERIALS AND METHODS

Cell Lines and Chemicals

Human colorectal cancer cell lines HCT116 (WT, p53-/-, p21-/-) were cultured in McCoy's 5A modified medium (HyClone) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Isogenic $p21^{-/-}$ and $p53^{-/-}$ HCT116 cell lines were described earlier (22). Millennium Pharmaceuticals Inc provided MLN4924, which was then dissolved in DMSO (Sigma). The concentration of Z-Vad-FMK (Calbiochem) used was 50μM. The concentration of Nocodazole (Sigma) used was 40ng/ml.

siRNA

Short interfering (siRNA) oligonucleotides (Invitrogen) were made to the following target sequences (sense): GL2 (control), AACGUACGCGGAAUACUUCGA; Cdt1, GCAAUGUUGGCCAGAUCAA; Cdc6, GAUCGACUUAAUCAGGUAU; Mcm7, GAUGUCCUGGACGUUUACA; Geminin (Gem), UGCCAACUCUGGAAUCAAA (12); Cdt2, GAAUUAUACUGCUUAUCGA. Transfections were performed with 20nM siRNA oligonucleotide duplexes with Lipofectamine RNAiMAX (Invitrogen) according to the instructions of manufacturer.

Antibodies and immunoblotting

Rabbit anti Cdt1, rabbit anti-geminin, and rabbit anti-Cdt2 were raised as described (9, 23). The purchased antibodies were mouse anti-p21 (Lab vision/Neomarkers); mouse anti-β-actin, mouse anti-Chk1, mouse anti-Chk2 (Sigma); rabbit anti-Chk1-P-S317, rabbit anti-Chk2-P-T68; rabbit anti-PARP, rabbit anti-H3-P-S10 (Upstate). Cells were

lysed as described (24), and western blot analysis was performed according to standard procedures.

Flow Cytometry Analysis (FACS)

Cells were harvested by trypsinization and fixed with 70% ethanol overnight at -80°C. Cells were then stained and analyzed as described before (12). For FACS analysis with both PI and BrdU double staining, cells were labeled with 10μM BrdU (Sigma) and then harvested as described earlier (12).

Timelapse Movie analysis

HCT116 cells were plated at 15,000 cells per well in 6-well culture plates (Becton Dickinson). For continuous treatment cells were treated with 1μM MLN4924 for 72 hours. For wash-out treatment HCT-116 cells were treated with 1μM MLN4924 for 8 hours then washed with fresh media to remove compound and maintained in fresh compound free media for 8 days. Timelapse movie images were taken at times indicated using an automated TE2000U microscope (Nikon Instruments, Melville, NY) with Hoffman-modulation optics, 20x objective, with environmental control, and an Orca-ER CCD camera (Hamamatsu, Bridgewater, NJ) controlled with MetaMorph imaging software (Molecular Devices, Downingtown, PA).

Measure Cell Growth and Clonogenicity

The number of viable cells was estimated with a cell proliferation assay (MTT) kit (Promega) according to the manufacturer's instructions. Cells were seeded into 96-well

plates at 500 cells per well, treated with DMSO or MLN4924 and incubated for 7 days before MTT assay. Cell clonogenecity assay was performed as described (25). Cells were seeded into 6-well plates at $3*10^3$ cells per well. DMSO or 1μM MLN4924 were added for 8 hours. Cells were washed twice with PBS and incubated in fresh medium after the wash-out. Medium was changed every 2-3 days and the Colonies were stained with crystal violet to show cell clonogenicity. OD595 was measured to quantify cell colony numbers and normalized to DMSO treated control sample to obtain cell survival rate.

SA-β-gal Staining Assay for Senescence

Senescence β-galactosidase staining assay was performed in a 6-well plate with staining kit (Cell Signaling Technology, #9860). Cells were washed with PBS, fixed and stained following manufacturer's instruction. Stained plates were checked under a microscope for development of blue color. For each sample, SA-β-gal positive and total cell numbers were counted from 5 different microscopic fields (roughly >200 cells per field).

RESULTS

Stabilization of Cdt1 protein is critical for MLN4924-induced re-replication in HCT116 cells

Consistent with previous results (21), we observed re-replication after 20 hours of treatment of HCT116 cells with MLN4924 (Fig. 1A). To investigate whether the

regulation of Cdt1 protein level plays a role in MLN4924-induced re-replication, HCT116 cells were treated with siRNA oligonucleotides targeting Cdt1 for 48 hours prior to the addition of MLN4924. After 20 hrs of MLN4924 treatment, Cdt1 protein level increased significantly as expected (Fig. 1B, lane 1,2) and more than 40% of cells were determined to have re-replicated, containing >4N DNA content. However, in the cells depleted of Cdt1 by siRNA, the percentage of re-replicating cells reduced to 15% (Fig. 1A). In these cells, Cdt1 protein expression was effectively repressed (Fig. 1B, lane 1,3 and 4), although at a higher exposure, Cdt1 protein level was observed to be modestly induced in MLN4924 treated cells (Fig. 1B, lane 3,4) indicating that the drug was still inhibiting its degradation, potentially explaining the 15% of cells with >4N DNA.

Re-replication has been shown to induce both single-strand and double-strand DNA breaks, resulting in activation of checkpoint pathways (24, 26). Indeed, we observed both Chk1 and Chk2 phosphorylation in MLN4924 treated cells (Fig. 1B, lane 1, 2). Cdt1 siRNA treatment not only decreased the percentage of cells undergoing re-replication, but also decreased the activation of Chk1 and Chk2 (Fig. 1B, lane 2, 4), indicating that MLN4924 induces DNA damage primarily through Cdt1-dependent re-replication.

To characterize whether other replication initiators contribute to the re-replication induced by MLN4924, we systematically depleted other components of the pre-RC. We treated HCT116 cells hypomorphic for ORC2 (27) with MLN4924 and compared the extent of re-replication with that of WT cells. As shown before, although we could detect ORC2 in 6ug of extract from WT HCT116 cells, it was hard to detect ORC in even 60µg of e83 HCT116 cells (Fig. S1A). Despite this, there was no significant difference in the

amount of re-replication between these cell types (Fig. 1C). Furthermore, we performed siRNA knockdown of both MCM7 and Cdc6 (Fig. S1B, S1C) and observed no difference in the amount of MLN4924 induced re-replication in both cases (Fig. 1D, 1E). Interestingly, we noticed that MLN4924 treatment could also induce Cdc6 protein expression (Fig. S1C), which indicated that Cdc6 could be a potential CRL substrate. Overall, these results demonstrate that the ubiquitin-dependent degradation of Cdt1 protein is the rate-limiting step in preventing re-replication, and that stabilization of this component of the pre-RC by MLN4924 induces re-replication in HCT116 cells. The re-replication leads to DNA damage and activates checkpoint pathways.

MLN4924 induces re-replication through the inhibition of CRL4^{Cdt2}

Because MLN4924 functions as a NAE inhibitor (21), it is expected to inhibit all cullins, including Cul1 and Cul4 ubiquitin ligases known to degrade Cdt1 (28-32). Cdt2 depletion, and thus inactivation of CRL4^{Cdt2}, in zebra fish, xenopus egg extracts and human cancer cells induces DNA re-replication (13, 33). If CRL4^{cdt2} inhibition is the primary mechanism by which MLN4924 causes re-replication, one would predict that there should be no synergy between MLN4924 and siCdt2 in induction of re-replication.

To determine whether MLN4924 acts through the same mechanism to induce re-replication as that of Cdt2 depletion, we compared the Cdt1 protein level in cells depleted of Cdt2 by siRNA or treated with MLN4924. As seen in Figure 2A, Cdt2 depletion caused less Cdt1 accumulation than MLN4924 alone (Fig. 2A, lane 2 and 3) and together the two stabilized Cdt1 more (lane 4). However, the extent of re-replication caused by siCdt2 was more than that observed in cells treated with MLN4924 alone, and adding the

two together did not increase re-replication (Figure 2B). This suggests that (a) pure $CRL4^{Cdt2}$ inhibition with siCdt2 is more effective at inducing re-replication than inhibiting all cullins by MLN4924, but (b) once Cdt1 level has crossed a certain threshold, there is no further increase in re-replication with more Cdt1. In addition, other substrates/pathways affected by MLN4924 may make cells die, thereby decreasing the extent of re-replication observed.

In contrast to the lack of synergy in induction of re-replication by siCdt1 + MLN4924, siGeminin (to activate Cdt1 in S through G2 phase) + MLN4924 caused more re-replication than either agent alone. This is particularly evident when one consider the proportion of cells with >6N DNA content (Fig. 2B).

These results suggest that removal of an inhibitor of Cdt1 (Geminin) will act additively with stabilization of Cdt1 by MLN4924 to cause more re-replication. In contrast, inhibition of $CRL4^{cdt2}$ by siCdt2 and MLN4924 does not additively cause more re-replication, even though there was more stabilization of Cdt1. This result is consistent with the hypothesis that MLN4924 causes re-replication primarily through the inhibition of $CRL4^{cdt2}$.

Transient exposure of HCT116 cells to MLN4924 induces re-replication

One advantage of using MLN4924 treatment to induce re-replication lies in its ability to act rapidly. Previous studies have shown that in as little as 5 minutes following MLN4924 treatment, the NEDD8 pathway is inhibited concurrent with the accumulation of Cdt1 protein (20, 21). Therefore we wanted to test whether transient treatment of MLN4924 is sufficient to induce re-replication in HCT116 cells.

Surprisingly, as shown in Fig. 3A, one-hour exposure to MLN4924 was sufficient to induce re-replication in 40% of the cells. The percentage of cells with re-replication increased with longer pulse of MLN4924, but was close to its maximum after 4 or 8 hours of treatment, with 60 % of cells re-replicating their DNA.

To determine the rate of Cdt1 turnover following MLN4924 wash-out, we treated HCT116 cells with MLN4924 for 4 hours, and harvested the cells after different time periods. Cdt1 protein level increased following MLN4924 treatment (Fig. 3B, W0). However, by 4 hours after wash-out, Cdt1 level decreased to basal levels (Fig. 3B, W4). These results indicate that although Cdt1 stability returns to normal in roughly 4 hours after the removal of MLN4924, this short period of Cdt1 stabilization is sufficient to induce irreversible DNA re-replication.

S phase cells are more susceptible to MLN4924 induced re-replication

Since a mere 4-hour pulse treatment of MLN4924 leads to re-replication, we decided to test which portion of the cell cycle was more susceptible to the drug exposure. HCT116 cells synchronized by nocodazole block/mitotic shake-off were exposed to MLN4924 at the indicated times post-mitosis (Fig. 4A). Cells were then collected for flow cytometry analysis (FACS). As shown in Fig. 4B, cells started to enter G1 around 4 hours after nocodazole release and began S phase after approximately 12 hours. This suggests that the majority of the cells were in M-G1 at T4 and in either S phase or at the G1/S transition at T12. We saw a similar increase in Cdt1 protein level after MLN4924 treatment in both populations (Fig. 4C). However, whereas only 10% of the cells re-replicated when Cdt1 was stabilized in M-G1 phase cells, 50% of the cells showed re-

replication upon stabilization of Cdt1 in S phase cells (Fig. 4D). These results demonstrate that S phase cells are more susceptible to MLN4924-induced DNA re-replication.

To ask whether cells must be in S-phase for MLN4924 to induce re-replication, we labeled the cells with bromodeoxyuridine (BrdU) for 40 minutes to mark cells in active S phase, washed out BrdU and added MLN4924 for 4 hours. Cells were collected for FACS after 20 hours (Fig. 4E). BrdU positive cells, which were actively replicating when exposed to MLN4924, showed 44% of cells re-replicating, whereas only 14% of the BrdU negative cells re-replicated (Fig. 4F). These results further demonstrate that actively replicating cells are more susceptible to MLN4924–induced re-replication.

Both checkpoint and apoptosis pathways are activated upon short exposure of cells to MLN4924

Re-replication induces DNA damage and checkpoint activation (24, 26, 34). The initiation of DNA re-replication by a short exposure to MLN4924 led us to test whether checkpoint pathways were similarly activated in those cells. We treated HCT116 cells with MLN4924 for 8 hours and harvested cells 24 or 72 hours after wash-out. Chk1 was activated 24 hours after drug wash-out while DNA re-replication was seen in 30 to 55% of cells (at 1 and 3μM MLN4924, respectively). The DNA damage checkpoint pathway still persisted even at 72 hours after wash-out, when re-replication was observed in 10 to 25% of cells (Fig. 5A, 5B). In addition, we noticed that PARP cleavage happened only at the later time point, suggesting that apoptosis was not activated until 72 hours after wash-out. This was further confirmed by the increase of sub-G1 population cells (Fig. 5B).

Overall, these results were consistent with the idea that even transient exposure of MLN4924 leads to re-replication, activates checkpoint pathways and eventually induces apoptosis following irreparable DNA damage.

Senescence is induced after transient exposure to MLN4924 through the induction of p53 and p21

When culturing cells after transient exposure to MLN4924, we noticed changes in cell morphology starting approximately 72 hours post-wash-out, including an increase in cell size, intracellular vesicle accumulation and flatness. As shown in the upper panel of Fig. 6A, after 48 hours or more of continuous exposure to MLN4924, cells shrank and became round, suggesting those cells were undergoing apoptosis. However, after a transient 8-hour exposure to MLN4924, cells exhibited the flattened, vesiculated morphology, often noticed when cellular senescence pathway is activated.

Senescence is marked by permanent withdrawal from the cell cycle. To test whether MLN4924 induces senescence, we first performed colony formation assays to determine the clonogenicity of the cells (35). We added MLN4924 to HCT116 cells for 8 hours and cultured cells for 7 days after wash-out for colony formation as measured by crystal violet staining (Fig. S2A). Quantitation of the optical density of staining (Fig. 6B) showed that MLN4924 treatment suppressed the clonogenicity, a characteristic of senescent cells.

Senescence Associated β-gal (SA-β-gal) staining is a well-accepted biomarker of senescence (36). Transient treatment of cells with MLN4924 increased the percentage of re-replicating cells (Fig. 3A) and the percentage of SA-β-gal staining (Fig. 6C, 6D). This

suggested that the senescence pathway was activated upon transient exposure to MLN4924. These results were consistent with the earlier findings that re-replication can activate the DNA damage response leading to cellular senescence (37).

We next examined whether reduction of re-replication by Cdt1 depletion could decrease senescence following MLN4924 treatment. We performed a similar assay as that displayed in Fig. 1A, except that the HCT116 cells were exposed to MLN4924 for only 8 hours and cells collected for FACS after 24 hours or SA-β-gal staining after 72 hours (Fig. 6E). Consistent with our hypothesis, Cdt1 depletion reduced both re-replication and senescence to a similar degree. Together, these data suggest that re-replication induced by transient exposure to MLN4924 leads to senescence. 8 hr exposure to MLN4924 also induced apoptosis, as measured by the cleavage of PARP (Fig. 5A). Thus transient treatment with MLN4924 induces senescence or apoptosis (also evident in Fig. 6A, 72hr/WO), while continuous treatment with the drug leads mostly to apoptosis (Fig. 6A, 72 hrs).

Both p53 and p21 can have a function in the cellular senescence pathway (38, 39). We therefore examined protein expression levels of p53 and p21 over 8 days following an 8-hour treatment with MLN4924. Both p53 and p21 were induced 24 hour post-wash-out and their expression persisted thereafter for the entire time course (Fig. 6F). We then performed the SA-β-gal staining assay in $p53^{-/-}$ or $p21^{-/-}$ HCT116 cells to determine the level of senescence in the absence of these proteins. As shown in Fig. 6G, the number of SA-β-gal stained cells was only half in the $p21^{-/-}$ HCT116 compared to those of WT HCT116, indicating that p21 plays an important role in the senescence pathway. p53 appeared to be less essential than p21, which was consistent with results from other

studies (40). The p16 gene is silenced in these cells (41), so the residual senescence in the $p21^{-/-}$ cells was most likely by a p21- and p16- independent pathway.

p21 and p53 deficient HCT116 cells are more sensitive to transient treatment with MLN4924

Since transient exposure to MLN4924 causes both senescence and apoptosis, but senescence is attenuated in the $p21^{-/-}$ cells, we could ask how important is the senescence for the toxicity of MLN4924 on cancer cells. We noticed that although p21- and p53-deficient cells exhibited less senescence after transient treatment with MLN4924, the total cell numbers observed were much less than in WT cells, which suggested that $p53^{-/-}$ or $p21^{-/-}$ HCT116 cells might be more susceptible to overall cell death or growth inhibition by MLN4924. Colony formation assays following an 8-hour treatment of MLN4924 in all three cell-lines confirmed this (Fig. 7A). Consistent with our previous results (Fig. 6B), colony formation was decreased by MLN4924 treatment in a dose-dependent manner in WT HCT116 cells, but the p53- and p21- deficient cells formed fewer colonies than WT cells, indicating that the absence of p53 or p21 sensitized the cells to the drug treatment. Since senescence is attenuated in the mutant cells (Fig. 6G), the result suggests that apoptosis pathways are important for cell killing after transient exposure to MLN4924.

We also performed an MTT cell growth assay to compare cell survival rate upon either transient or 72-hour continuous exposure to MLN4924. After 8-hour treatment, the IC_{50} for WT, $p53^{-/-}$ and $p21^{-/-}$ cells were 0.9μM, 0.18μM and 0.25μM, respectively (Fig. 7B), which was consistent with the results of the colony formation assays. However, the

difference was much smaller upon 72-hour continuous exposure: IC_{50} of 0.08μM, 0.07μM and 0.07μM in WT, $p53^{-/-}$ and $p21^{-/-}$ cells, respectively (Fig. S4A).

To confirm that cells with mutant p53 were more susceptible to cell death by MLN4924, we performed MTT assay in MCF7 cells after MLN4924 transient exposure. After p53 knockdown, IC_{50} decreased from 0.68μM to 0.4μM (Fig 7B, S4B). Similar results were obtained when we compared IC_{50} in two lung cancer cells NCI-H23 (p53 mutant, IC_{50} 0.28μM) and NCI-H460 (wild type p53, IC_{50} 1.5μM) (Fig 7B). Intriguingly, when we compared IC_{50} in more than 20 cancer cell lines upon 72hr MLN4924 treatment, after dividing them into p53 WT (wild type) group and p53 MT (mutant) group, we found the median value of WT group was significantly higher than MT group (465nM vs. 280nM, Fig. S4C) despite all the different genetic backgrounds. It must be noted, however, that there are other genetic factors that affect MLN4924 sensitivity besides p53 status. For example we obtained opposite results when comparing MCF7 (WT p53) with MDA-MB-231 (MT p53) cells where the MCF7 cells were more susceptible to MLN4924 (Fig. S4D). Despite this exception, our data suggests that p53 mutant cells are generally more susceptible to MLN4924.

Thus there is a clear therapeutic advantage of transient MLN4924 treatment, particularly considering that the p53 mutant cells are more susceptible to cell death than the p53 WT cells. Since up to 50% of human tumors have a mutant p53 gene, our results suggest that the kinetics of MLN4924 administration might alter the therapeutic index.

DISCUSSION

As a potential anti-cancer drug, MLN4924 was discovered to inhibit NAE activity, inhibit cullins, increase the expression of CRL substrates, induce re-replication and cause cell death (21). In this paper, we demonstrated that the regulation of Cdt1 protein level is the rate-limiting step for the induction of re-replication upon MLN4924 treatment. It is noteworthy that even transient exposure of HCT116 colon cancer cells to MLN4924 leads to DNA re-replication. Once re-replication is induced, DNA damage checkpoint pathways are activated, which then lead to apoptosis and cellular senescence. We found that $p53^{-/-}$ and $p21^{-/-}$ HCT116 cells are both more sensitive to MLN4924 exposure than wild type cells, indicating that cancer cells with p53 mutations, are likely more susceptible to transient exposure to the drug.

Various CRL substrates accumulate upon MLN4924 treatment, including Cdt1, p27, NRF2(21) and possibly Cdc6 (Fig. S1C). However, our data suggests that the deregulation of Cdt1 protein level plays an essential role in DNA re-replication induction, demonstrated by the decline in re-replication when Cdt1 is knocked down. That depletion of Orc2, MCM7 and Cdc6 did not prevent re-replication should not be interpreted to say that pre-RC components are not required for re-replication. The more likely hypothesis is that these proteins are in vast excess and so do not become rate limiting for re-replication after siRNA depletion.

We noticed a high G2 peak and a residual 15% of cells re-replicating after MLN4924 treatment in Cdt1 depleted cells (Supplementary Fig. S1C). The 15% of cells labeled as re-replicating could arise from the tail of the large G2/M peak observed and may not be real re-replication that leads to DNA damage, as there was no activation of either Chk1 or Chk2 in these cells (Fig. 1B). Taken together, these data show that

MLN4924 cause a G2/M arrest, consistent with the report that siCdt2 can induce G2/M arrest (13). This hypothesis was further confirmed by the increased phosphorylation of Cdc2 on Y15 and the loss of phosphorylation of H3 on S10 in cells treated with MLN4924 (Supplementary Fig. S1E), indicating that cells cannot enter mitosis. None of these changes, increase in G2 population, increase in Cdc2-P-Y15, and decrease of H3 phosphorylation, were relieved by decreasing Cdt1. Thus unlike re-replication, the G2/M block seen with MLN4924 may be due to stabilization of substrates other than Cdt1.

In vivo data suggested that Cdt1 protein level peaked at 2-4 hours after injection of MLN4924 into tumor-bearing mice and started to decrease by 4-8 hours post-injection (21). Therefore we wished to evaluate the effect of transient exposure of cancer cells to MLN4924. Amazingly even one-hour exposure was sufficient to induce re-replication in 40% of a colon cancer cell population in culture. With short treatment, we discovered that S phase cells were more susceptible to MLN4924 induced re-replication, which is consistent with the idea that S phase cells have already licensed origins (and fired many of them), so that relicensing by transient stabilization of Cdt1 would cause re-replication. The observations that transient exposure can lead to re-replication and S phase cells are more susceptible to this exposure are positive indicators for the clinical usefulness of this compound.

In addition to activation of apoptosis, we observed activation of senescence pathway after transient exposure of MLN4924. As previously stated, this was not due to a reduction in re-replication, as HCT116 cells displayed an equivalent increase in cells with a >4N DNA content even after short treatment with MLN4924 compared to continuous treatment (Fig. 3). This re-replication subsequently led to DNA damage and activated

checkpoint and apoptosis pathways (Fig. 5). Unexpectedly, we observed that the senescence phenotype did not appear in continuously treated cells (Fig. 6A). Although there was barely any difference in the extent of re-replication between the two treatments, DNA damage signals (DDS) were possibly different, resulting in a different choice of cell fate between apoptosis and senescence (42). Upon short exposure, no new DDS occurred from persistent origin re-firings, which likely occurred in the continuously treated cells. This lower level of DNA damage signaling perhaps is not great enough in duration or extent to trigger cells apoptosis, though it is sufficient to induce p21 and p53.

One remaining question is what activates senescence. Is it related to re-replication induced DNA damage? Previous papers suggested DNA damage caused by re-replication could activate the senescence pathway (37, 43, 44). In our hands, depletion of Geminin or Emi1 in HCT116 cells similarly induced senescence after 3-4 days (data not shown). Consistent with this idea, decrease in re-replication by depletion of Cdt1 reduced cellular senescence (Fig. 6E). Thus the senescence is triggered by the re-replication induced DNA damage. Another intriguing question is, once cell fate has been determined, is it reversible? We treated cells with Z-VAD-FMK together with MLN4924 to inhibit cells from entering apoptosis (Supplementary Fig. S3). However, there was no significant increase in senescence, which suggested an irreversible commitment to apoptotic, non-senescent pathways.

It has already been suggested that p53 and p21 level are increased during cellular senescence (45, 46). Multiple studies have shown that the p53-p21 pathway is critical for senescence to occur in human fibroblasts and cancer cells (38, 42, 47, 48). However, some researchers have observed that although p53 and p21 are positive factors in

senescence they are not necessary (49). Our results suggest that p53 and p21 have important functions in initiating cellular senescence upon MLN4924 treatment in tumor cells, but they are dispensable given that $p53^{-/-}$ or $p21^{-/-}$ cells showed decreased but not absent SA-β-gal staining (Fig. 6G).

Although both $p53^{-/-}$ and $p21^{-/-}$ HCT116 cells underwent less senescence than WT cells, both were more susceptible to cell death after transient treatment with MLN4924 (Fig. 7), suggesting a shifting of the balance towards a more apoptotic phenotype upon intermittent treatment in those cells. This p53-independent susceptibility to MLN4924 is potentially critical for clinical applications, where nearly half of human tumors have mutated their p53 gene. Conventional chemotherapy is less effective in p53 mutant cells. Thus MLN4924 is exceptional in its ability to target p53 mutant tumors.

ACKNOWLEDGMENTS

We are grateful to Dr. Fred Bunz for providing us isogenic p21-/- and p53-/- HCT116 cells. We wish to thank members of the Dutta lab for reading the manuscript and helpful discussions.

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FIGURE LEGENDS

Figure 1. Cdt1 protein level is important for MLN4924 induced re-replication in HCT116 cells

(A) HCT116 cells were transfected twice with siGL2 or siCdt1 at 0- and 24-hour time point and incubated for a total of 48 hours before the addition of 0.3μM MLN4924 or DMSO. Cells were harvested for PI FACS after 20 hours of treatment. The percentage of cells containing >4N DNA was shown. (B) Total cell lysates from (A) were blotted with indicated protein antibodies. (*: non-specific band) (C) HCT116 WT or e83 cells were treated with DMSO or 0.3μM MLN4924 for 20 hours before harvested for FACS. The percentage of cells containing >4N DNA was shown. (D) Similar assay as described in (A) was performed with siMcm7. The percentage of cells containing >4N DNA was shown. (E) Similar assay as in (A) was performed with siCdc6.

Figure 2. MLN4924 induces re-replication through inhibition of $CRL4^{Cdt2}$

(A) HCT116 cells were tranfected with GL2, Cdt2, Geminin siRNA and treated with MLN4924 as described in Fig. 1(A). Cell lysates were harvested and blotted with indicated antibodies. (B) DNA contents of the cells treated in (A) were determined using FACS and plotted in horizontal bar graph. Representative FACS data from siGL2 treated cells indicating different DNA contents measured.

Figure 3. Transient exposure to MLN4924 induces re-replication in HCT116 cells

(A) HCT116 cells were treated with DMSO or 1μM MLN4924 for indicated hours. Cells were washed with PBS twice, incubated in fresh medium and harvested 24 hours after initial addition of the chemicals. Percentage of cells containing >4N DNA contents was plotted. (B) HCT116 cells were treated with DMSO or 1μM MLN4924 for 4 hours. Cells were then washed and harvested at 0, 2, 4 and 20 hours after the wash-out as indicated. Cell lysates were blotted with Cdt1 or Actin antibodies.

Figure 4. S phase cells are more susceptible to MLN4924 induced re-replication

(A) Schematic of experimental procedures of (B) to (D). (B) FACS profiles of control samples harvested at indicated time points. (C) Total lysates from cells harvested 20 hours after the drug wash-out were blotted with indicated antibodies. (D) FACS profiles from above cells were shown. The percentage of cells with >4N DNA is plotted in the bar graphs below. (E) Schematic of experiment of (F). (F) FACS profiles were shown as indicated. (Dashed line: DMSO; solid line: MLN4924) Percentages of re-replicating cells after MLN4924 treatment are indicated.

Figure 5. Both checkpoint and apoptosis pathways are activated upon short exposure of cells to MLN4924

(A) HCT116 cells were treated with DMSO, 1μM or 3μM MLN4924 for 8 hours. Cells were harvested 24 or 72 hours after the drug wash-out. Cell lysates were blotted with indicated protein antibodies. (*: nonspecific band) (B) Cells from above were

harvested for PI FACS. Percentages of cells containing <2N (left) or >4N DNA (right) are shown.

Figure 6. The senescence pathway is induced in HCT116 cells after transient exposure to MLN4924

(A) HCT116 cells were treated with 1μM MLN4924 continuously (top panel) or only for 8 hours (middle and lower panels). Movie images of the cells at indicated time points are shown. (B) HCT116 cells were treated as described in the text. Cell survival rate in the colony formation assay is shown. Error bar represents three independent experiments. (C) HCT116 cells were treated with 1μM MLN4924 for indicated hours before wash-out. SA β-Gal staining assay was then performed after 72 hours. Positive stained cells were counted and plotted as percentage of total cell numbers. Mean±standard deviation of three different experiments. (D) Representative SA-β-gal staining for indicated samples. (E) HCT116 cells were transfected twice with siGL2 or siCdt1 as described in Figure 1(A). 48 hours after initial transfection, cells were treated with 1μM MLN4924 for 8 hours. Cells were either harvested for FACS analysis after 24 hours, or subjected to SA-β-gal staining assay after 72 hours. (F) HCT116 cells were treated with 1μM MLN4924 8 hours. Cells were harvested for western blots of p53 and p21 at different time points after wash-out. (G) HCT116 WT, p53-/- or p21-/- cells were treated with 1μM MLN4924 for 8 hours. SA-β-gal staining assay was performed 72 hours after the wash-out. Percentage of positive stained cells is shown. Mean±standard deviation of 3 experiments. * indicates statistical significance ($p<0.01$).

Figure 7. p53 mutant cells are susceptible to transient treatment with MLN4924

(A) HCT116 WT, p53-/- or p21-/- cells were treated with 0, 0.1, 0.3, 0.9, 1.8 or 2.7μM of MLN4924. Cell survival rates were measured as described in Figure 6(B). Mean and standard deviation from triplicates. * indicates statistical significance ($p<0.01$). (B) Viable HCT116, MCF7 (MLN4924 treatment 24hrs after control or p53 siRNA), H460 and H23 cells after 8hr MLN4924 treatment at different doses were measured using MTT assay as described. The points indicate mean and standard deviation of triplicates. * indicates statistically significant difference at various MLN4924 concentrations between the two cell lines ($p<0.01$).

Figure 1. Cdt1 protein level is important for MLN4924 induced re-replication in HCT116 cells.

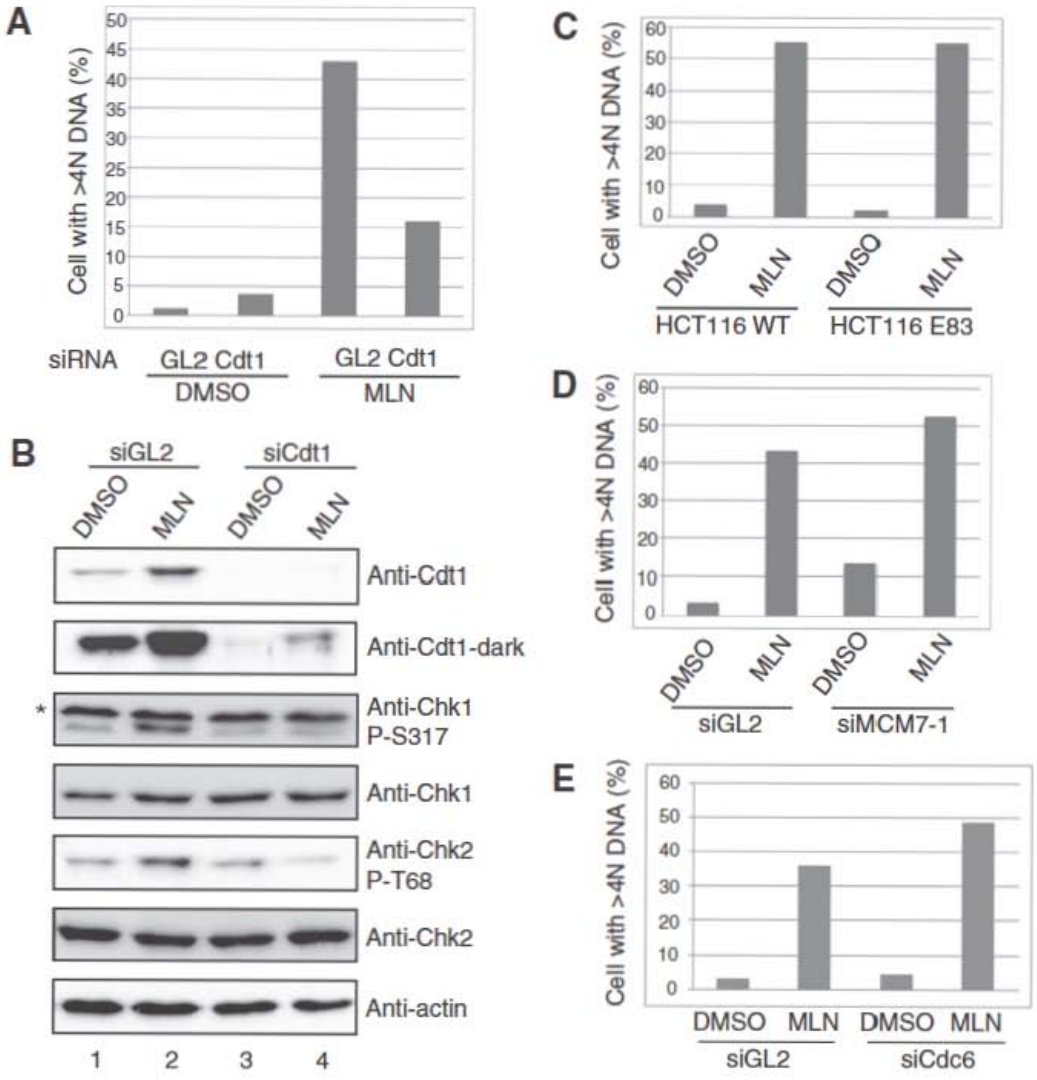


Figure 2. MLN4924 induces re-replication through inhibition of $CRL4^{Cdt2}$.

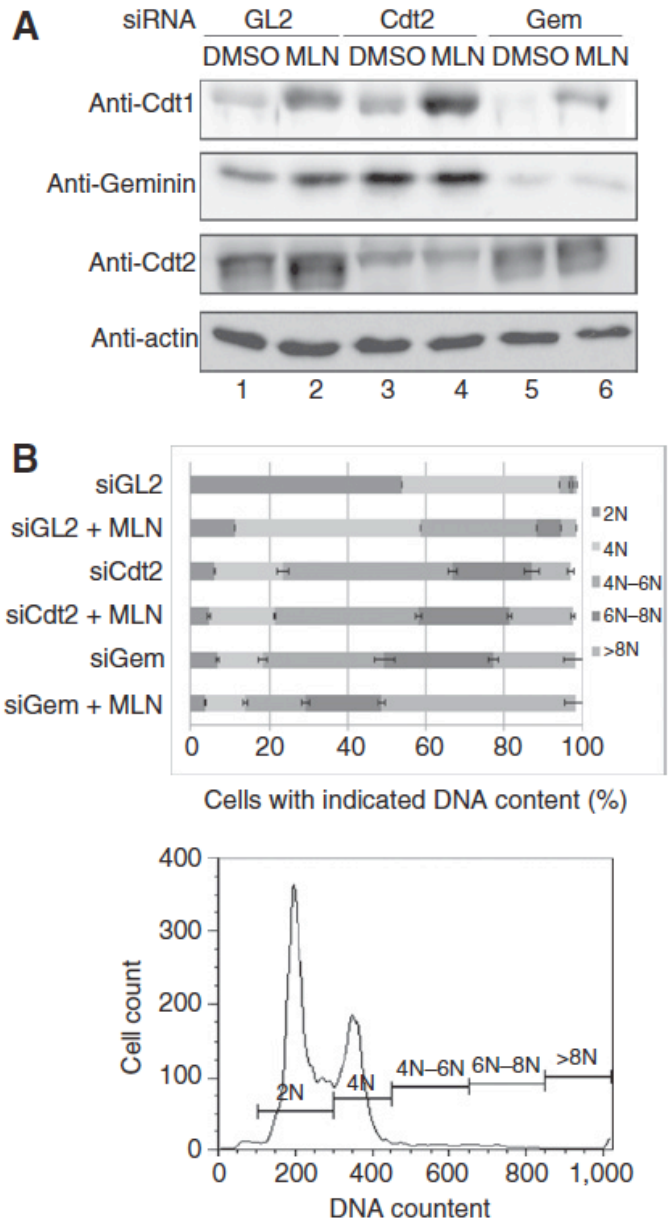


Figure 3. Transient exposure to MLN4924 induces re-replication in HCT116 cells.

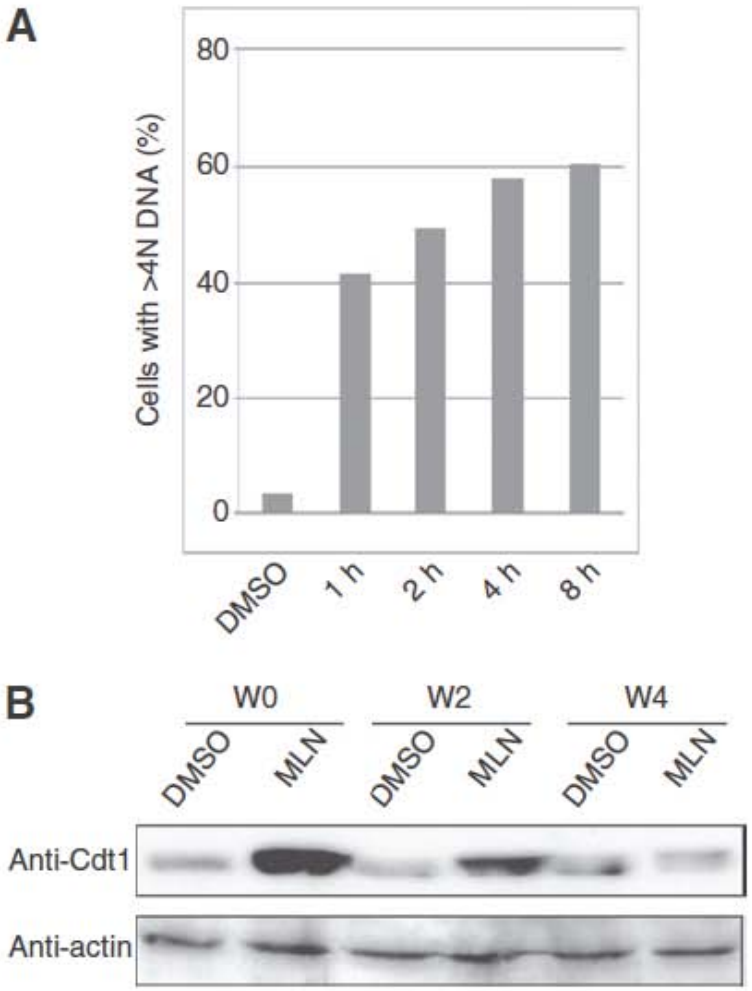


Figure 4. S phase cells are more susceptible to MLN4924 induced re-replication.

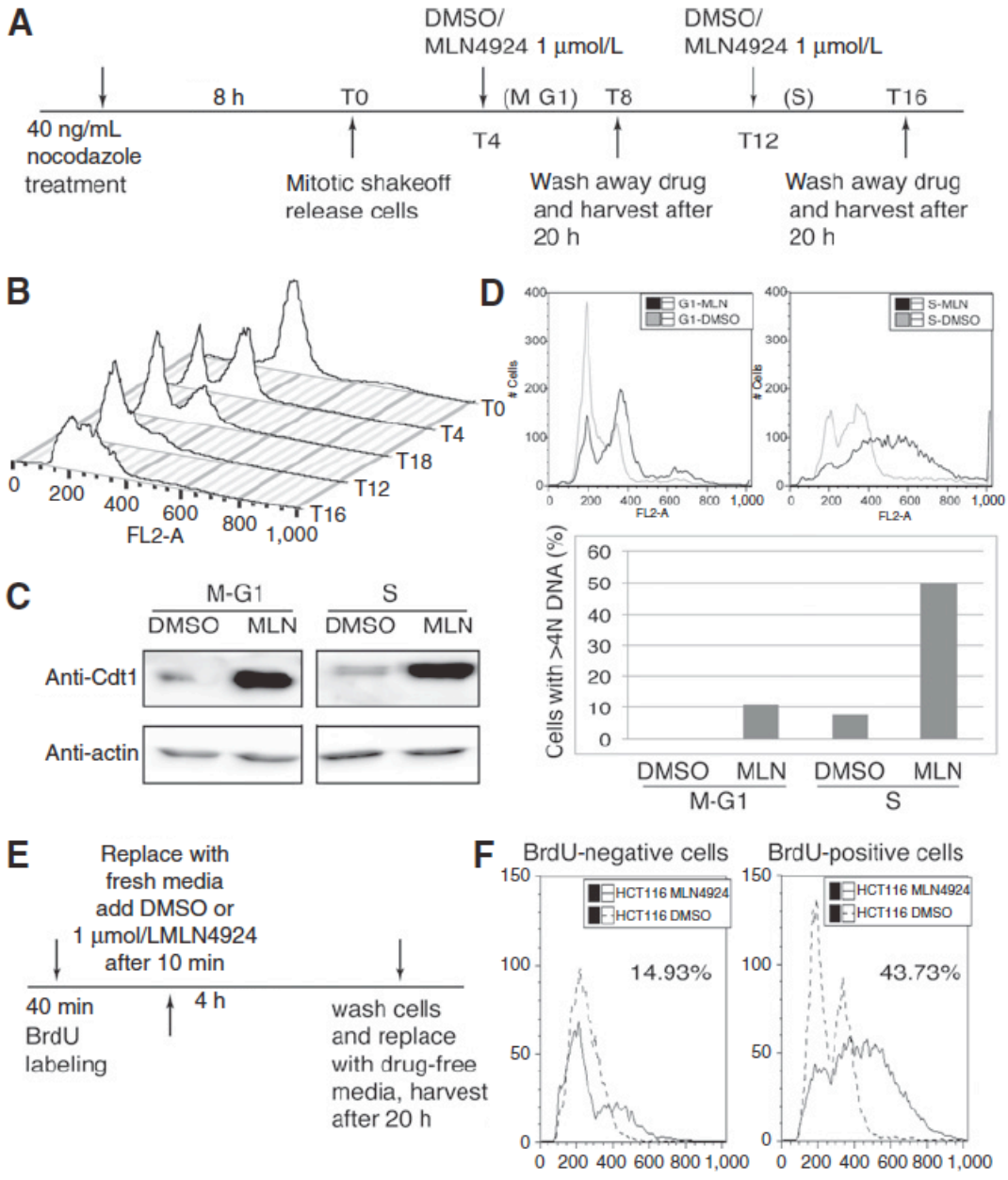


Figure 5. Both checkpoint and apoptosis pathways are activated upon short exposure of cells to MLN4924.

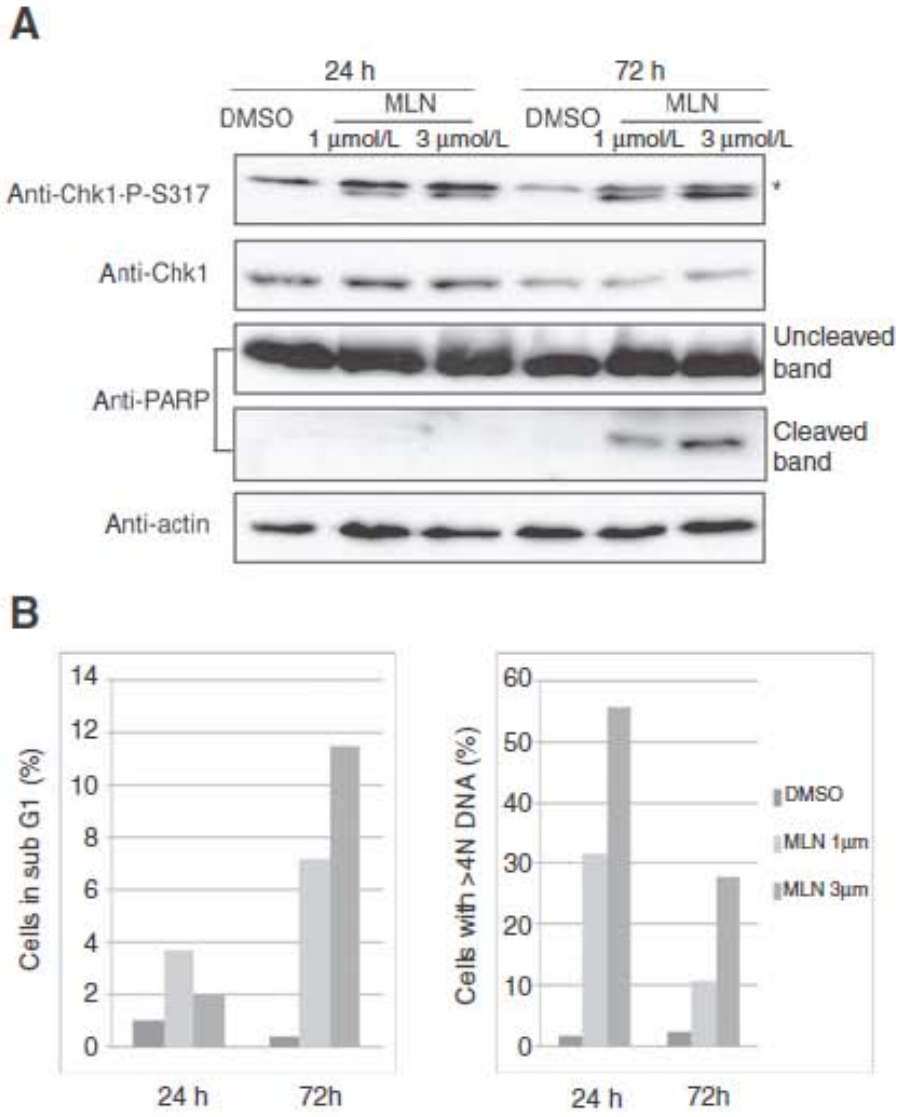


Figure 6. The senescence pathway is induced in HCT116 cells after transient exposure to MLN4924.

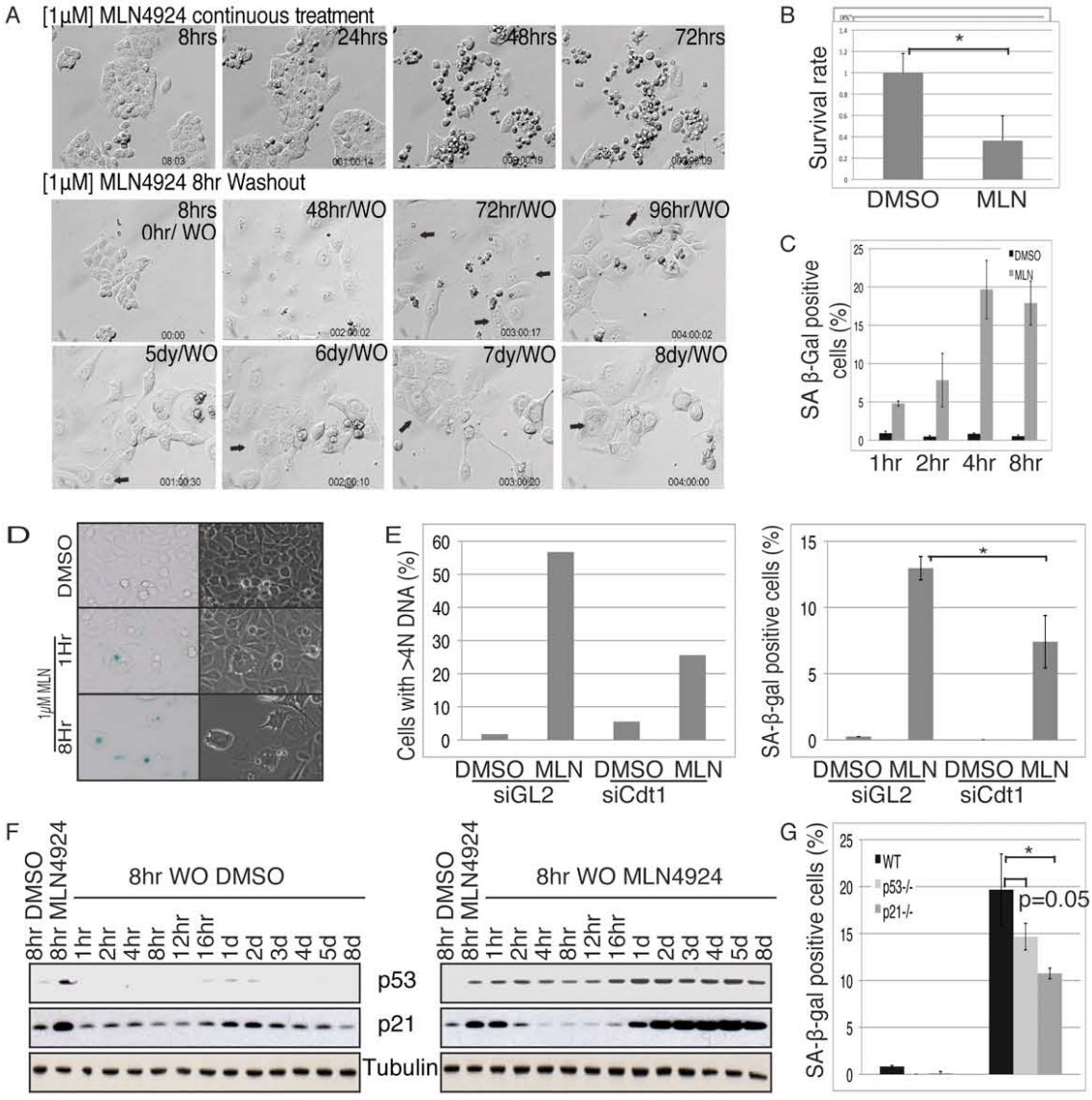
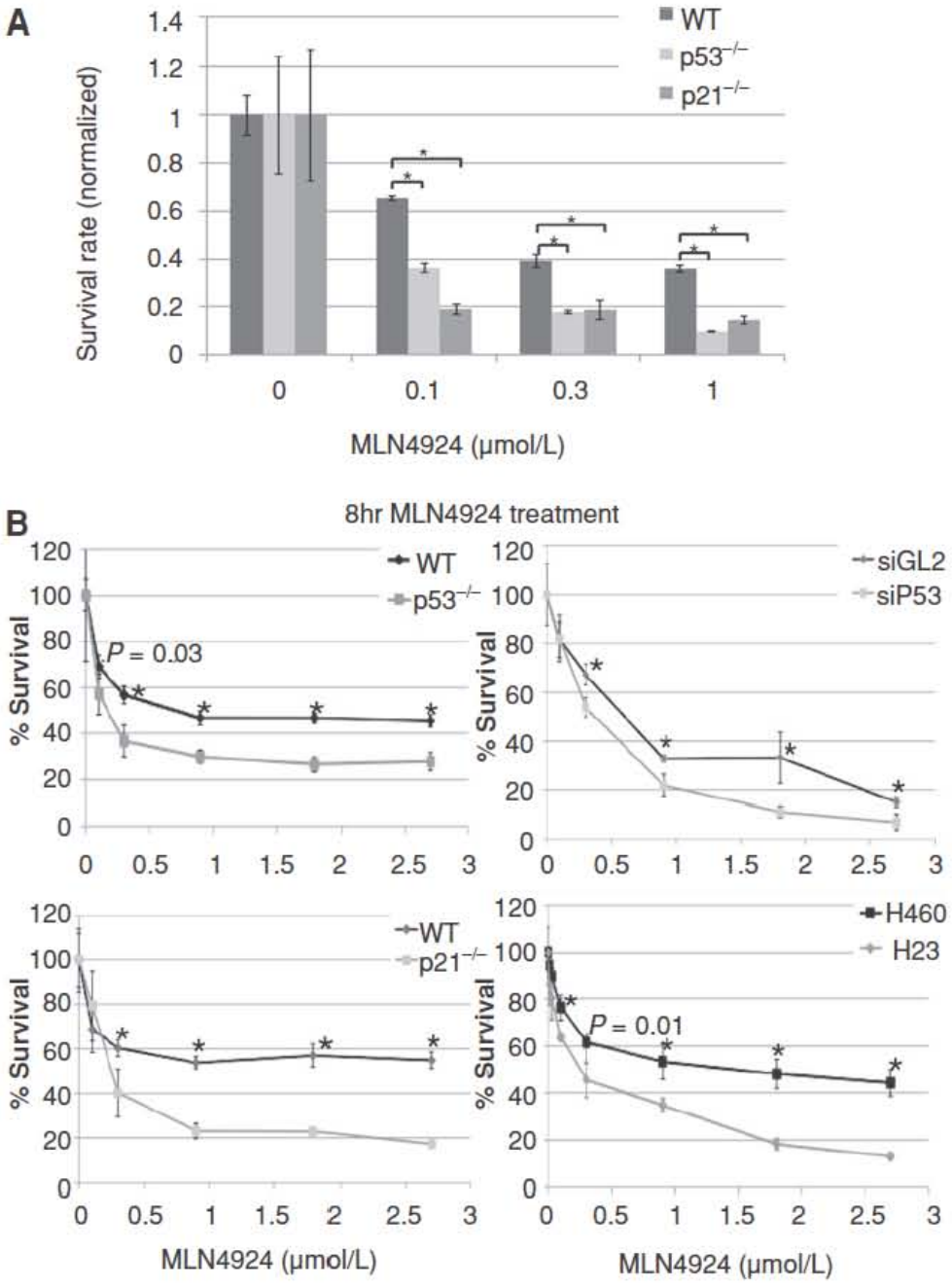


Figure 7. p53 mutant cells are susceptible to transient treatment with MLN4924.



SUPPLEMENTARY MATERIALS

Supplemental methods

Cell viability assays were performed by Southern Research (Birmingham, Alabama). Exponentially growing cell suspensions were seeded at 3,000–8,000 cells per well in 96-well culture plates and incubated overnight at 37ºC. MLN4924 was added to the cells in complete growth media and incubated for 72 hours at 37ºC. Cell viability was determined using the ATPlite assay (Perkin Elmer, Waltham, MA).

Supplemental figure legends

Figure S1. Cdt1 protein level is important for MLN4924 induced re-replication but not G2/M arrest in HCT116 cells

(A) Different amounts of cell lysates from Fig. 1C were immuno-blotted with indicated antibodies. (B) HCT116 cells were treated as described in Fig. 1D. The levels of Mcm7 were shown. Actin was shown as a loading control. (C) Cell lysates from Fig. 1E were immuno-blotted with indicated antibodies. (D) FACS profiles of samples described in Figure 1(A). (E) Total cell lysates of the above samples were blotted with indicated antibodies to show the G2/M arrest. Total cdc2 proteins were immunoprecipitated before blotting with cdc2-P-Y15 antibody to show Y15 phosphorylated cdc2.

Figure S2. p21 plays an important role in causing cellular senescence following transient exposure to MLN4924

(A) HCT116 cells were treated as described in Fig. 6B. Colonies were stained with crystal voilet to show cell clonogenicity. (B) HCT116 WT, p53-/- or p21-/- cells were treated with DMSO or 1μM MLN4924 for 8 hours. SA-β-gal staining assay was performed 72 hours after the washout. Representative pictures were shown.

Figure S3. Apoptosis inhibitor Z-VAD-FMK cannot reduce MLN4924 induced senescence

HCT116 cells were treated with DMSO or 1μM MLN4924 for 8 hours and incubated in fresh medium for 24 hours before treated with 50μM Z-VAD for 48 hours. Fresh Z-VAD was added every 24 hours. Cells were then stained with SA-β-gal. SA-β-gal positive cells were counted and plotted as a percentage of total cells.

Figure S4. p53 status is one of the factors that affect MLN4924 induced cell death

(A) Viable HCT116 cells after 72hr MLN4924 treatment at different doses were measured using MTT assay. The points indicate mean and standard deviation of triplicates. * indicates statistically significant difference at various MLN4924 concentrations between the WT and the mutant cells ($p<0.01$). (B) MCF7 cells were transfected with siGL2 or siP53 36 hours before MLN4924 exposre. Cell lysates were harvested before MLN4924 treatment and blotted with p53 and actin. These are the cells used in Fig. 7B. (C) 3,000–8,000 cells were seeded per well in 96-well culture plates and incubated overnight at 37ºC. MLN4924 was added to the cells and incubated for 72 hours. Cell viability was determined using the ATPlite assay. Median values were

indicated with lines. The cells are classified based on their p53 status: wild type or mutant. (D) Viable MCF7 (p53 WT) and MDA-MB-231 (p53 mutant) cells after 8hr MLN4924 treatment at different doses were measured using MTT assay. The points indicate mean and standard deviation of triplicates.

Figure S1. Cdt1 protein level is important for MLN4924 induced re-replication but not G2/M arrest in HCT116 cells.

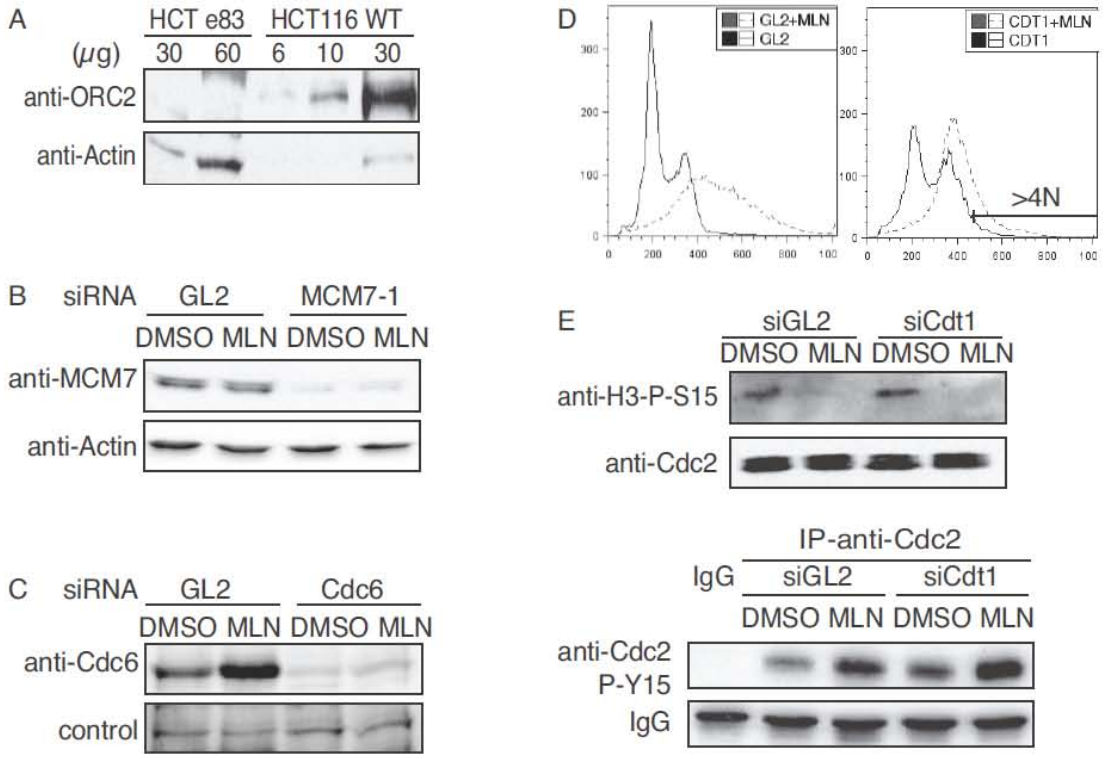


Figure S2. p21 plays an important role in causing cellular senescence following transient exposure to MLN4924.

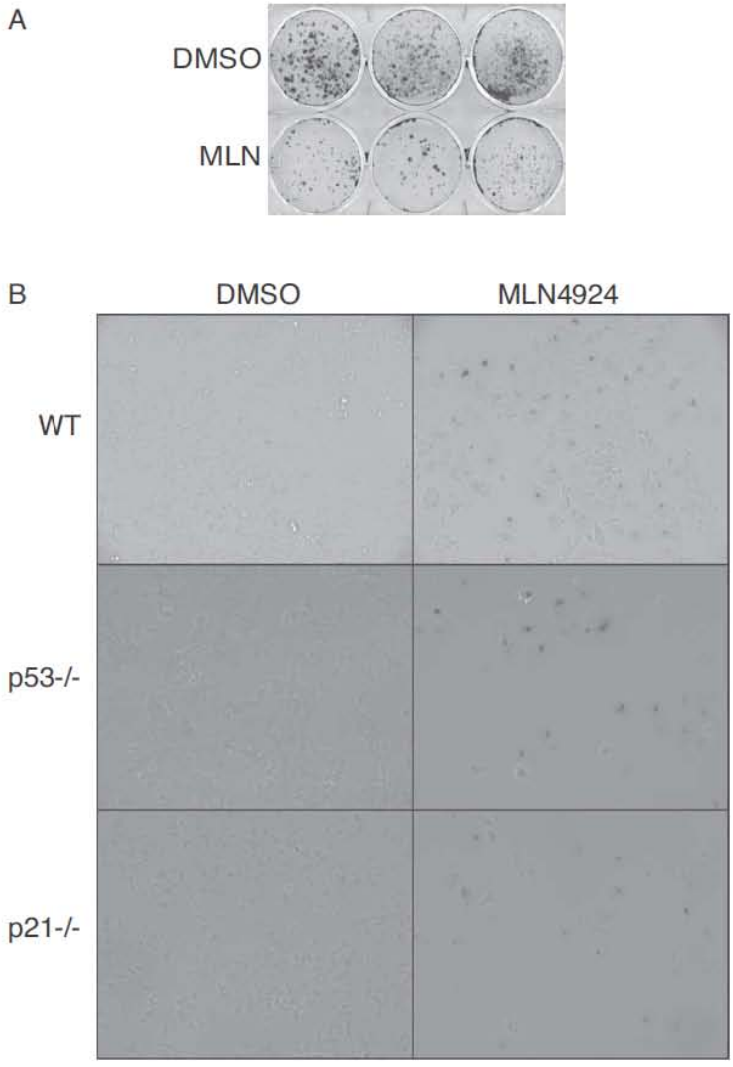


Figure S3. Apoptosis inhibitor Z-VAD-FMK cannot reduce MLN4924 induced senescence.

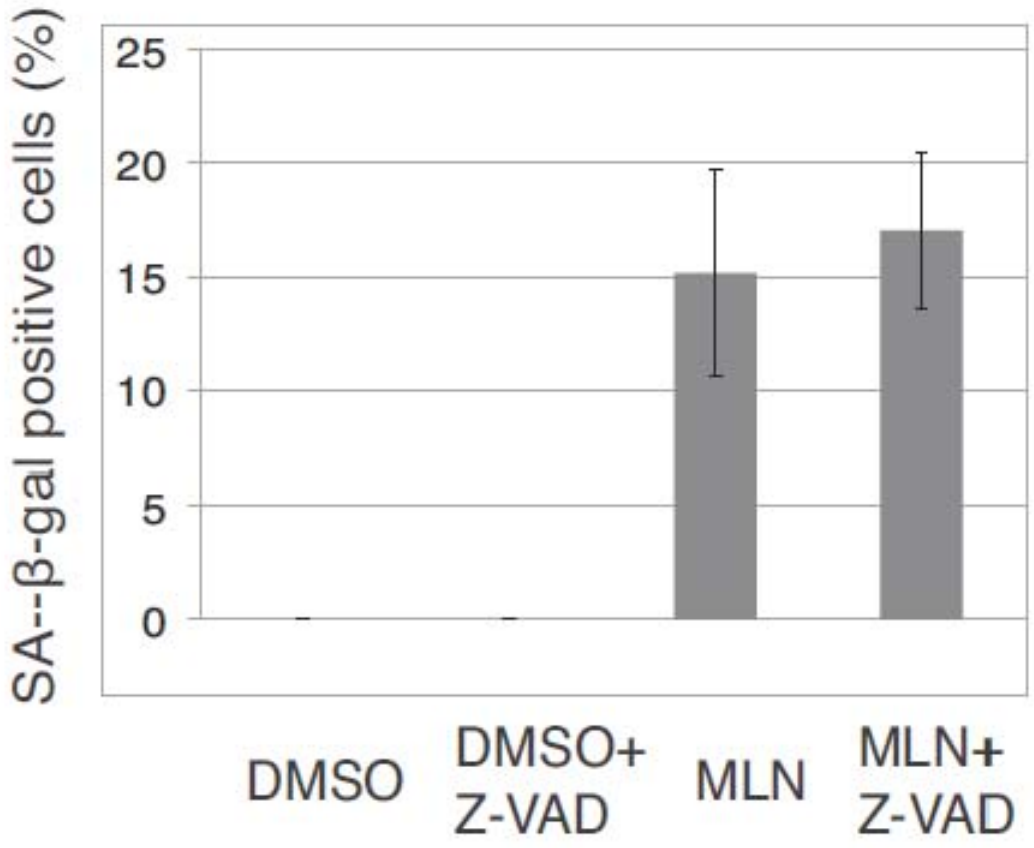
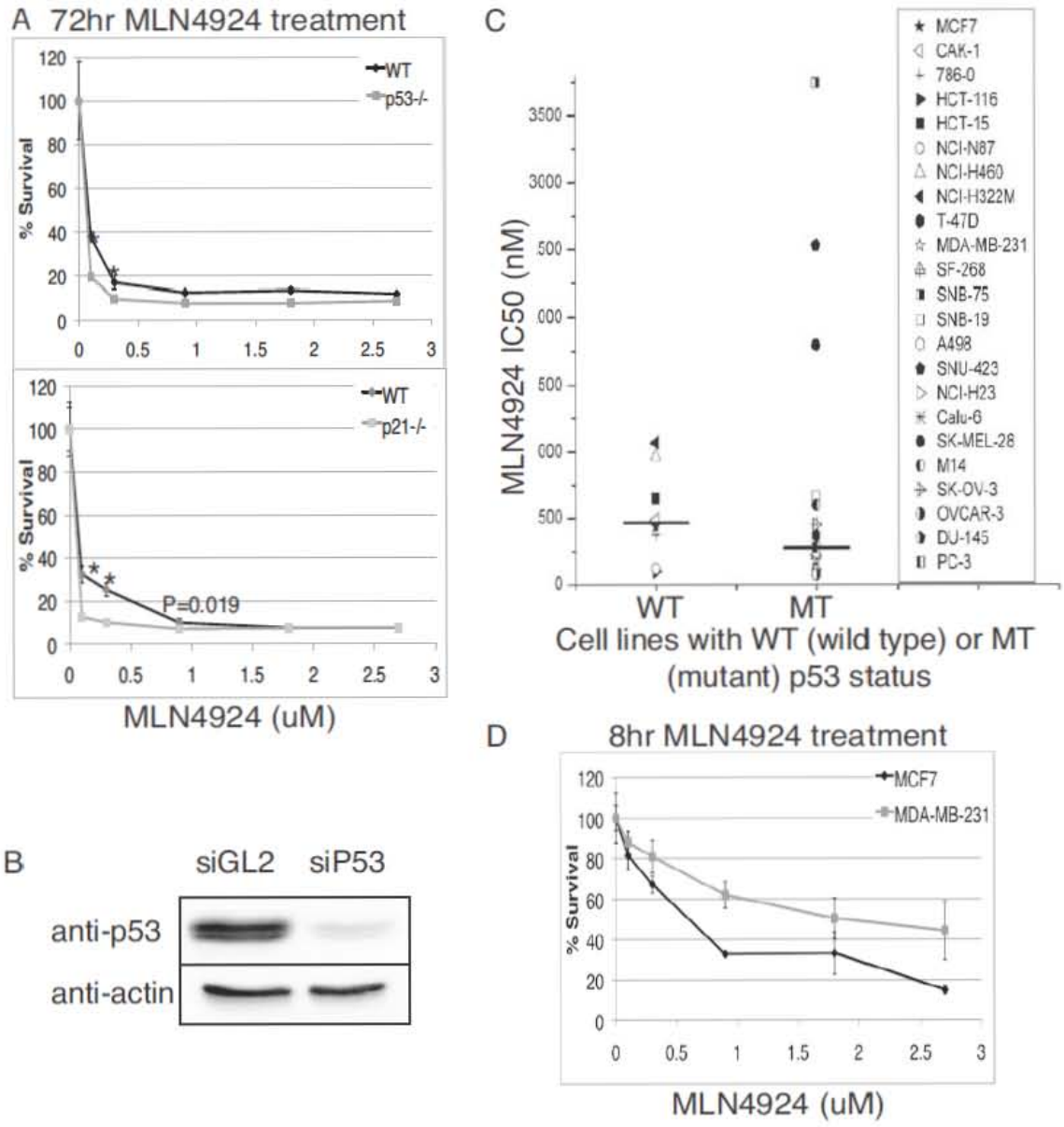


Figure S4. p53 status is one of the factors that affect MLN4924 induced cell death.



CHAPTER FOUR

UNPUBLISHED WORK

CHAPTER FOUR: UNPUBLISHED WORK

In this chapter, two different pieces of work are described that have not been published. The first section describes the discovery of a new player in the G2/M checkpoint pathway induced by re-replication. The second section describes the test of a hypothesis that re-replication induces gene amplification and stimulates tumorigenesis.

4.1 HDAC6, A NEW PLAYER IN THE G2/M CHECKPOINT PATHWAY INDUCED BY RE-REPLICATION

4.1.1 Introduction

HDAC6 (histone deacetylase 6) belongs to the HDAC super family, a class of enzymes that remove acetyl groups from lysine amino acids on proteins. In mammals, HDACs deacetylate histones and are components of transcriptional repressor complexes (1). In addition, they can associate with non-histone proteins and play roles in other biological pathways (2).

The substrates of HDAC6 include tubulin, Hsp90, and cortactin (3). In general HDAC6 stays in the cytoplasm and performs several key regulatory functions (4). It can deacetylate non-dynamic and highly acetylated tubulin, facilitate the reorganization of microtubules and increasing cell motility (5). The C-terminal zinc finger of HDAC6 binds to polyubiquitinated proteins, targets them to aggresomes and promotes their degradation. This process can protect cells from cytotoxic cell death when the usual

ubiqutin-targeted proteasome system is impaired or overwhelmed (6). HDAC6 is also associated with Hsp90 and involved in chaperone function to facilitate the proper folding and assembly of Hsp90 clients (7). In addition, HDAC6 is able to shuttle between cytoplasm and nucleus depending on different signals (8). It has been reported that HDAC6 can also act as a classic nuclear co-repressor (7). However, it is not clear what signaling pathways may result in the change of HDAC6 cellular localization.

As described in earlier chapters, DNA re-replication induced by Geminin depletion can lead to G2/M checkpoint activation. After the loss of Geminin, ATR/Chk1 pathway is activated at an early time point by the generation of ssDNA (single-stranded DNA). This pathway is considered the primary pathway that plays an essential role in G2/M arrest and re-replication accumulation (9).

Previous research in lab found that UV induced Chk1 phosphorylation was decreased after HDAC6 depletion by siRNA in multiple cancer cell lines. In addition, HDAC6 interacted with Chk1, Claspin, ATR and ATRIP after its overexpression in 293T cells (data not shown). Taken together, HDAC6 is suspected to function in UV-induced ATR/Chk1 pathway. Therefore, we decided to test the involvement of HDAC6 in re-replication-induced checkpoint pathway.

4.1.2 Results

HDAC6 Protein Is Required for G2/M Checkpoint Activation after Geminin Depletion

We wanted to find out whether HDAC6 is involved in cell cycle checkpoints activated by DNA re-replication. As described earlier, Geminin depletion in HCT116

cells induces DNA re-replication, which activates the ATR/Chk1 mediated checkpoint pathway. Therefore we performed RNA interference to silence HDAC6 protein expression in control or re-replicated cells (Fig. 1).

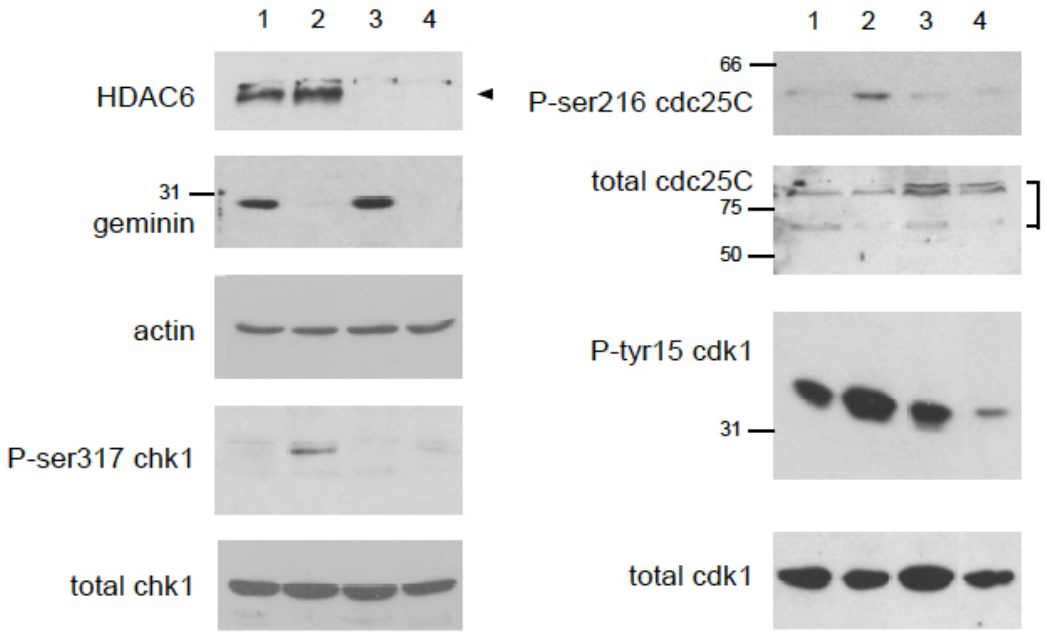


Figure 1. HDAC6 protein is required for G2/M checkpoint activation after Geminin depletion. HCT116 cells were transfected with siRNAs to luciferase (GL2) (lane 1, 2) or HDAC6 (lane 3, 4) on day 1. On day two, cells were transfected with either siGL2 (lane 1, 3) or siGeminin (lane 2, 4). The cells were harvested 72 hours after the first transfection and protein extracts were immunoblotted for the indicated antibodies. Total Cdc25C runs as multiple bands with the upper species being the mitotic form. 1. siGL2; 2. siGeminin; 3. siHDAC6; 4. siGeminin+HDAC6.

Co-depletion of the checkpoint components such as ATR or Rad17 with Geminin can suppress the accumulation of re-replicated cells because the checkpoint arrests cell progression through G2 to M thereby giving more time for re-replication and allowing the cells to survive with excess DNA (9, 10). By FACS (flow cytometry analysis), we

observed that the percentage of re-replicated cells (with >4N DNA) ranged from 28.1-41.7% in Geminin depleted cells (Table. 1). HDAC6 siRNA itself did not cause any re-replication. However, HDAC6 and Geminin co-depletion decreased re-replication by 50% after subtracting background, suggesting that HDAC6 loss decreases the accumulation of re-replicated cells, probably because HDAC6 is a component of the ATR/Chk1 signaling pathway.

Experiment	Geminin siRNA	Gem. siRNA + HDAC6 siRNA	GL2 (Luciferase siRNA)	HDAC6 siRNA
1	28.1	17.7	5.3	7.2
2	36.8	17.7	2.7	2.8
3	41.7	24.9	4.2	4.0

Table 1. Re-replication induced by geminin depletion decreases upon co-depletion of HDAC6. HCT116 cells were treated as described in Figure 1. Cells were harvested and stained with PI (propidium iodide) for DNA content before FACS. Column numbers denote the percentage of cells with >4N DNA when transfected with the indicated siRNAs.

Biochemical analysis showed that the activation of checkpoint proteins was decreased in Geminin knockdown cells when HDAC6 was co-depleted. As expected, the loss of Geminin led to Chk1 phosphorylation on Ser317, Cdc25C phosphorylation on Ser216 and Cdc2 phosphorylation on Tyr15 (Fig. 1, lane 2). In the absence of HDAC6 and Geminin, phosphorylation of Chk1 and inhibitory phosphorylations of Cdc25C and Cdc2 were decreased (Fig. 1, lane 4). Interestingly, HDAC6 depletion here specifically decreased Chk1 phosphorylation without decreasing Chk1 protein levels. We have not yet tested Claspin levels. Thus HDAC6 is important for the G2/M checkpoint activation upon DNA re-replication.

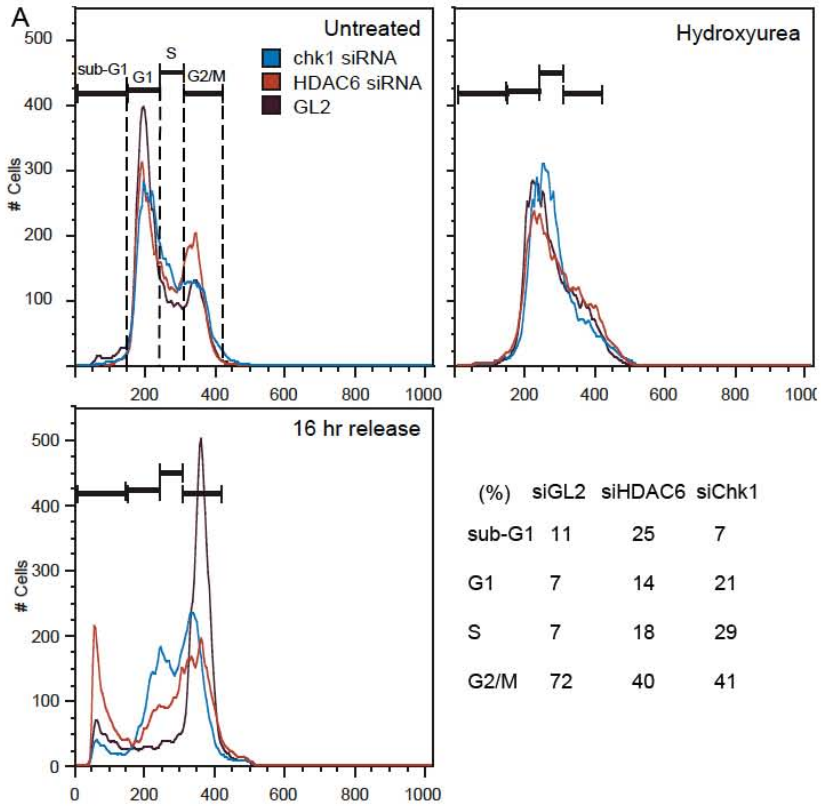
HDAC6 is Important for Cell Cycle Re-entry after Replication Arrest

ATR/Chk1 signaling pathways are not only important for the S and G2/M checkpoints, they also play a crucial role in maintaining replication fork stability and recovery from cell cycle arrest after replication inhibition (11). Therefore, we decided to test whether HDAC6 was important for cell cycle re-entry after HU (hydroxyurea) treatment.

HDAC6 or Chk1 was depleted in U2OS cells using siRNA. Cells were subsequently treated with HU (1mM) for S phase arrest. HU was washed out after 15 hours and cells were allowed to re-enter the cell cycle in the presence of nocadazole for mitotic arrest. As shown in Fig. 2, in untreated or HU treated cells, the cell cycle profiles were not changed after siRNA of Chk1 or HDAC6 (Fig. 2A, top panel). After HU washout, 72% of control cells accumulated in G2/M phase, indicating the successful re-entry to normal cell cycle. However, in Chk1 or HDAC6 depleted cells, only 40% of them entered G2/M phase (Fig. 2A, lower left), implying that Chk1 and HDAC6 were both required for cell cycle re-entry after replication inhibition by HU.

The knockdown efficiency of HDAC6 and Chk1 was shown in Fig. 2B by immunoblotting. Claspin is a protein that interacts with Chk1 and facilitates Chk1 activation. Consistent with previously reported results, Claspin was destabilized by Chk1 depletion (12, 13). In addition, Claspin was degraded during DNA damage recovery (14-16). However, siHDAC6 did not decrease the Claspin or Chk1 levels in HU arrested cells, nor did it affect the degradation of Claspin after release from HU. We suspect (but have not tested) that the poor recovery from HU arrest in HDAC6 depleted cells was due

to failure to activate Chk1. However, the reason for this failure cannot be ascribed to loss of Claspin or loss of Chk1.



(%)	siGL2	siHDAC6	siChk1
sub-G1	11	25	7
G1	7	14	21
S	7	18	29
G2/M	72	40	41

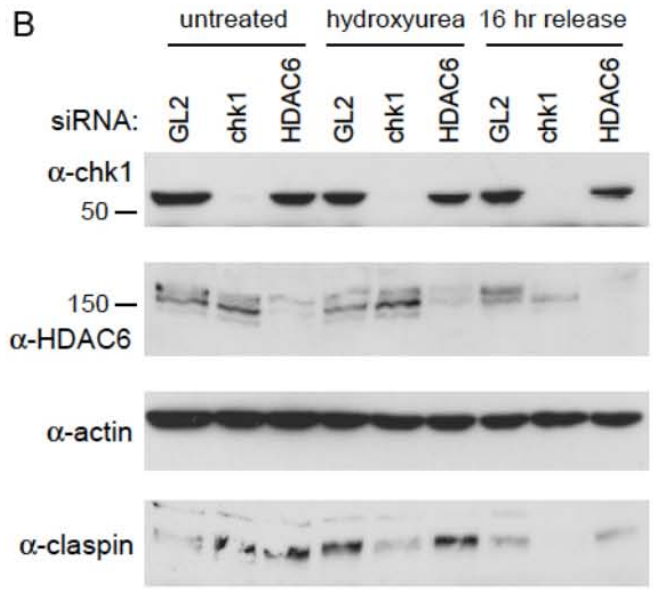


Figure 2. HDAC6 is important for cell cycle re-entry after replication arrest by HU. (A) U2OS cells were transfected with indicated siRNAs for 28 hours before the addition of 1mM HU for 15 hours. After HU washout, cells were allowed to recover in the presence of nocodazole (40ng/mL) for 16 hours. Cells were subsequently harvested and stained with PI for DNA content before FACS. The histograms were shown with x-axis indicating PI fluorescence and y-axis indicating cell number count. The percentage of cells in each cell cycle phase after release from HU was quantitated at lower right. (B) Protein extracts from the cells treated in (A) were immunoblotted using the indicated antibodies.

siHDAC6 Can Decrease Both Chk1 Protein and Chk1 Phosphorylation

In response to UV, Chk1 is phosphorylated on Ser317 and Ser345 by ATR (17, 18). Phosphorylation of Chk1 leads to its conformational change and an increase in the kinase activity (19, 20). It was mentioned earlier that HDAC6 depletion decreased UV induced Chk1 phosphorylation in multiple cell lines including U2OS, PC3 and 293T (also shown in Fig. 3). Further studies in HCT116 cells validated that HDAC6 was important for ATR/Chk1 checkpoint activation upon DNA re-replication (Fig. 1). Combined with the data that HDAC6 interacted with important components of the pathway, such as Chk1, Claspin, ATR, and ATRIP (data not shown), HDAC6 seemed to directly function in ATR/Chk1 pathway. However, the story became more complicated when we found that siHDAC6 could destabilize Chk1 protein as well.

In Fig. 3A, Chk1 was phosphorylated on Ser317 after UV treatment in PC3 cells (lane 1 and 2). Upon HDAC6 knockdown by four different siRNAs, we observed a dramatic decrease of the UV responsible phosphorylation relative to the GL2 control. However, a decrease on total Chk1 protein level was also noticed after siHDAC6, especially after si6A and si6B (Fig. 3A, lane 3-6). In addition, Chk1 mRNA was reduced by siHDAC6 to a similar extent, suggesting that Chk1 transcription was affected by

HDAC6 (data not shown). Despite this, Fig. 3B showed that after normalized to total Chk1, the UV induction of phospho-Chk1 declined in HDAC6 depleted cells. Overall, HDAC6 seems to be required for both Chk1 mRNA transcription and Chk1 protein phosphorylation.

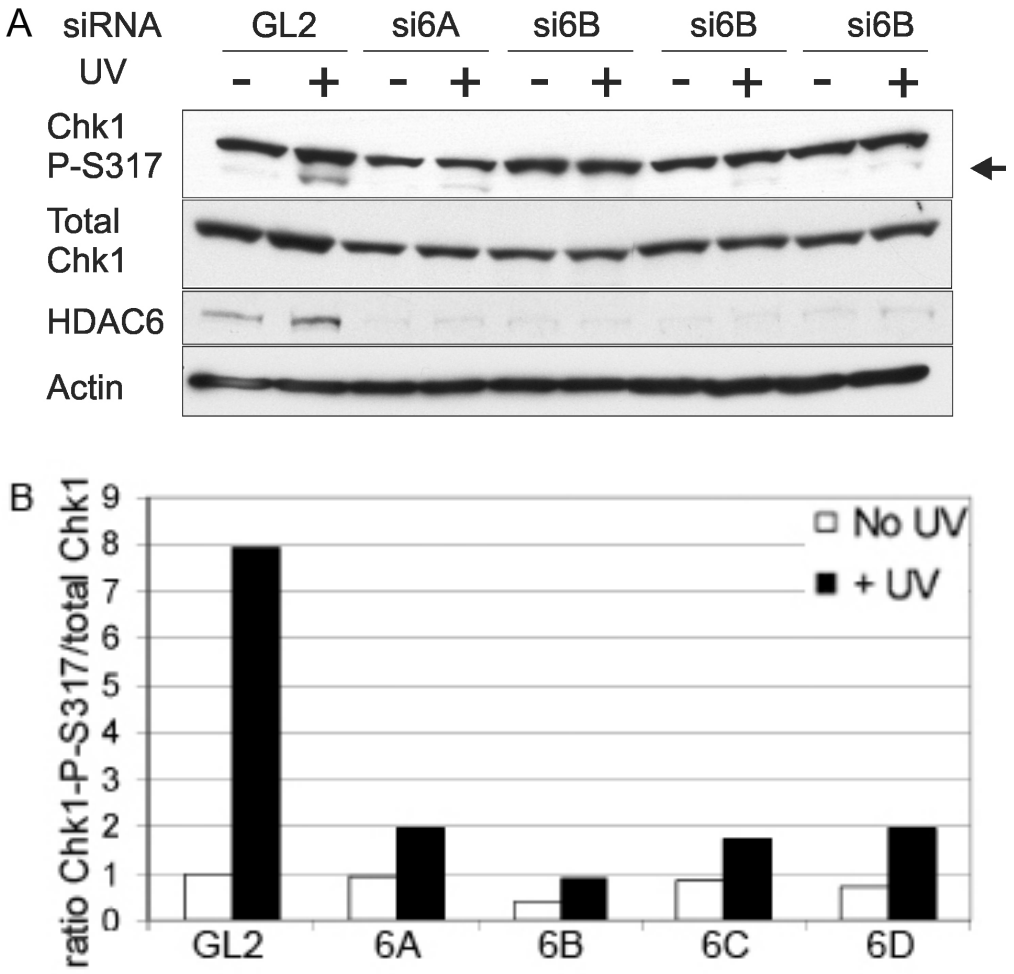


Figure 3. siHDAC6 can decrease both Chk1 protein and Chk1 phosphorylation. (A) PC3 cells were transfected with siGL2 or indicated HDAC6 siRNA. After 72 hours, cell lysates were made before or 1 hour after a 40J/m^2 dose of UV and immunoblotted with HDAC6, Chk1-P-S317 and total Chk1 antibodies. Arrow: Chk1-P-S317 band. (B) The quantitation of western blots using ImageJ was shown in the bar graphs. The value of phospho-Chk1 to total Chk1 in

GL2 minus UV cells was set to 1 and other samples were normalized to this. The data in this figure was generated by Dr. Etsuko Shibata.

Geminin Depletion Does Not Change HDAC6 Localization

Endogenous HDAC6 is mostly localized in cytoplasm in many cell lines (4, 21, 22). In addition, HDAC6 shuttles between cytoplasm and nucleus depending on different signals (8). We wanted to test whether the role of HDAC6 in ATR/Chk1 pathway requires translocation of the protein into the nucleus upon DNA re-replication. However, HDAC6 remained in the cytoplasm after Geminin depletion in HCT116 cells (Fig. 4). The giant nuclei formed after siGeminin indicated that DNA re-replication was occurring (Fig. 4, lower panel). Taken together, these data suggest that although HDAC6 knockdown affects Chk1 function after DNA re-replication, HDAC6 exerts its function while remaining mostly in the cytoplasm.

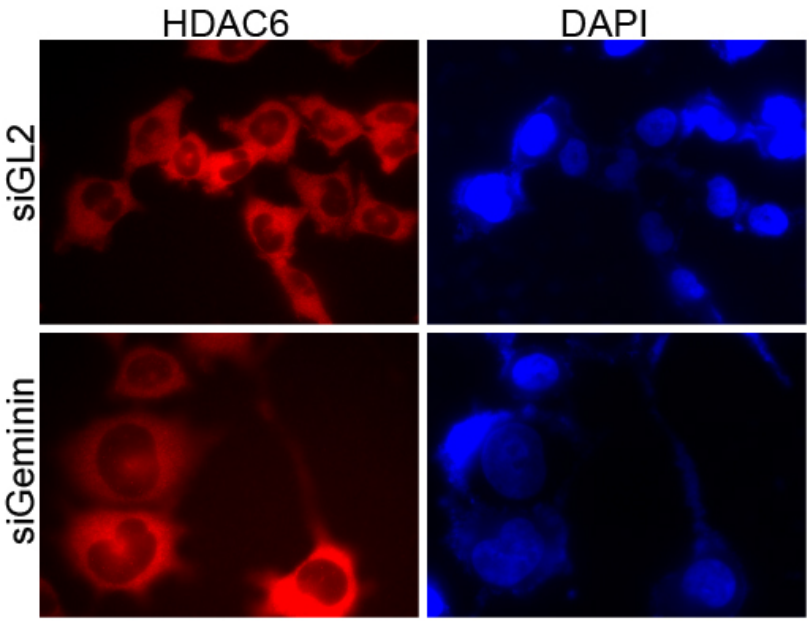


Figure 4. Geminin depletion does not change HDAC6 localization. HCT116 cells were transfected siRNA to GL2 or Geminin and harvested for immnofluorescence after 72 hours with indicated antibodies. Red: HDAC6; Blue: dapi stained nuclei.

4.1.3 Discussion

We have shown that siHDAC6 decreased Chk1 phosphorylation and activation after DNA re-replication and UV treatment (Fig. 1 and 3). SiHDAC6 also affect recovery from HU arrest. To further validate the function of HDAC6 in checkpoint activation, we treated HDAC6 knockout MEFs (mouse embryonic fibroblasts) with UV and measured Chk1 phosphorylation. However, we did not observe any decrease of Chk1 phosphorylation in those MEFs (data not shown). This result is opposite to our earlier findings in human cell lines (Fig. 1 and 3) and suggests that the effect of HDAC6 on Chk1 activation could be indirect and variable. The conclusion is further supported by the observation that HDAC6 remains in the cytoplasm after DNA re-replication (Fig. 4).

The ATR/ATRIP kinase complex has to be activated by Rad17/RFC and the Rad9-Hus1-Rad1 (9-1-1) complex before ATR can phosphorylate Chk1. ATR can also phosphorylate other substrates such as Rad17. HDAC6 appears to affect the phosphorylation of Chk1 but not factors upstream of Chk1. In 293T cells stably expressing shHDAC6 (short hairpin RNA against HDAC6), Rad17 phosphorylation after UV treatment was not decreased. In addition, Chk1 and Claspin association remained the same (data not shown).

The subsequent question is, how can HDAC6 specifically regulate Chk1 phosphorylation and whether the effect is direct or indirect? The mechanism may be complicated.

We have shown that siHDAC6 decreased Chk1 mRNA and protein stability to a similar extent in PC3 cells (Fig. 3). After normalization to Chk1 level, UV induced Chk1 phosphorylation was still decreased. Further studies demonstrated that Claspin mRNA and protein level declined in siHDAC6 cells as well (data not shown). Taken together, HDAC6 knockdown diminished ATR/Chk1 activation in several different ways, including (a) reducing Chk1 and Claspin mRNA and protein level, and (b) decreasing Chk1 phosphorylation and activation.

When we looked in the literature for the connection between HDAC6 and Chk1, one protein named Hsp90 (heat shock protein 90) came into sight. Hsp90 is a ubiquitously expressed molecular chaperone. It is an essential component of a multiprotein chaperone complex. Hsp90 and its cofactors function together in the folding, assembly, maturation, and stabilization of specific proteins called client proteins (23). HDAC6 has been shown to interact with Hsp90 and regulate its chaperone activity by deacetylation (24). Indeed after HDAC6 inhibition, Hsp90 is hyperacetylated, dissociates from the cochaperone p23, and loses the chaperone activity. In addition, many important proteins, such as cell cycle kinases and p53, have been identified as clients of Hsp90 (23). Chk1 happens to be a client protein of Hsp90 (25, 26). Chemical inhibition of Hsp90 destabilizes Chk1 and disrupts Chk1 signaling pathway during replication stress. All together, depletion of HDAC6 is implicated to suppress Hsp90 chaperone activity, and thus could indirectly decrease Chk1 protein and activity.

To test whether Hsp90 is the missing link in our study, we can add Hsp90 inhibitor into the cells after UV treatment or re-replication. If the phenotype mimics that of siHDAC6, it suggests that HDAC6 knockdown affects the ATR/Chk1 pathway

through Hsp90. If the phenotype is not exactly the same, other factors may be involved in the regulation. For example, it is difficult to explain why Hsp90 inhibition decreases Chk1 phosphorylation even after normalization to Chk1 protein level in Fig. 3.

In summary, it is not clear how HDAC6 is involved in ATR/Chk1 pathway. It is possible that the requirement of HDAC6 is partially through Hsp90 chaperone function. However, further work is needed to elucidate the mechanism.

4.2 DNA RE-REPLICATION AND GENE AMPLICATION

It is believed that DNA re-replication can lead to gene amplification and contribute to tumorigenesis. However the theory had not been experimentally tested when I started testing the hypothesis.

4.2.1 Introduction

Gene amplification is a cellular process characterized by an increase in copy number of certain chromosome regions (27, 28). Amplification is common in cancer cells and significantly contributes to tumorigenesis (28-31). There are various forms of gene amplification in mammalian cells, including extrachromosomal copies named double minutes, tandem arrays within a chromosome with cytologically visible HSR (homogeneously staining region), and distributed insertions across the genome. Free DNA ends rising from an incorrectly repaired DSB (double-stranded break) can initiate gene amplification only in cells deficient in DNA damage checkpoints (32, 33).

Moreover, although duplications of chromosomal regions can be detected in the genomes of individuals and many cancers, no amplification is normally observed in normal cultured mammalian cells, suggesting the presence of inhibitory mechanisms (which are probably related to DNA damage checkpoints) (34-36). One of the models for gene amplification is that it is initiated by DNA re-replication (37, 38).

Replication of chromosomal DNA is a key event in the cell cycle. Chromosomes must be copied precisely once and only once per cell cycle. In mammalian cells, the negative regulation of Cdt1 seems to be a major focus to prevent DNA re-replication (39). Cdt1 is degraded in a replication dependent or a S-Cdk (cyclin dependent kinase in S phase) dependent manner, by distinctive E3 ligases. In addition, Geminin inhibits Cdt1 from recruiting MCM2-7 proteins. Both Cyclin A (which binds S phase Cdk2 and is required for S phase progression) and Geminin are substrates of APC (anaphase promoting complex), an E3 ligase that is normally active in mitosis and early G1 phase (40). APC is kept in an inhibited state by an F-box containing protein, Emi1 (41, 42). In previous studies, we have demonstrated that depletion of Emi1can prematurely activate APC, cause the simultaneous degradation of Geminin and Cyclin A, and inducing DNA re-replication in various cell lines, including MCF10A breast epithelial cells (43).

It has been shown that DNA re-replication can generate both ssDNA and DSBs, and lead to checkpoint activations (9, 44, 45). Hypothetically, DSBs are potential substrates for gene amplification in cells that are able to continue the cell cycle with the unrepaired DNA (34-36). However, whether re-replication can truly induce gene amplification and stimulate tumor growth has not been tested experimentally.

Therefore, we induced DNA re-replication by Emi1 depletion in various breast cancer cells. We showed checkpoint activation by DNA re-replication in those cells. Simultaneously, we used MTX (methotrexate) to inhibit DHFR (dihydrofolate reductase) and cell proliferation (46). DHFR is an enzyme that reduces dihydrofolic acid to tetrahydrofolic acid and is essential for the synthesis of purines, thymidylic acid, and certain amino acids (47). If DHFR gene is amplified, cells will survive MTX. Therefore we used LD50 (lethal dose, 50%) of MTX to estimate DHFR gene amplification after DNA re-replication. However, gene amplification was not detected in re-replicated cells, even after checkpoint inactivation. Furthermore, no significant difference was found in tumor growth between xenografts of control and re-replicated cells.

4.2.2 Results

Emi1 Depletion Induces DNA Re-replication and Checkpoint Activation in Various Breast Cancer Cells

To test the hypothesis that re-replication can induce gene amplification in breast cancer cells, we first determined (a) which cell lines to use and (b) whether siRNA to Emi1 could induce DNA re-replication in those cells.

As stated earlier, gene amplification can only occur in DNA damage checkpoint deficient cells (32, 33). P53 is known as an important downstream target of checkpoints, functioning as a barrier to tumorigenesis. P53-deficient cells are supposedly more susceptible to genetic alterations and developing MTX resistance (48). Therefore, we chose three different p53 mutant breast cancer cell lines, Sk-Br-3, T47D, and Mda-Mb-468 cells, to perform these experiments (Fig. 5).

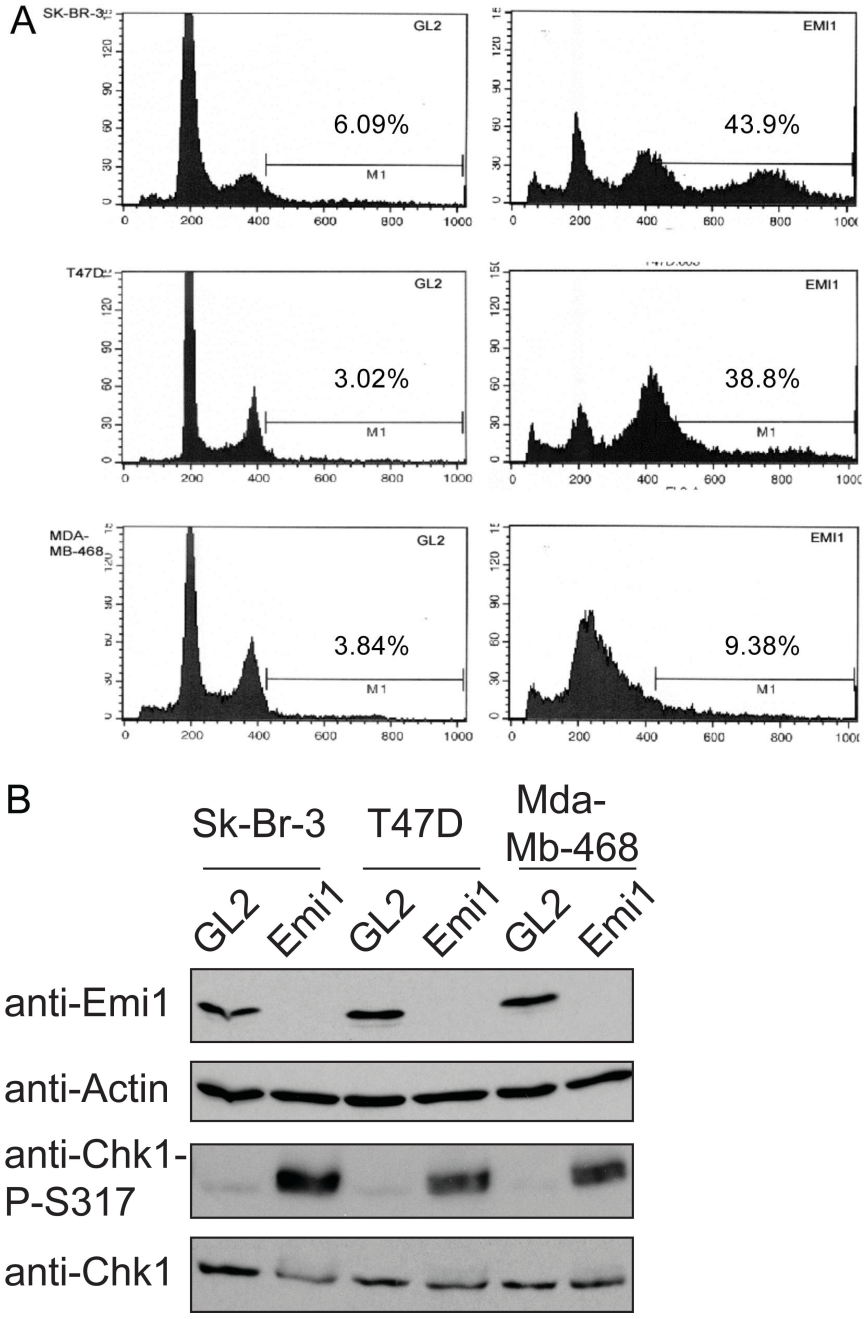


Figure 5. Emi1 induces DNA re-replication and activates checkpoint in various breast cancer cells. Sk-Br-3, T47D and Mda-Mb-468 cells were transiently transfected with control siRNA GL2 or Emi1 siRNA. Cells were harvested after 72 hours, fixed by 70% ethanol and stained with PI solution. The labeled cells were then analyzed by flow cytometer. The percentages of cells with >4N DNA content are indicated in (A). The western blots with indicated antibodies are shown in (B).

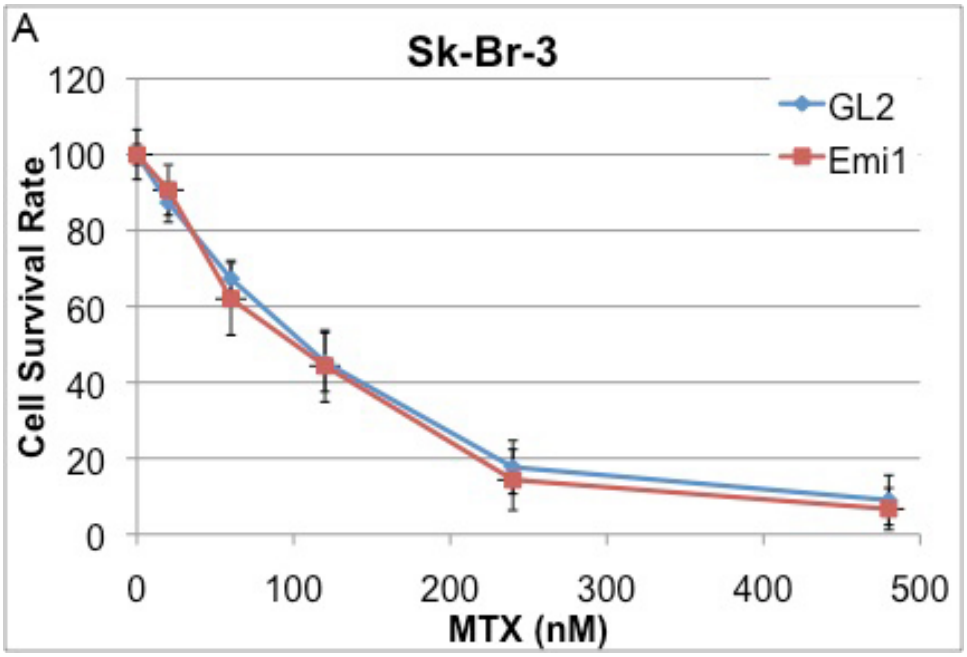
Emi1 siRNA or GL2 siRNA was transiently transfected in these cancer cells. FACS was performed after 72 hours to analyze the percentage of re-replicated cells (Fig. 5A). In both Sk-Br-3 and T47D, around 40% cells contain >4N DNA, indicating that 40% cells undergo DNA re-replication. However, upon Emi1 depletion, Mda-Mb-468 seemed to have an S-phase arrest with less than 10% cells undergoing re-replication. Consistent with previous results that DNA re-replication activates checkpoint pathways, Chk1 phosphorylation on Ser317 can be detected by immunoblotting in protein lysates made from Emi1 depleted cells, including Mda-Mb-468 (Fig. 5B). Taken together, Emi1 depletion can induce DNA re-replication and activate checkpoint pathways in Sk-Br-3 and T47D cells. Although the loss of Emi1 activates S checkpoint pathways in all cells, Mda-Mb-468 seems to be the only one that responds by suppressing DNA replication so that not much re-replication is seen.

MTX LD50 Was Not Changed in Re-replicated Breast Cancer Cells

To test whether gene amplification occurs after DNA re-replication, we decided to use MTX to examine the ability of these cells to amplify the DHFR gene. Cells that overexpress the DHFR protein by either DHFR amplification or certain mutations can all survive MTX. To ensure that we were looking at bona fide amplification events, we planned to use southern blot to compare the DHFR gene copy number after we found difference in MTX resistance between control and Emi1 depleted cells.

Indicated cells were transfected with siRNA of control (GL2) or Emi1 on day 1, 4 and 7. Colony formation assays were subsequently performed to measure cell survival after MTX treatment. After plotting percentages of survived cells in control (siGL2) and re-replicated (siEmi1) cells, we did not observe any changes in LD50 of all three cell-

lines tested (Fig. 6). One explanation could be that all the cells that undergo re-replication are arrested by protective checkpoint pathways or directed to apoptosis before initiation of gene amplification. Therefore, we decided to inactivate checkpoint pathways by adding UCN-01 and inhibit p53 independent apoptosis by adding caspase inhibitor Z-VAD-FMK, respectively (49, 50). However, no significant difference was noticed even after the addition of the two chemicals (data not shown).



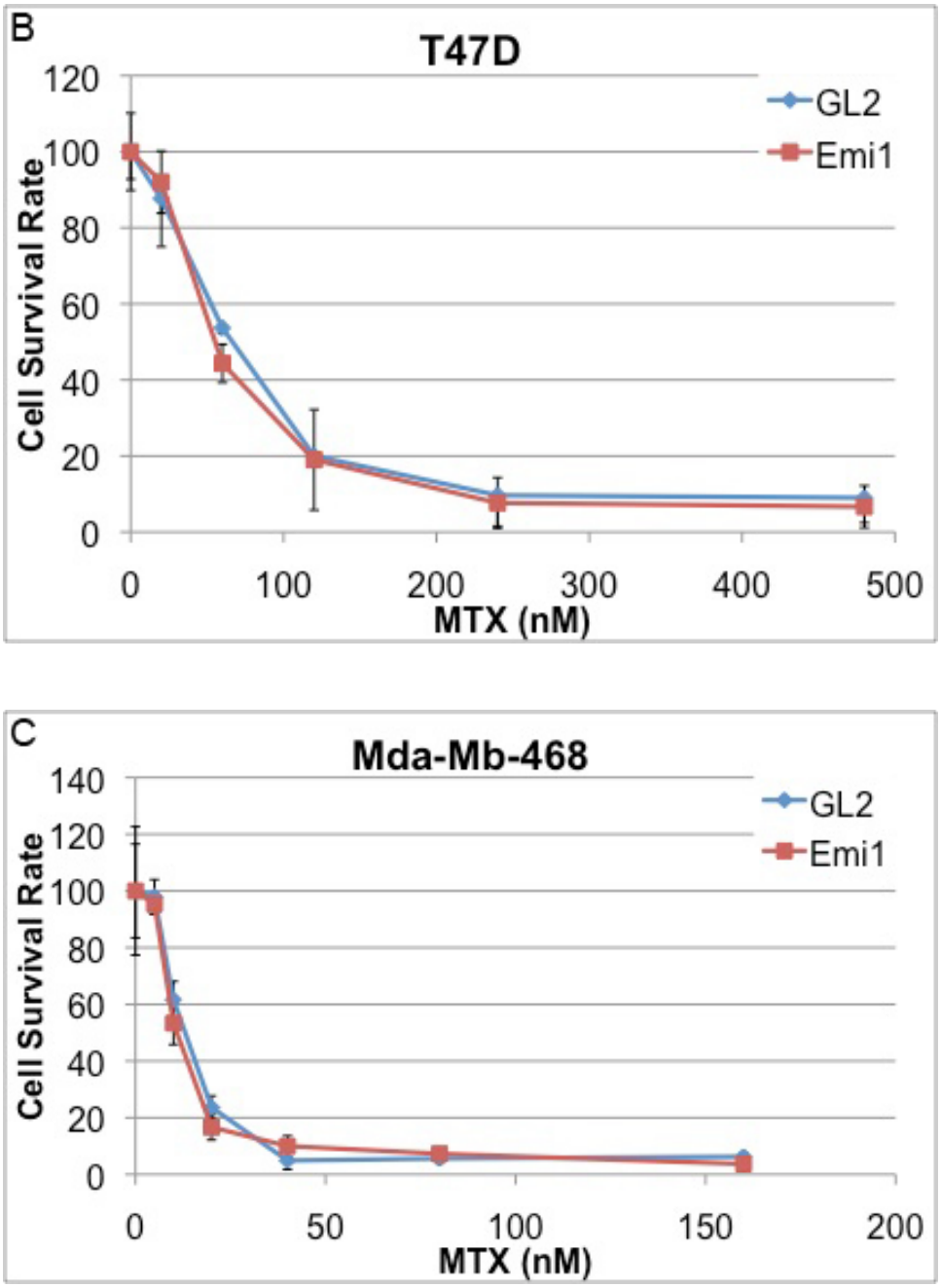


Figure 6. MTX LD50 was not changed in re-replicated breast cancer cells. Sk-Br-3 (A), T47D (B), and Mda-Mb-468 (C) cells were transfected with siGL2 (control) or siEmi1 on day 1, 4 and 7. Cells were subsequently seeded in 6-well plates for colony formation assay as described in "Materials and Methods". Cell were harvested on day 9 after seeding, fixed and stained to estimate the percentage of cell survival.

No Difference in Tumor Growth Was Detected Between Xenografts of Control (siGL2) and Re-replicated Cells (siEmi1)

The ultimate goal of the work is to test whether DNA re-replication can be directly connected with gene amplification and tumor growth. Although no difference of MTX resistance has been observed, it does not necessarily mean that gene amplification does not occur after DNA re-replication. MTX resistance only measures DHFR amplification. We hypothesized that re-replication could induce gene amplification on a genome-wide scale and thus a growth advantage of re-replicated cells could be observed in an *in vivo* system. Therefore, we decided to perform xenograft experiment using control or re-replicated MCF10A breast epithelial cells and T47D breast cancer cells, with or without the addition of MTX.

To prepare the cells for xenografts, MCF10A and T47D were treated as described in Fig. 6 to induce DNA re-replication. Cells were then cultured in MTX-containing media for 7 days before being collected for xenograft experiment. Five nude mice per group, a total of 40 mice (two cell lines: MCF10A and T47D; four groups each cell line: MTX-GL2, MTX+GL2, MTX-Emi1 and MTX+Emi1) were injected. In mice injected with MTX+ cells, MTX was subsequently injected on day 1 and 8 for further inhibition. The tumors were measured twice a week and the average tumor size for each group on day 51 was plotted in Fig. 7. In MCF10A cells, small palpable nodules were formed instead of real tumors, consistent with the previous report (51). SiEmi1 did not stimulate tumor growth as we expected. There was an increase in tumor size when siEmi1 treated MCF10A was placed under MTX selection. However, the difference was not significant.

Tumors were formed in T47D cells. Unfortunately, there was no significant difference between control (siGL2) and re-replicated (siEmi1) group, before or after MTX addition.

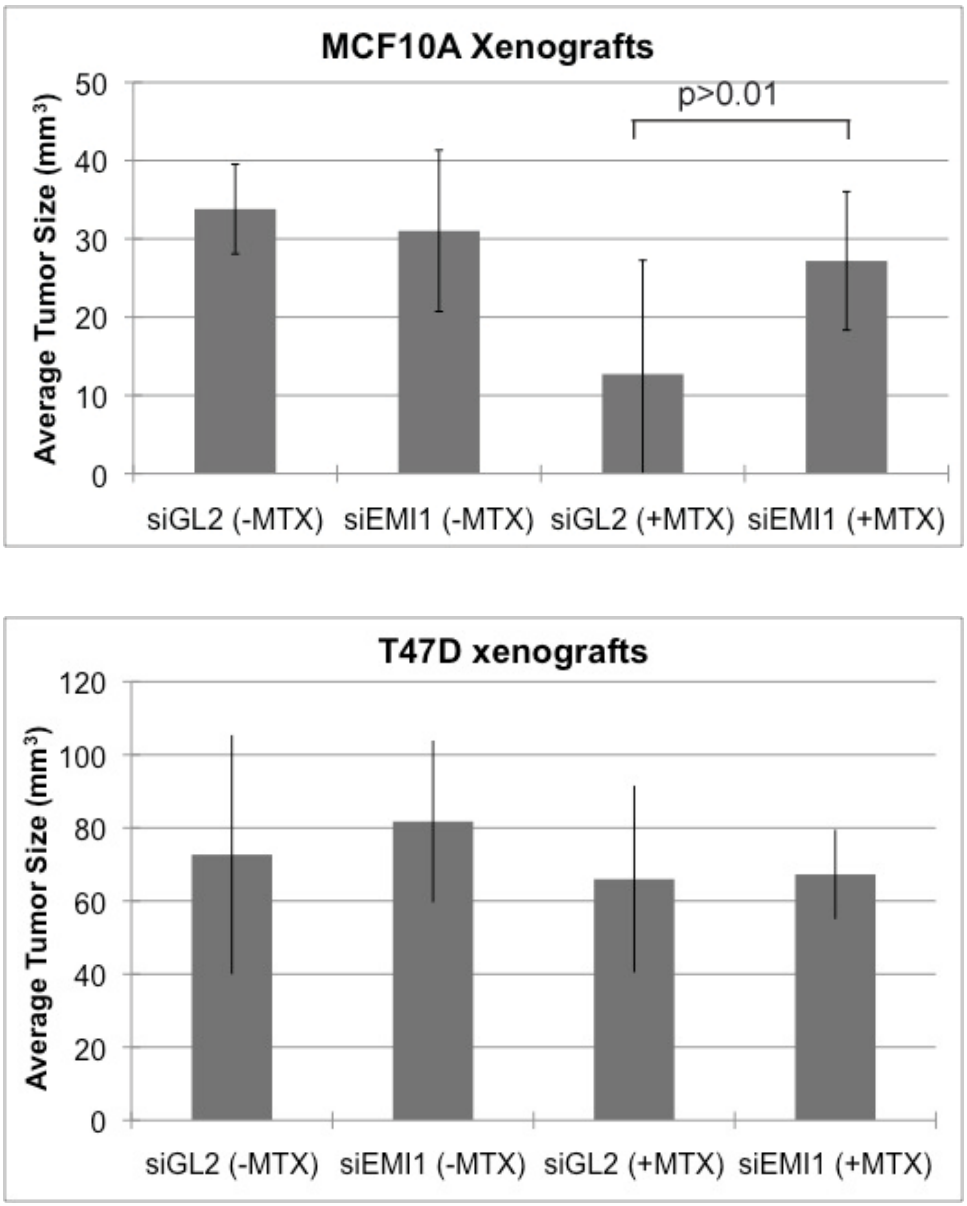


Figure 7. No difference in tumor growth was detected between xenografts of control (siGL2) and re-replicated Cells (siEmi1). MCF10A or T47D cells were transiently transfected with GL2 or Emi1 siRNA on day 1, 4 and 7. Culture cells in normal growth media containing no MTX or 20nM MTX for an additional 10 days before xenograft experiments. Cells were injected into nude mice as described in "Materials and Methods". The average tumor size

from different mice was plotted. Error bar stands for standard deviations from the 5 mice in each group.

4.2.3 Discussion

We were disappointed at the result that DNA re-replication could not induce gene amplification in breast cancer cells. Green *et al.* demonstrated in a different experimental system that re-replication is a potent mechanism of amplification (52). In their system, *Saccharomyces cerevisiae*, re-replication can be predominantly induced from a single origin (ARS317) by misregulation of Cdc6 and MCM2-7 proteins (53). Although it is unknown why *ARS317* reinitiates so efficiently, the origin can be used to build a link between re-replication and gene amplification. Gene amplification was measured in re-replicated cells with colony color. A selected gene (the *ade3-2p* allele) was combined with *ARS317* in a re-replicating reporter cassette that was integrated at desired loci. Yeast cells containing one copy of *ade3-2p* are pink, and cells containing two copies are red. By simply looking at the percentage of colonies with red sectors, Green *et al.* could determine that gene amplification has occurred. They found a 42-fold increase in the % of colonies with red sectors. In addition, aCGH (array comparative genomic hybridization) was performed with the red sectors, confirming the amplification of *ade3-2p* containing region. Moreover, aCGH results showed that yeast amplicons were huge in size (135-470kb). They were usually tandemly arrayed in head-to-tail orientation (Fig. 8), which probably resulted from NAHR (nonallelic homologous recombination), with boundaries of repetitive DNA sequences.

The work in budding yeast demonstrated that DNA re-replication could lead to gene copy number increase possibly by a mechanism illustrated in Fig. 8. It suggested that the DNA structure formed during re-replication might initiate amplification even in mammalian cells. Bearing this hypothesis in mind, the question we want to raise is, why did we not detect gene amplification in our study? There are two possible reasons. First, we lacked an efficient system to screen for amplification. It is difficult to find a well-defined origin like *ARS317* that is known to preferably refire after re-replication induction in mammalian cells. Moreover, there is no easy technique to screen copy number increase in human cells like in yeast. Secondly, no amplification occurred in our system. Previous studies showed DNA re-replication caused checkpoint activation and apoptosis (44, 54). Furthermore, re-replication was suggested to induce cellular senescence pathway (55, 56). Checkpoint, apoptosis and senescence pathways are all suggested to be important barriers to tumorigenesis (57, 58). Therefore, it is possible that the three pathways inhibited the critical initiation step from re-replication to gene amplification. It is worth testing whether gene amplification can take place after the inactivation of checkpoint, apoptosis and senescence pathways in re-replicated cells.

In summary, although gene amplification was not detected in re-replicated breast cancer cells, we do not conclude that re-replication cannot induce amplification. Indeed, more efforts should be made in future to create a better experimental system in mammalian cells for screening for gene amplification.

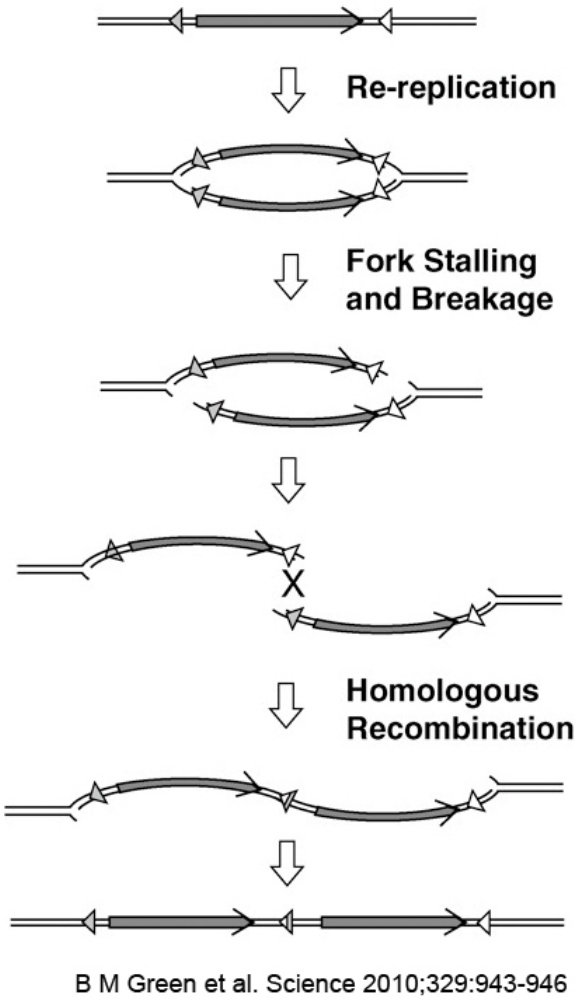


Figure 8. The potential mechanism of how re-replication might stimulate NAHR and lead to gene amplification in *S. cerevisiae*. Arrowheads, nonallelic or hybrid recombinant repetitive element; arrows, amplified segments.

4.3 MATERIALS AND METHODS

Antibodies and chemicals

Hydroxyurea, nocodazole, methotrexate and crystal violet were obtained from Sigma. Z-Vad-FMK (Calbiochem) and UCN-01 (Sigma) were used at 50μM and 25nM,

respectively. Rabbit anti-Geminin was raised as described earlier (59). Mouse anti-Chk1, mouse anti-β-actin, (Sigma), rabbit anti-phospho-Chk1 (Ser317), rabbit anti-phospho-Cdc2 (Tyr15), rabbit anti-phospho-Cdc25C (Ser216) (Cell Signaling), mouse anti-Cdc2 (sc-54), rabbit anti-Cdc25C (sc-6950) (Santa Cruz) and rabbit anti-Claspin (A300-266A) (Bethyl) antibodies were used in immunoblotting. Rabbit anti-HDAC6 antibody (FH21) was raised by Covance using the last 20 amino acids of human HDAC6. FH21 was used in both immunoblotting and immunofluorescence.

SiRNA and shRNA

Short interfering (siRNA) oligonucleotides (Dharmacon and Invitrogen) were made to the following target sequences: geminin (sense): 5'UGCCAACUCUGGAAUCAAA 3'; HDAC6A: 5' CAAGCCUCCUCAACUAUGA 3' (5' UTR); 6B: 3' CGGAUGACCACACGAGAAA 5' (3' UTR); 6C: 5' GGUAAAGAAGAAAGGCAAA 3' (coding); 6D: 5' CCGCUAUGCUCAACAGAAA 3' (coding); Chk1: 5' UCGUGAGCGUUUGUUGAAC 3'; Luciferase (GL2): 5' CGUACGCGGAAUACUUCGA 3'; Emi1: 5' GAGAAUUUCGGUGACA GUCUA 3'. Transfections were performed with 20nM siRNA oligonucleotide duplexes with Lipofectamine RNAiMAX (Invitrogen) according to the instructions of the manufacturer.

FACS analysis

Cells were collected by trypsinization and fixed with 70% ethanol overnight in −80℃. Cells were centrifuged and stained with PI solution (0.05% Nonidet P-40, 50ng/ml PI, and 10μg/ml RNaseA) after fixation. The labeled cells were analyzed on a Becton Dickinson flow cytometer with Cellquest Pro software.

Immunoblotting

Cells were lysed in lysis buffer containing 0.1% NP-40, 50mM Tris-HCl 7.4, 150mM NaCl, 5mM EDTA, 50mM NaF, 1mM Na_3VO_4, and protease inhibitor mixture (Sigma). Equal amounts of cell lysates were resolved on SDS-PAGE, and then proteins were transferred and blotted with indicated antibodies.

Hydroxyurea arrest and cell cycle re-entry

U2OS cells were transfected with siRNAs to luciferase (GL2), chk1, or HDAC6 (6D siRNA). 28 hours later, cells were treated with 1 mM hydroxyurea for 15 h. Cells were washed 3 times and allowed to recover in fresh media containing 40ng/mL nocodazole. Cells were harvested for FACS analysis and protein expression before hydroxyurea treatment (untreated) after hydroxyurea treatment, and after a 16 h recovery from arrest.

Immunofluorescence

HCT116 cells were transfected with indicated siRNA. 72 hours later, cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature and permeabilized with 0.5% ice-cold triton-X100 in PBS for 10 min. Cells were blocked for 1 hour in 5% goat serum in TBST (0.1% triton-X100 in TBS) and incubated with primary antibody in TBS with 3% BSA overnight. Cells were then incubated with secondary antibody (Alexa Fluor, Invitrogen) for 1 hour. Cells were mounted with solution containing 4′, 6′-diamidino-2-phenylindole (DAPI) (Vector Laboratories) before examination under the microscope.

Colony formation assay

After cells were transfected with siGL2 or siEmi1 for three times, cells were trypsinized and different amount of cells were seeded in 6-well plate. $3*10^4$ cells were

seeded per well for Sk-Br-3, $6*10^4$ per well for T47D, and 1000 per well for Mda-Mb-468. The above numbers were decided by earlier experiments. 24 hours after seeding, media containing no MTX or indicated concentrations of MTX was added. The media was replaced every three days. On day 9, cells were harvested as described (60). OD595 was measured to quantify cell colony numbers and normalized to untreated sample to obtain cell survival rate.

Xenograft experiment

40 athymic nude mice (female, 5-6 week old) were used in this study with five per group. MCF10A and T47D cells were transfected with siGL2 or siEmi1 as indicated above. These cells were trypsinized and resuspended in PBS. The cells were mixed with an equal volumn of matrigel to get a 50% matrigel solution with $5*10^7$ cells/ml. 100µl of this solution was injected as a subcutaneous inoculum into the flank of mice. The mice were treated with MTX or PBS (for control) on day 1 and 8 after tumor implantation, with a dosage of 50mg/kg at the first time and 100mg/kg at the second time. The mice were weighed and tumor size measured with calipers twice weekly. The mice were humanely euthanized two months after the initial injection. The average tumor size on day 51 was plotted as shown.

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CHAPTER FIVE

CONCLUDING REMARKS

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5.1 SUMMARY

Deregulation of DNA replication initiation can lead to re-replication in many systems (1-5). Clearly, it is important to investigate the consequences of DNA re-replication in order to understand its biological significance.

The depletion of Geminin in colon cancer cells causes DNA re-replication, activates an ATR/Chk1 mediated checkpoint and arrests cells in G2/M phase. The cell cycle checkpoint is essential for the accumulation of re-replicated cells, which can further activate ATM/Chk1 pathway. The elimination of any components in ATR/Chk1, but not ATM/Chk2, pathway can inactivate the G2/M checkpoint and abolish DNA re-replication. The results suggest that the DNA damage created initially by re-replication is ssDNA, which leads to the preferential activation of ATR/Chk1 pathway. At later stages, fork collapse and replication across single-stranded nicks generate dsDNA breaks, and activate ATM/Chk2, p53 and possibly apoptosis. Such mechanism can give cells a chance to repair minor DNA damage, and induce apoptosis only when re-replication becomes extensive and unbearable. The sequential generation of ssDNA and dsDNA breaks in re-replication is further confirmed by other groups (6).

Similarly, MLN4924, an experimental cancer drug, can stabilize Cdt1 and induce DNA re-replication in various human cancer cells. Transient exposure of MLN4924 is sufficient to cause re-replication. S phase cells are more susceptible to DNA re-

replication, suggesting that MLN4924 is more toxic on highly proliferating cells. Furthermore, short exposure of MLN4924 can activate apoptosis and senescence pathways. Apoptosis is more important for the MLN4924 anti-proliferative effect although both pathways are contributive. More intriguingly, p53 negative cancer cells are equally sensitive to MLN4924 treatment, suggesting its potential ability in clinical applications.

Overall, our study showed DNA re-replication can activate checkpoint pathways, apoptosis, and cellular senescence, phenomena that can be used for killing cancer cells by drug-induced re-replication. The exciting aspect is that this mode of therapy is effective in p53 negative cancer cells, which are usually resistant to most DNA damaging chemotherapy agents.

In unfinished work, I have also shown that the HDAC6 deacetylase has an unexpected role in activating checkpoint pathways following re-replication. The reason for this requirement is unclear, but Chk1 protein level and Chk1 phosphorylation are both decreased following HDAC6 depletion.

One concern about re-replication is that it may provide the seeds of gene amplification. However, I did not see any evidence of this, most likely because re-replication is so toxic that the cells do not survive to allow gene amplification. Of course, this is good news for drugs like MLN4924 that cause re-replication, but negative results have to be treated with caution.

5.2 OUTSTANDING QUESTIONS IN THE FIELD

5.2.1 Re-replication, DNA Damage Response, and Senescence Pathway

DNA re-replication is shown to induce checkpoint activation. It is suggested that the abnormal DNA structures generated during re-replication trigger checkpoint pathways (7). However, it is not clear how DNA re-replication induced by different mechanisms activates different pathways. For example, re-replication seen after Cdt1 overexpression leads to a protective S phase checkpoint (5). In contrast, re-replication activated by Geminin depletion activates a G2/M checkpoint and causes the accumulation of re-replicated cells (4, 8). One explanation of the different outcomes is that the different studies used distinct experimental systems. Another explanation is that Geminin plays an important role in M phase. Geminin is known to be a dual function protein with roles in regulation of both replication initiator Cdt1 and transcription (9, 10). Furthermore, Geminin level increases at G1/S transition and remains high in G2 and M phase when Cdt1 is absent, suggesting its potential roles in G2/M. Indeed, Geminin was shown in a study to participate in the promotion of proper cytokinesis (11). Thus Geminin depletion may not have exactly the same effect as Cdt1 overexpression.

In addition to checkpoint pathways, cellular senescence is activated by re-replication. Our study has shown that both p53 and p21 are important but not essential in the pathway. What are other factors involved in the pathway? $P16^{INK4a}$ or Rb could be involved. Both the proteins are suggested to function in senescence pathway (12). Co-depletion of p53, p21, $p16^{INK4a}$ and Rb proteins can be tested in our system to examine whether all of them are required. If we can make cells escape senescence and apoptosis, it will be possible to answer what happens when cells re-enter the cell cycle after irreparable re-replication. It is a critical question to understand the biological significance

of DNA re-replication, particularly if we are to investigate whether gene amplification results from re-replication.

An equally important question is, whether low level of re-replication happens in normal cells and the effect of this anomaly. Massive re-replication can be catastrophic and cause inevitable cell death. However, if only very few re-initiation events occur in the cells, the resulting abnormal DNA structures may be under the threshold of checkpoint activation. Thus cells bearing extra DNA may proceed to mitosis and enter the next cell cycle. This can possibly lead to inheritable genomic instability. To test the hypothesis, it is necessary to develop more sensitive methods to detect DNA re-replication.

5.2.2 Re-replication and Gene Amplification

As described in earlier chapters, DNA re-replication is a potent mechanism of gene amplification in budding yeast (13). It suggests that the abnormal DNA structures formed during re-replication can induce gene amplification. However, in order to verify the hypothesis in human cells, we need to answer the following questions. (a) In human cells, does re-replication happen in a site-specific manner? Several papers showed increased copy numbers of known origins, such as *LaminB2* and *β-globin*, are detectable in re-replicated cells, suggesting a site-specific re-replication in human cells (4, 14). (b) Can we find a suitable screen to identify amplified sites? If we confirm certain regions are specifically amplified during re-replication, we can tag those sites with GFP or antibiotic selective genes to screen for gene amplification in cell culture. (c) Can re-replicated cells escape cell cycle arrest, apoptosis and senescence? To answer this

question, we need to find the essential factors in these response pathways, especially senescence. With chemical inhibitor or siRNA depletions, we can override the pathways and may then observe gene amplification after re-replication. Or with more sensitive methods to observe re-replication (like FISH, fluorescence *in situ* hybridization), we can ensure that we are seeing re-replication while keeping the re-replication level below the threshold for detection by checkpoint machinery, so that there is no cell death.

As discussed earlier, in normal cells, if cells bearing re-replicated DNA enter the following cell cycles, can re-initiation happen again at the same site? Can the resulting structures lead to gene amplification? Short inverted repeats in mammalian genome are shown to initiate gene amplification (15). In yeast, amplicons are bound with repetitive DNA sequences (13). Therefore, it is worth investigating whether we can induce re-replication from artificial origins bounded by short inverted repeats and then determine whether the re-replication leads to tumorigenesis.

5.2.3 Re-replication and tumorigenesis

Currently there is no direct evidence for the correlation of re-replication with tumorigenesis, although Cdt1 overexpression can stimulate tumorigenesis (16, 17). In addition, increased Cdt1 and/or Cdc6 proteins are observed in tumors and cancer cell lines (18-20). Of course it is possible that the oncogenic ability of Cdt1 and Cdc6 is not dependent on their functions in replication initiation. For example, Cdc6 can be recruited to INK4/ARF locus, recruit histone deacetylases, and repress all three tumor suppressors $p15^{INK4b}$, $p16^{INK4a}$, and ARF (18). However, definitive experiments with mutant Cdt1 or Cdc6 should be done to answer whether mutants that cannot support replication initiation

can still support re-replication. Indeed, Jamie Teer in the lab, has shown that mutants of Cdt1 that cannot interact with MCM2-7 (and thus should not support DNA replication) can still promote re-replication (21).

DNA re-replication causes cell cycle arrest, apoptosis and senescence, all of which are barriers to tumorigenesis (22-24). Is it possible that re-replication can stimulate tumorigenesis in cells deficient in those pathways? Indeed, oncogene activation in normal human cells induces DNA re-replication, DNA damage response, and senescence. Inactivation of checkpoint abrogates senescence and stimulates tumor growth (24). It has been suggested that this oncogene induced re-replication is the primary selection for checkpoint inactivation during cancer progression. It is noteworthy that p53 deletion synergizes with elevated Cdt1 and Cdc6 to increase genomic instability and tumor formation, suggesting re-replication can induce tumorigenesis in cells deficient in checkpoint, apoptosis and senescence pathways (17, 20). If the theory is true, how does re-replication stimulate tumorigenesis after escaping cell death? Gene amplification, which is prevalent in some tumors, is a possible mechanism (25-27). Besides amplification, the stalled forks in re-replicated cells can initiate other forms of genome instability such as deletion and chromosomal rearrangements.

It is also worth noting that cells with re-replication often show over-replication of the centrosomes (28). Excess centrosomes lead to multipolar spindles that have been shown to give rise to asymmetric segregation of chromosomes and aneuploidy (29). Thus, the elevated Cdt1 and Cdc6 seen in cancer can also be responsible for aneuploidy, another possible mechanism of oncegenesis.

Given the connection between re-replication and tumorigenesis, it remains to be seen whether low doses of MLN4924 give rise to tumors. This is not an unexpected side effect of many therapies used in cancer, starting from radiation to alkylating agents like doxorubicin. Agents that kill cancers by DNA damage are expected at low doses to produce enough genomic instability to promote cancers.

5.3 FUTURE PLANS

In order to answer the above questions and understand the biological significance of DNA re-replication, we first need to find out specific re-replication sites in the human genome. Using new technology like next generation sequencing, we can compare copy number change in re-replicated and control cells, and discover regions that are over-replicated. However, global increase in DNA cannot be picked up by this method. We need to measure the total DNA yield per cell and compare the DNA content of control and that of re-replicated cells. Once amplified sites are identified, FISH with the desired probes can be used to detect microscopic re-replication. The consequences of microscopic re-replication can also be tested by knocking in GFP or antibiotic resistant genes into the hot-spots of re-replication. Xenografts with these cells will then find out whether gene amplification is induced and tumor growth is stimulated. It is worth examining whether it is necessary to inactivate checkpoint and senescence pathways in this scenario. To eliminate cellular senescence, more work is necessary to identify essential players in re-replication-induced senescence pathway. On the other hand, it is

important to test whether spontaneous re-initiation events happen in normal cells on re-replication specific sites using FISH. If yes, can it eventually lead to gene amplification under selective conditions? And is it contributing to tumor development? The answers to these important questions will lead to a better understanding of the roles of DNA replication control and re-replication in human body and in diseases.

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