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Regulation of MDC1 during Mitotic DNA Damage

By

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Doctor of Philosophy

Graduate Division of Biological and Biomedical Science
Biochemistry, Cell and Developmental Biology

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Abstract Cover Page

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B.S., China Agricultural University, 2007

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Abstract

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Genetic instability is a hallmark of human cancers. DNA double strand break (DSB), if improperly repaired, results in genetic instability and tumorigenesis. Previous research has established γH2AX as a bona fide marker of DSBs and that hierarchical foci assembly of DNA damage response (DDR) proteins at DSB sites is required for efficient repair. Recent findings from our group demonstrated that during either spontaneous or induced prolonged mitosis, cancer cells acquire DSBs, which lead to further chromosomal abnormality (1). Deciphering the mechanism of such DSB accumulation may provide new insights into the mechanism of genetic instability. Several recent studies suggest a partial DDR during mitosis, wherein the DSBs are marked by γH2AX, but repair only takes place after mitotic exit (2, 3). We hypothesize that the increase in DSBs during prolonged mitosis is due to the lack of efficient DDR. To this end, we focus on the protein Mediator of DNA Damage Checkpoint 1 (MDC1), an important mediator in DSB repair. MDC1 directly binds to γH2AX, serves as a platform to accumulate/retain downstream DDR proteins at DSB sites and concomitantly initiates cell cycle arrest through its multiple interaction domains. In this dissertation, I present evidence that demonstrates Cyclin-dependent kinase 1 (CDK1) inhibits MDC1- γH2AX interaction and

provide new insights into mechanisms in response to DNA damage during mitosis. Additionally, my results suggest F-Box and WD Repeat Domain-Containing 7 (FBW7), the substrate recognition component of the E3 ligase complex SCF (complex of SKP1, CUL1 and F-box protein) as a negative regulator of MDC1 in human cancer cells.

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Brook University Medical Center. Carla has been instrumental in scheduling and setting up my committee meetings over the years. Thank you, Carla, for being a true friend, whom I can rely on.

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Table of Contents

Abstract

Genetic instability is a hallmark of human cancers. DNA double strand break (DSB), if improperly repaired, results in genetic instability and tumorigenesis. Previous research has established γH2AX as a bona fide marker of DSBs and that hierarchical foci assembly of DNA damage response (DDR) proteins at DSB sites is required for efficient repair. Recent findings from our group demonstrated that during either spontaneous or induced prolonged mitosis, cancer cells acquire DSBs, which lead to further chromosomal abnormality (1). Deciphering the mechanism of such DSB accumulation may provide new insights into the mechanism of genetic instability. Several recent studies suggest a partial DDR during mitosis, wherein the DSBs are marked by γH2AX, but repair only takes place after mitotic exit (2, 3). We hypothesize that the increase in DSBs during prolonged mitosis is due to the lack of efficient DDR. To this end, we focus on the protein Mediator of DNA Damage Checkpoint 1 (MDC1), an important mediator in DSB repair. MDC1 directly binds to γH2AX, serves as a platform to accumulate/retain downstream DDR proteins at DSB sites and concomitantly initiates cell cycle arrest through its multiple interaction domains. In this dissertation, I present evidence that demonstrates Cyclin-dependent kinase 1 (CDK1) inhibits MDC1- γH2AX interaction and provide new insights into mechanisms in response to DNA damage during mitosis. Additionally, my results suggest F-Box and WD Repeat Domain-Containing 7 (FBW7), the substrate recognition component of the E3 ligase complex SCF (complex of SKP1, CUL1 and F-box protein), as a negative regulator of MDC1 in human cancer cells.

Chapter I

Introduction and literature review

DNA damage response

The human genome is constantly exposed to DNA-damaging agents, such free oxygen species as a result of inherent cellular metabolism. Also, cells may be temporarily exposed to external sources such as cosmic radiation or environmental toxins (4). Among all types of DNA damage, DNA double-strand breaks (DSBs), the most detrimental form of DNA damage (5), can be induced by ionizing radiation or radiomimetic chemicals, both are frequently used as cancer therapies (6, 7). DSBs are also introduced in physiological processes such as meiosis and immune system responses (8). Due to the absence of intact template strand, DSBs can lead to genomic rearrangements if not faithfully and efficiently repaired. To combat this, cells have evolved highly sophisticated repair mechanisms, coined DNA damage response (DDR) (9-11).

DNA damage repair foci formation

The hallmark of mammalian DDR is the prompt deployment of repair proteins to the damage sites on the DNA of the affected cells. These complex structures, which can be visualized by standard immunofluorescent staining, are commonly known as ionizing

radiation-induced foci (IRIF) (Fig1). While some proteins are directly involved in lesion repairs, others initiate signaling cascades that result in cell cycle arrest or apoptosis. Some DDR factors possess inherent affinity to free DNA ends, which are aberrant DNA structures found at DSB sites (12). In human cells, the MRE11/RAD50/NBS1 (MRN) complex has been shown to be a conserved DDR sensor, which rapidly detects and binds to broken DNA strands (13). MRN is also required for efficient activation of Ataxia Telangiectasia Mutated (ATM), a major kinase marking DSBs, by phosphorylation (14, 15). In human cells, the core regulator of IRIF formation is the histone H2A variant X or H2AX, an integral component of nucleosomes that accounts for 10-15% of total H2A(16, 17). Upon DSB induction, a conserved serine residue (S139) on the carboxyl terminus of H2AX is rapidly phosphorylated by activated ATM (pATM) (18-20). This phosphorylation of H2AX, termed as γH2AX, spreads in regions flanking the DSB sites, confined to up to two megabases in either direction (17). γH2AX serves as an epigenetic marker of damaged chromatin sites (17). Mediator of DNA damage checkpoint protein 1 (MDC1), a large nuclear protein then binds γH2AX, acting as a landing platform for (i) MRN-pATM to accelerate γH2AX formation spreading to chromatin regions flanking DSB sites (21-23); and (ii) recruiting other DDR factors to participate in damaged chromatin remodeling, initiation of repair pathway and activation of DNA damage checkpoint (24). An example is MDC1-mediated histone ubiquitination by E3 ligases ring finger protein 8 and 168 (RNF8 and RNF 168), thereby amplifying ubiquitination on histone H2A/H2AX, to promote the recruitment of downstream repair factors, such as p53-binding protein 1 (53BP1) and breast cancer 1, early onset (BRCA1) (25, 26). The final effects are repair

pathway activation, aided by temporary cell cycle arrest and if necessary- apoptosis (27).

Major DSB repair pathways

There are two major repair pathways that repair DSBs: non-homologous end joining (NHEJ) and homologous recombination (HR). In NHEJ, the two free DNA ends are directly ligated independent of a homologous template, resulting in a swift, but error-prone, repair (28). NHEJ requires DNA-binding Ku complex, DNA-dependent protein kinases (DNA-PKs), DNA-end-processing enzymes and the XRCC4-ligase IV complex (29). NHEJ pathway is active in all cell cycle phases. In contrast to NHEJ, HR is limited to S and G_2 phase, only when an intact sister chromatid is available(30). HR ensures faithful repair as information is copied from an intact homologous DNA template. HR consists of several steps. First, MRN complex promotes the initial resection of DNA ends by Retinoblastoma binding protein 8 (RBBP-8/CtIP) to produce short 3' overhangs, followed by further resection involving Dna2 and Exo1 nucleases to extend the overhangs (31, 32). Then replication protein A (RPA) recognizes and binds to 3' overhangs and is then replaced by radiation sensitive 51 (Rad51). The Rad51-bound single stranded DNA then invades into homologous double-stranded DNA (33). The extension of the strand invasion leads to the formation of Holliday junctions, which are subsequently resolved in order to complete an error-free DSB repair (34). How damaged cells facilitate the optimal repair pathway is unclear. However, recent findings suggest that an antagonistic

effect of NHEJ on HR. Basically, NHEJ mediator 53BP1 inhibits DSB resection and Rad51 recruitment in BRCA1-deficient cells (35, 36).

Cell cycle checkpoints

DDR activation leads to different outcomes depending on the severity or type of DNA damage and cell cycle stages. The major responses can be categorized into DNA damage repair, cell cycle arrest to ensure adequate time, global transcriptional regulation, senescence or apoptosis when damage is beyond proper repair (Fig.1). DNA damage checkpoint activation can result in cell cycle arrest. Such temporary delay of cell cycle progression allows time for damage repair and prevents replication of damaged DNA. In S phase, DSB activates intra-S phase checkpoint, resulting in cessation of DNA synthesis. Cells without efficient checkpoint can proceed with DNA replication, incorporating mutations arisen from DSBs. In G_2 phase, the G_2-M checkpoint prevents cells with damaged chromatin from entry into mitosis. In the presence of DSBs, the major effector kinase Checkpoint kinase 2 (CHK2) arrests the cell cycle by inactivating phosphatases of the Cell division cycle 25 (CDC25) family through mechanisms that include catalytic inactivation, nuclear exclusion, and proteasomal degradation (37, 38). This, in turn, prevents CDC25 from dephosphorylating and activating Cyclin-CDK complexes, thereby initiating G_1/S and G_2/M cell cycle checkpoints.

Mitosis, though short, is particularly vulnerable to genomic instability, due to the

challenge to accurate chromosome segregation into daughter cells. Eukaryotic cells have evolved a sophisticated spindle assembly checkpoint mechanism (SAC), which monitors the correct attachments of all chromosomes to microtubule spindle apparatus via their kinetochores (39, 40) (Fig2). When not correctly attached to the spindle, kinetochores send a "wait" signal to SAC, which in turn blocks cell cycle progression. Once all kinetochores are stably attached to the mitotic spindle, the checkpoint is inactivated, which alleviates the inhibition on E3 ligase anaphase promoting complex (APC). Activated APC then degrades Cyclin B and securin. Cyclin B is required for sustained CDK1 activity, essential to maintain mitotic state and securin inhibits separase, which allows separation of sister chromatids at the onset of anaphase (41). Thus, either disruption of spindle dynamics or the kinetochore complex can activate SAC, introducing prolonged mitosis or mitotic arrest (42). Previously, our laboratory has demonstrated that spontaneous mitotic arrest is a common feature in cancer cells. Consistently, induced mitotic arrest results DNA damage and chromosomal aberrations (1, 43).

MDC1, the master organizer of DDR factors in IRIF

Apart from sensor and transducers, there is a group of DDR proteins that lack enzymatic activity, yet are vital to proper DDR. Mediator of the DNA damage checkpoint 1 (MDC1) is a large scaffolding protein that specifically binds to γH2AX and serves as a landing platform for downstream DDR factors. Biologically, MDC1 knockout mice displayed a variety of phenotypical defects similar to H2AX knockout mice, thus substantiating the

vital role of MDC1 at early stage of DDR (44, 45). Furthermore, reduction or loss of MDC1 is associated with increased tumorigenesis in mice as well as a significant proportion of human carcinomas (46).

At the molecular level, MDC1 is composed of several functional domains (Fig. 4). The functions of MDC1 can be divided based on individual domains, since each of them seems to specifically recognize interacting partners that aggregate at the damaged chromatin site. Over the past 10 years, the molecular mechanism of the MDC1-γH2AX interaction has been extensively explored, while MDC1-mediated localization of several DDR factors is gradually unveiled (47). Phosphorylation-dependent protein-protein interactions appear to be the central mechanism of these processes.

MDC1- mediated γH2AX foci formation

Mechanistic studies reveal that the BRCA1 C Terminus (BRCT) domain in MDC1 is the critical requirement for MDC1-γH2AX interaction and MDC1-mediated γH2AX foci formation. Shang et al. showed that the GFP-tagged MDC1 protein lacking BRCT domain failed to accumulate in γH2AX foci. The ectopic expression of BRCT region abrogated endogenous MDC1 and γH2AX foci (48). Oriented phosphopeptide library screening identified the optimal phosphopeptide-binding motif for the MDC1 BRCT tandem domain, which selectively bound to C-terminus γH2AX peptides (49, 50). Consistently, biochemical, X-ray structural, and functional studies confirmed that MDC1 directly

interacts with γH2AX via the BRCT domain (51-53). Point mutations disrupting either the phosphopeptide binding cleft of the BRCT tandem domain, or the H2AX C-terminus, abrogated the MDC1–γH2AX interaction *in vitro*. Abrogating MDC1-γH2AX interaction also disrupted foci formation of many other key DDR factors, such as MRN, pATM, 53BP1 and BRCA1, resulting in elevated radiosensitivity in cells (54). These data strongly suggest that one major biological function of the MDC1 in DDR is the initial recognition of the γH2AX and acting as a landing platform for other DDR factors to concentrate at the repair complex.

MDC1 does not only recognize the DSBs marked by γH2AX, but also promotes γH2AX foci formation. Reduction of total MDC1 or artificial disruption of the MDC1–γH2AX interaction reduces H2AX phosphorylation and γH2AX IRIF size (52, 54). This indicates that a positive feedback loop of γH2AX formation mediated by MDC1-γH2AX interaction. Since H2AX phosphorylation in response to DSBs is confined to chromatin regions flanking the lesion site, it was proposed that MDC1 might be required for γH2AX spreading along the damaged chromatin fiber (54, 55). In this model, MDC1 recognizes and binds to γH2AX in proximity to DSB sites and either directly or indirectly to promote the recruitment of activated ATM (pATM) in the damaged chromatin region. H2AX phosphorylation by pATM could speed up and spread more distal to the initiating lesion, consequently forming microscopically discernible foci. However, this simple model of MDC1-mediated self-reinforcing H2AX phosphorylation loop might not be the only explanation.

One of the major caveats in quantifying γH2AX foci formation is the lack of "resolution." Until recently, microscopy was the only way to study the dynamic assembly of the γH2AX foci. While these microscopic techniques have the intrinsic advantage to measure the kinetics of the γH2AX foci assembly and DDR factor aggregation, they are incapable of yielding information on the density of H2AX phosphorylation and the distance of γH2AX spreading along the damaged chromatin. To circumvent this problem, Savic et al. combined chromatin immunoprecipitation (ChIP) and the usage of endonuclease with known targeting sites in the genome, to create site-specific DSBs. Using primary mouse lymphocytes, they measured the γH2AX density and range at DSB sites generated by recombination activating gene 1/2 (RAG1/2) endonucleases during the Variable, Diverse, and Joining gene (VDJ) recombination. Since the genomic locations of RAG- generated DSBs are known, these regions could be specifically isolated by ChIP and subsequently measured for γH2AX density and range at DSB sites. Surprisingly, these data revealed that MDC1 is only required for maintaining γH2AX marks proximal to the break site, but not in the spreading process (56). Whether these findings also apply to the general response to DSBs remains to be determined.

Alternatively, MDC1 may control the dephosphorylation of γH2AX by shielding the phospho-epitope at C-terminus of H2AX. This model was based on the evidence that the purified MDC1 BRCT domains could efficiently protect γH2AX phosphopeptides from

phosphatase activity *in vitro*. Furthermore, overexpression of the MDC1 BRCT region in U2OS cells increased basal H2AX phosphorylation(50). Presently, it was known that the phosphatases PP2A, PP4 and wild-type p53-induced phosphatase 1 (Wip1) are involved in dephosphorylating of γH2AX in mammalian cells (57, 58). Thus, it will be interesting to examine how MDC1 interact with these phosphatases *in vivo*.

SDT repeat mediated MDC1–MRN interaction

The SDT region of MDC1 is characterized by conserved patches of 8–10 amino acids encompassing serine (S) and threonine (T) residues typically with an aspartate (D) in between. The SDT interacts with the MRN complex in a phosphorylation-dependent manner (23, 59, 60). Although the initial MRN recruitment and ATM activation are MDC1 independent, accumulation and retention of MRN complex at the damaged chromatin regions are strictly MDC1-mediated events (61-63). Expression of MDC1 lacking the SDT regions results in aberrant MRN micro-IRIF rather than fully developed MRN foci, occupying same region as γH2AX (23). This strongly suggests that initial recruitment of MRN complex is MDC1-independent, but its retention at the lesion site requires MDC1. Interestingly, MDC1 and MRN constitutively reside in the same complex even in undamaged cells. Further studies revealed that this interaction is mediated by acidophilic casein kinase 2 (CK2), which phosphorylates the SDT motifs (21, 59). Only doubly phosphorylated pSDpT motifs are functional for mediating MDC1's interaction with Nijmegen breakage syndrome 1 (NBS1), a component of the MRN complex (21).

Doubly phosphorylated pSDpTD peptides interact with both the FHA and BRCT domains of human NBS1 since only mutations in both domains effectively abolished such interaction (64). Moreover, disruptions in either forkhead-associated (FHA) or BRCT domain impair the MRN complex localization to IRIF (59, 65). Functionally speaking, the NBS1 FHA/BRCT region is required for the activation of the intra-S-phase and the G_2/M DNA damage checkpoints. Consistently, abrogation of MDC1- MRN interaction impairs checkpoint activation (59). Collectively, these data strongly indicate that MDC1 is upstream of MRN-mediated checkpoint activation during DSBs.

PST repeat-mediated MDC1 -DNA-PK interaction

The region between amino acids 1141 and 1662 in human MDC1 consists of 13 consecutive imperfect repeats, rich in proline, serine and threonine, hence the name PST (rich) repeats. The PST repeat in MDC1 is conserved in vertebrate species, though the number of repeats varies greatly from seven in mouse to thirteen in human (66). Primary sequence analysis revealed neither known structural or functional motifs, nor any potential homolog protein. To this day, the functional significance of the PST repeat remains an enigma. However, several observations indicate that the PST repeat is neither required for MDC1 recruitment (48), nor for MDC1-mediatd accumulation of 53BP1, BRCA1, and the MRN complex in damaged chromatin compartments (67, 68). Surprisingly, overexpression of a MDC1 mutant lacking PST repeats triggers partial NHEJ and defective HR repair, indicating a role of PST region in both DSB repair pathways (67,

68).

MDC1 - CHK2 interaction

It has been shown that the MDC1's FHA domain is important in the DDR: absence of this domain leads to many DDR defects, including defective G_2/M DNA damage checkpoint, inefficient DSB repair by HR, and radioresistance (52, 69-71). CHK2 is a major effector in DDR, facilitating checkpoint activation and cell cycle arrest. Not surprisingly, phosphorylated CHK2 was proposed as a putative MDC1–FHA interacting factor (72). Moreover, the MDC1 FHA domain was required for this interaction. CHK2, phosphorlyated on Thr68 (pThr68), stably interacts with MDC1 as shown in co-immunoprecipitation and peptide-binding experiments. Consistently, oriented phosphopeptide library screening demonstrated that the MDC1 FHA domain bound selectively to certain peptides, which closely matched the sequence surrounding the Thr68 of CHK2 (73). These lines of evidence suggest pThr68 CHK2 is a bona fide binding partner of the MDC1 FHA domain. However, CHK2 does not co-localize with MDC1 in DSB sites, but rather remains dispersed throughout the nucleus (74). This implies that the interaction between MDC1 and phosphorylated CHK2 is very transient in nature. Furthermore, it was recently discovered that pThr68-dependent dimerization of CHK2 effectively occluded the phospho-Thr68 motif, thus preventing its interaction with the MDC1 FHA domain (75). However, CHK2 autophosphorylation produces monomeric CHK2, which would expose the phospho-Thr68 motif (75, 76). This mechanism could

cause a rapid shift of abundance in the phosphorylated CHK2 between MDC1-free dimer and MDC1-bound monomer. This may explain the observation of unchanged CHK2 localization pattern in response to DNA damage (74).

The TQXF cluster-mediated chromatin modification

In contrast to the MRN complex, whose recruitment involves direct interaction with MDC1, recruitment of BRCA1 and 53BP1 depends on MDC1-mediated chromatin ubiquitination. This function is executed through a region rich in Phosphatidylinositol 3-kinase-related kinase (PIKK) consensus phosphorylation Ser/Thr-Gln (S/TQ) sites, followed by the phenylanaline residue at the +3 position, hence the name TQXF cluster. A subset of the TQXF motifs in MDC1 have been shown to be bona fide ATM targets in response to IR (77, 78). These phosphorylation events mediate the recruitment of the E3 ubiquitin ligase RNF8, through direct interaction of its FHA domain with the phosphorylated TQXF region (77, 78). Disruption of such interaction impairs histone ubiquitination, as well as 53BP1 and BRCA1 recruitment, whereas γH2AX, MDC1, and MRN IRIF remain unaffected. The current working model is that RNF8, in collaboration with another E3 ligase the RNF168, ubiquitinate H2A and H2AX around the DSB (77-80). Such ubiquitination creates binding sites for BRCA1 and mediates further histone methylation to recruit 53BP1.

MDC1 in Mitotic progression

Besides mediating DDR, MDC1 is also shown to be a novel regulator in mitotic progression. The anaphase-promoting complex (APC) has been identified as a binding partner of MDC1. APC-mediated degradation of Cyclin B plays a central role in mitotic progression. Specifically, active APC binds MDC1, as the APC activator protein Cdh1 was also retrieved with the APC-MDC1 complex (81). Depletion of MDC1 blocks mitotic cells at metaphase, indicating MDC1 is required for metaphase-anaphase transition, independent of DNA damage (82). Consistently, the absence of MDC1 results in a compromised APC activity *in vitro*. Mechanistically, MDC1 seems to influence APC activity by interacting with cell-division cycle protein 20 (CDC20), which regulates APC substrate specificity. Curiously, CDC20 appears to bind to the same MDC1 region required for direct binding of γH2AX. This suggests a potential competition mechanism for MDC1 between CDC20 and γH2AX.

MDC1 degradation mechanism

MDC1 foci assembly is important to DDR. At the same time, foci turnover also appears to be just as vital to efficient repair foci establishment. Shi et al. first reported that MDC1 is degraded via ubiquitin-proteasome pathway following DNA damage and the removal of MDC1 foci precedes the establishment of BRCA1 foci (83). However, their approach using proteasome inhibitor MG132 weakened their conclusion, since MG132 may affect a host of processes during DDR. Lou et al. elegantly identified the SUMO-

directed E3 ligase RNF4 as the direct regulator of MDC1 foci removal (84). Mutating the SUMOylation site K1840 significantly stabilized MDC1 foci and delayed BRCA1 recruitment. Consistently, RNF4 deregulation impairs DSB repair, rendering cells hypersensitive to DSB inducing agents (85, 86). Another regulatory mechanism is caspase 3 cleavage during apoptosis-induced DNA damage. MDC1 is inactivated by caspase 3 and unable to promote DSB repair foci. This cleavage-mediated inactivation is also consistent with previous studies showing the cleavage of ATM and DNA-PK, two major kinases in DDR, after apoptotic induction (37, 38, 87). Despite these recent findings, how MDC1 is regulated in undamaged cells requires further elucidation.

Current view on mitotic DNA damage repair

In contrast to interphase, DSB repair in mitosis remains mostly an uncharted territory. Potential roadblocks to repair include condensed chromosomes and high CDK1 activity. The onset of mitosis is visualized by nuclear envelope breakdown and the regulated compaction of chromatin into mitotic chromosomes, which are to be faithfully separated as sister chromatids in anaphase. Interestingly, vertebrate cells can delay mitotic entry, even reverse mitotic progression if damaged by IR during antephase (late G_2 to mid prophase), when chromatins are undergoing compaction (88). However, once cells have passed antephase, they are committed to mitosis even in the presence of DSBs (89). In contrast to canonical damage checkpoints, DNA damage at the onset of mitosis does not hinder mitotic progression (89, 90). CDK1 activity remains unaffected

once cells have passed a “point-of-no-return” in early mitosis (91). Consistently, the CDK1 inhibitor Wee1 becomes inactive upon mitotic entry (92). Severe DNA damage that involves chromosome fragmentation or disruption of kinetochore–spindle attachments triggers the spindle assembly checkpoint (93, 94). Nevertheless, mitotic DSBs do not pass through mitosis unnoticed. Several laboratories, including ours, have reported the presence of γH2AX foci in mitotic cells exposed to IR, suggesting intact early stage of DDR (1, 88, 95). However, late stage DDR only takes place once cells proceed through mitotic division until late telophase, as seen by the foci formation of a panel of DDR factors (96). Such marking of DSBs is essential for proper DSB repair in G_1 phase following mitosis. Several DDR factors, such as MDC1 and 53BP1, become hyperphosphorylated during mitosis, presumably by CDK1(3). Consistently, CDK1 is shown to promote MRN-CtIP dependent resection of DSB ends, while inhibiting Rad51 chromatin assembly. In this way, CDK1 inhibits both NHEJ and HR pathways in mitotic cells exposed to DNA damage(97). Collectively, these data suggest that partial activation of DNA damage checkpoint is integrated into normal mitotic progression. The precise mechanism of lack of repair is unclear.

Goal of this dissertation

The work presented in this dissertation sought to investigate two ideas relevant to understanding the regulation of MDC1 in human cancer cells. We have previously demonstrated that prolonged mitosis induces DNA damage (1). Recent advances in

mitotic DDR support our overarching hypothesis that such DNA damage is due to lack of efficient repair. First, we tested the hypothesis that MDC1-γH2AX interaction is attenuated in mitosis. To do so, we performed biochemical and morphological studies of MDC1-γH2AX colocalization during mitosis in human cell lines. This investigation revealed that, MDC1-γH2AX interaction is weakened during mitosis and that CDK1 activity is attributed to such regulation. Thus, CDK1 downregulates mitotic DDR, partly by inhibiting stable MDC1 localization to DSB sites. Moreover, this phenomenon suggests that cells prioritize timely completion of mitosis rather than repair during this critical stage. This study is the subject of Chapter II.

Due to the importance of MDC1 in DSB repair in human cells, we next investigated the potential regulator of MDC1 in undamaged cells. Particularly, we tested the hypothesis that F-box and WD repeat domain containing 7 (FBW7) downregulates MDC1. To do so, we measured MDC1 protein level in response to enhanced or suppressed FBW7 activity. This investigation suggested that FBW7 is a negative regulator of MDC1. This study is the subject of Chapter III.

Taken together, these studies describe novel mechanisms of MDC1 regulation during mitotic DNA damage and under normal conditions.

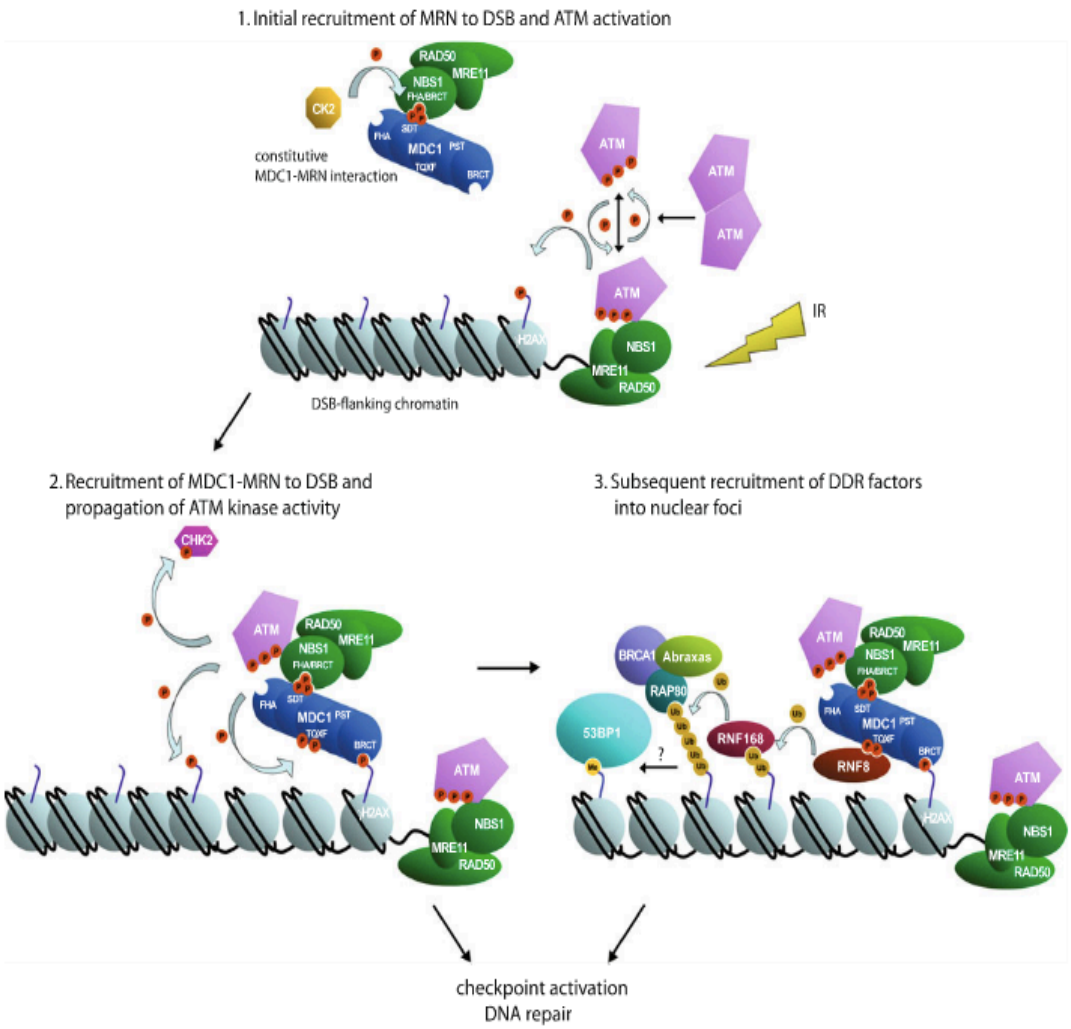

Figure 1. Schematic illustration of IRIF foci formation

Adapted from Jungmichel S, Stucki M. Chromosoma. 2010 Aug; 119(4): 337-49

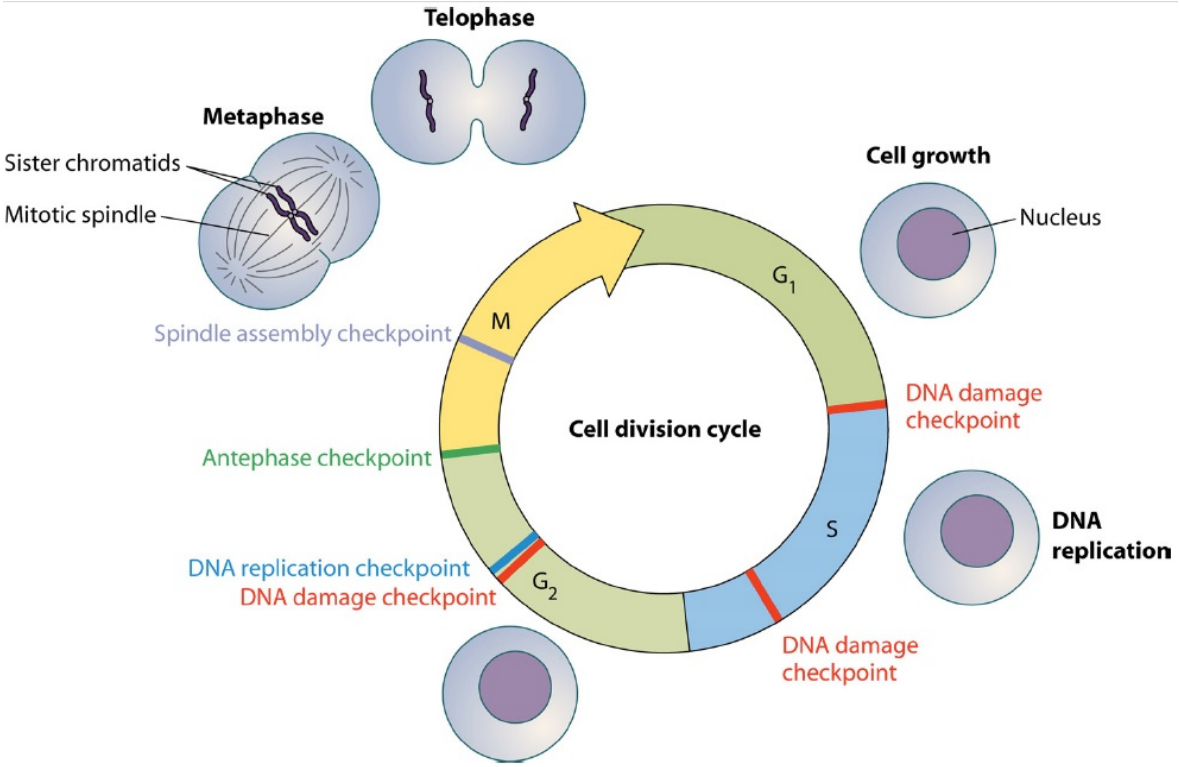

Figure 2. Cell cycle checkpoints

Adapted from Chin CF, Yeong FM. Mol Cell Biol. 2010 Jan;30(1):22-32.

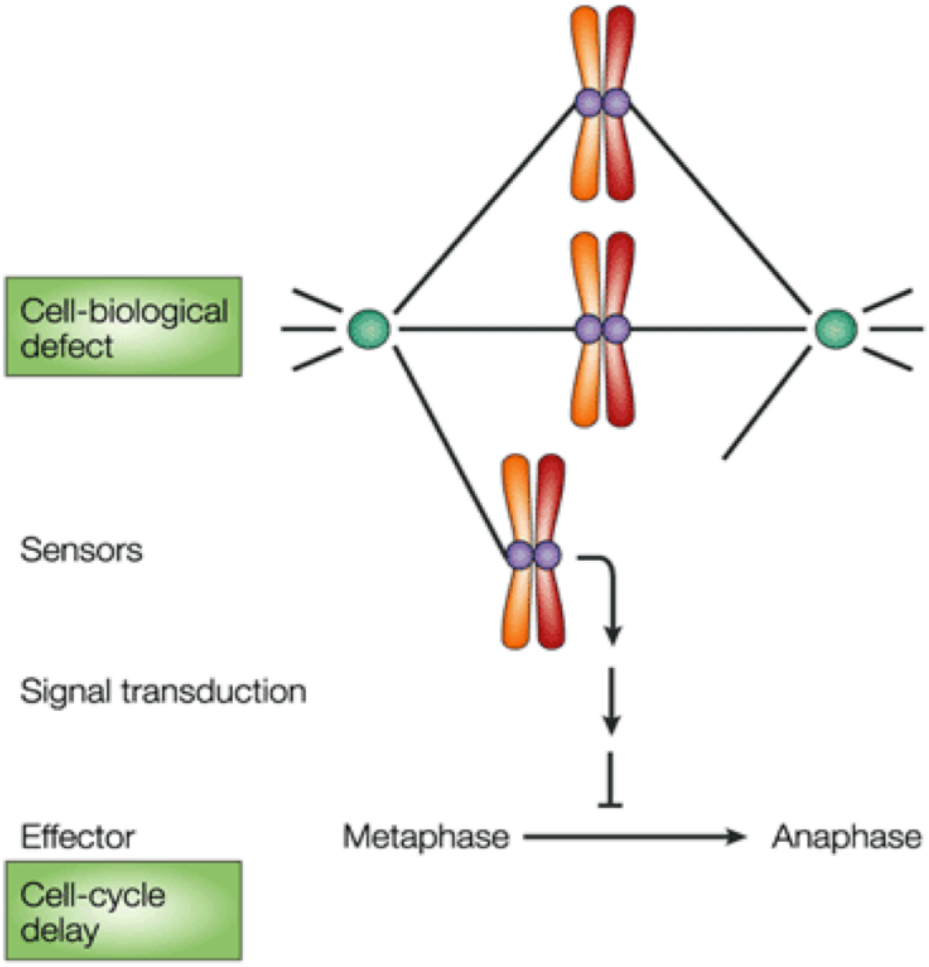

Figure 3. Spindle Assembly Checkpoint

Adapted from Andrea Musacchio & Kevin G. Hardwick Nature Reviews Molecular Cell Biology 2002 Oct;3, 731-741

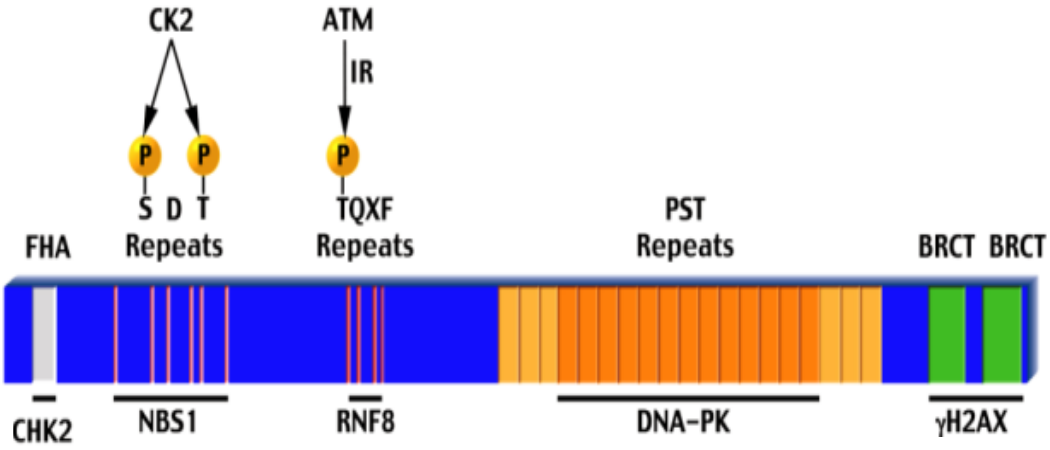

Figure 4. MDC1, a hub in DSB repair

Adapted from Gideon Coster, *et al*. Nucleus. 2010 Mar–Apr; 1(2):166-178

Chapter II

CDK1 Regulates Mediator of DNA Damage Checkpoint 1 (MDC1) During Mitotic DNA Damage

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Abstract

Cells engage sophisticated programs of DNA damage response (DDR) and repair to guard against genetic mutations. While there is significant knowledge concerning DDR in interphase cells, much less is known about these processes in mitosis. Direct interaction between MDC1, a master DDR organizer, and a marker of DNA damage, histone γH2AX, is required to trigger robust repair. Here we show that the DNA damage-induced interaction between MDC1 and γH2AX is attenuated in mitosis. Furthermore, inhibition in the activity of the core mitotic regulator CDK1, either by pharmacological inhibition or siRNA attenuation, enhances MDC1-γH2AX colocalization in mitosis. Our findings offer key new insights into how DDR is controlled during mitosis.

Introduction

Among various types of DNA damage, the DNA double strand break (DSB) is the most detrimental type, as no intact strand is left as a template for repair unless cell processes duplicate genomic material during proliferation. Eukaryotic cells have evolved an intricate DNA damage response (DDR) system to ensure the error-free duplication and separation of genomic information during the cell cycle. A primary sensor for DSB is H2AX, a variant of histone H2A, which is a component of the nucleosome (98). Upon DSB, the MRE11-RAD50-NBS1 (MRN) complex recognizes free ends of DNA and recruits activated ataxia telangiectasia-mutated (ATM) to phosphorylate H2AX on Serine 139.

This phosphorylated H2AX, termed γH2AX, directly interacts with an adaptor protein, mediator of DNA damage checkpoint 1 (MDC1), to initiate a cascade of DDR, amplifying and transducing the signal to downstream effectors to induce cell cycle arrest and DNA repair (53). Alternatively, cells can undergo apoptosis or permanent senescence to prevent the propagation of mutated daughter cells (99).

One hallmark of DDR is the accumulation and retention of repair proteins, which form microscopically discernible foci at the lesion sites. Such foci formation is hierarchical and depends on direct interaction between upstream and downstream factors (12). Over the past decade, extensive studies have generated a wealth of information on the mechanism of DDR in interphase cells; however, the precise mechanism regulating DDR during mitosis has yet to be unveiled. Previously, our lab has reported mitotic arrest as a novel source of DSBs (1). We hypothesize that the increase in DSBs during prolonged mitosis is due to lack of adequate DDR and accumulation of spontaneous inherent DNA damage. Supporting our hypothesis, recent research suggests an interesting model of incomplete mitotic DDR, wherein lesions are marked but downstream DDR is delayed until after mitotic exit (3). Accordingly, major mitotic kinases have been shown to actively inhibit DNA damage checkpoints (100). In particular, cyclin-dependent kinase 1 (CDK1) exhibits inhibitory effects on DSB repair shown in yeast and cell-free *Xenopus laevis* systems (101, 102). However, how the master organizer MDC1 is regulated during mitosis is unknown. On the one hand, MDC1 colocalizes with γH2AX in human

osteosarcoma U2OS cell line after γ-irradiation (3). On the other hand, MDC1 physically binds to anaphase-promoting complex/cyclosome (APC/C) complex and promote activation thereof (81). Consistently, siRNA downregulation of MDC1 blocks metaphase-anaphase transition (103). The dual role of MDC1 in mitosis indicates an unknown regulator. Here, we report that CDK1 activity reduces MDC1-γH2AX interaction during mitosis.

Materials and Methods

Cell culture and treatments

HCT116 and HT29 were obtained from the American Type Culture Collection. Early passage cells were used for experimentation and maintained in McCoy's 5A medium, supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum. Nocodazole and RO3306 (Sigma-Aldrich) were used at 200 nM and 2 μM, respectively. γ-irradiation was done with a Cs-137 Gamma cell. CDK1 knockdowns were performed using Stealth Select siRNA by RNAiMax transfection (Life Technologies). Final concentration of siRNA oligonucleotides was 20 nM, unless otherwise indicated. All analyses and further treatments were done 48 h post transfection.

Immunofluorescent staining

Cells received 2 Gy of γ-irradiation and recovered for 30 min before fixation with 2%

formaldehyde/PBS at room temperature (RT), washed and blocked with staining buffer (3% BSA, 0.2% Triton™ X-100 [(Sigma-Aldrich) in PBS]. Samples were incubated with primary and secondary antibodies for 1 h each at RT before Hoechst counterstaining. Confocal microscopy images were taken from Zeiss LSM Meta 510.

Immunoprecipitation

For co-immunoprecipitation, pre-IP lysates were prepared with Nuclear Complex Co-IP Kit (Active Motif) with minor modifications. For denaturing immunoprecipitation, whole cell extracts were denatured by boiling in denaturing buffer before immunoprecipitation.

Results and Discussion

Considering that foci formation is required for efficient repair of DSB, we first examined MDC1-γH2AX colocalization patterns in various phases of mitosis. Asynchronous HCT116 were subjected to 2 Gy of γ-irradiation, which efficiently induced DSBs without preventing mitosis (104). In contrast to the formation of distinct foci in interphase and anaphase cells, there was a progressive loss of colocalization of MDC1 with γH2AX during prometaphase and metaphase, as evidenced by the increased diffuse immunostaining of MDC1 (Fig. 1A). The colocalized MDC1-γH2AX foci returned upon commencement of anaphase. The distinct localization pattern of MDC1 coincided with dynamics of CDK1 activity, which reaches its peak in late prophase and declines in

anaphase (105). The relatively short time frame in which the MDC1 foci reappeared in anaphase suggests that their formation is posttranslationally regulated.

To confirm such colocalization indeed decreases in prometaphase, we introduced prometaphase block by nocodazole treatment and investigated MDC1-γH2AX colocalization. Consistently, MDC1 failed to colocalize with γH2AX in mitotically arrested cells (Fig. 1B; 16 h), whereas such colocalization was restored in postmitotic cells (Fig. 1B; 40 h). We also collected cells from the corresponding time points and measured MDC1 protein levels, which were not decreased in cells arrested in mitosis at 16 h (Fig 1C; Suppl. Fig. S1). These observations suggest that the decreased colocalization of MDC1-γH2AX occurs in prometaphase and is likely due to posttranslational modification of MDC1.

To confirm results of the morphological study, we biochemically determined the affinity between MDC1 and γH2AX in mitotic cells. HT29 cells were synchronized in mitosis by nocodazole treatment before γ-irradiation and shake off; the latter successfully separated mitotic cells from interphase cells that remained attached (Fig. 2A). The purity of the mitotic population was confirmed by FACS analysis (Suppl. Fig. S2). We then immunoprecipitated the H2AX immune complexes using a pan-H2AX antibody that does not abrogate MDC1-γH2AX binding. Compared to attached cells (Fig. 2B; lanes 4 and 6), significantly less amount of MDC1 was immunoprecipitated with γH2AX in

mitotic cells (Fig. 2B; lanes 3 and 5). This result demonstrates that the recruitment of MDC1 to γH2AX is reduced in prometaphase cells, independent of additional γ-irradiation. Furthermore, MDC1 in mitotic cells exhibited retarded mobility upon gel electrophoresis (Fig. 2B; lane 3 and 5, input), suggesting that posttranslational modification weakened MDC1-γH2AX interaction. This observation is in agreement with a previous report showing a reduction of MDC1 pull-down by a γH2AX peptide from mitotic cell lysates compared to non-mitotic cell lysates (3). This decrease of MDC1-γH2AX interaction is also consistent with the finding that APC/C and γH2AX binds to the same MDC1 domain, providing a model of competitive binding to MDC1 between APC/C components and γH2AX (81). Functionally speaking, MDC1 is required for metaphase-anaphase transition through APC/C activation during normal mitosis (103). Taken together, these observations provide an explanation for reduced MDC1-γH2AX interaction in mitosis, indicating that the role of MDC1, as a mitotic regulator, precedes that of a DDR mediator. Specifically, modified MDC1 dissociates from DSB sites in order for timely APC/C activation and weakens mitotic DDR due to insufficient bound MDC1.

Next we sought to identify the regulator(s) of MDC1 localization in mitosis. Previous studies reveal that phosphorylation is a major mechanism of MDC1 regulation (47). Since CDK1 is the definitive mitotic kinase (106), we tested whether MDC1 was phosphorylated by CDK1 in mitosis. We synchronized HCT116 cells in mitosis by nocodazole treatment for 16 h. The CDK1 inhibitor RO3306 was included as a negative

control. To exclude contaminating proteins binding to MDC1, we denatured whole cell extracts before immunoprecipitating with an MDC1 antibody. We then used an MPM2 antibody to detect phosphorylation of CDK1 motif(s) in immunoprecipitated MDC1 protein. As shown in Fig. 2C, mitotic cells had a higher level of MPM2 activity than asynchronous cell population. Consistently, CDK1 inhibition reduced both MPM2 abundance and mitotic index to the baseline (Fig. 2C; Suppl. Fig. S3). Our result is consistent with the mitotic phosphoproteomics study, suggesting CDK1 as the candidate kinase of MDC1 in mitosis (107).

Having established that CDK1 is the putative kinase regulating MDC1, we examined the effect of CDK1 inhibition on MDC1/γH2AX colocalization in mitotic cells. Unlike other CDK1/CDK2 dual inhibitors, RO3306 exhibits 10-fold selectivity for CDK1 over CDK2 (108). As shown in Fig. 3A, RO3306-treated HCT116 cells demonstrated a significant increase in MDC1-γH2AX colocalization in prometaphase and metaphase, judging by the intensified MDC1 foci compared to control cells. A similar effect was observed in HCT116 cells at a higher irradiation dosage (10 Gy) (Suppl. Fig. S4). We also performed the experiment in HT29 cells, which are proficient in mismatch repair in contrast to HCT116 (109), and showed that CDK1 inhibition too strengthened MDC1-γH2AX colocalization in mitotic cells (Suppl. Fig. S5). Furthermore, we tested whether CDK1 inhibition could enhance MDC1-γH2AX colocalization in prometaphase-blocked HCT116 (Fig. 3B). Indeed, additional treatment with RO3306 restored the colocalization compared to nocodazole

treatment alone (Fig. 3C). These findings indicate that CDK1 activity induces the loss of MDC1-γH2AX interaction during mitosis.

Finally, to confirm the result of pharmacological studies, we used siRNA to downregulate CDK1 in HCT116 cells. CDK1 was effectively knocked down (Suppl. Fig. S6), although the remaining CDK1 activity was sufficient for cell division according to nuclear morphology. Consistently, CDK1 knockdown significantly enhanced MDC1-γH2AX colocalization in prometaphase and metaphase cells compared to control siRNA treatment (Fig. 4A). To test whether low CDK1 activity protects MDC1-γH2AX colocalization in prolonged mitosis, we knocked down CDK1 before nocodazole treatment (Fig. 4B). Interestingly, sustained low CDK1 activity protected MDC1-γH2AX colocalization even when prometaphase was prolonged (Fig. 4C). These data suggest that MDC1 foci disassembly requires a higher CDK1 activity threshold than that necessary for the maintenance of mitosis.

In this study, we demonstrated that MDC-γH2AX interaction decreases as a cell traverses through two critical phases of mitosis, prometaphase and metaphase. This observation suggests that MDC1-γH2AX interaction is inversely correlated with CDK1 activity. Simona Giunta *et al*. reported that MDC1 colocalized with γH2AX in U2OS cells during normal mitosis (3). Although our results do not completely agree with theirs, we did observe residual MDC1 foci in prometaphase in the colon cancer cells we used. This

discrepancy could be due to that duration of normal prometaphase is not sufficient for a complete MDC foci disassembly, shown as smaller foci accompanied with increased diffuse immunostaining. Supporting this idea, we observed that prometaphase block by nocodazole could further diminish MDC1-γH2AX colocalization. This suggests MDC1 foci disassembly starts at prometaphase, presumably due to the maximized CDK1 activity. Moreover, CDK1 activity reduction, by either pharmacological or genetic manipulation, restored MDC1-γH2AX colocalization in both normal and prolonged mitosis. These data suggest that high CDK1 activity constitutively promotes MDC1 foci disassembly. However, whether phosphorylation of MDC1 by CDK1 induces foci disassembly requires further elucidation. Recently, Zhang et al. reported that high CDK1 activity suppressed mitotic DDR (2). Although we agree with their over-arching hypothesis, the utilization of alsterpaullone, a nonspecific CDK1 inhibitor, as a sole approach to downregulate CDK1 activity weakened their conclusion. In contrast, we used multi-angled approaches to demonstrate the inhibitory role of CDK1 in MDC1 foci formation. Since the sustained MDC1-γH2AX interaction is required to consistent downstream DDR activation, decreased MDC1-γH2AX interaction in mitosis partially explains the incomplete DDR.

In summary, our findings provide new insights into the regulation of DDR in mitosis. In the context of unperturbed mitosis, inherent DSBs are unlikely to sever chromosomes, due to their super-condensed structure. Others and we have shown that DSBs induced during mitosis, either by mitotic poisons or γ-irradiation, are actively repaired in

postmitotic cells, as shown by late-stage DDR protein foci staining (5, 6). Thus, completion of mitosis is prioritized over DSB repair, which supports our previous findings that prolonged mitosis induces genomic instability. Human cells benefit from timely progression through mitosis rather than repair. To ensure this, a subgroup of MDC1 dissociate from γH2AX foci in a CDK1-dependent manner to promote normal metaphase-anaphase transition.

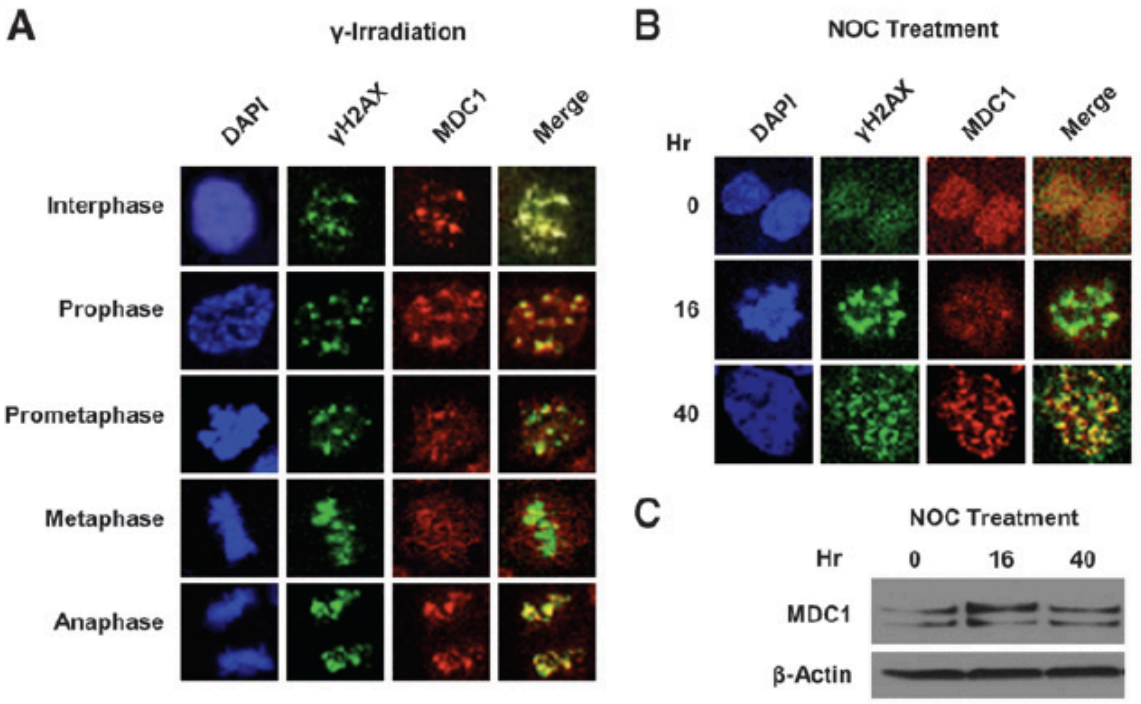


Figure 1. MDC1-γH2AX colocalization decreases during mitosis

(A) Immunofluorescent staining of MDC1 and γH2AX in HCT116 cells following γ irradiation. (B) Immunofluorescent staining of MDC1 and γH2AX during nocodazole (NOC) treatment. Representative prometaphase (16 h) and post-mitotic (40 h) cells were shown. The mitotic indices at the corresponding times points are shown in Suppl. Fig. S1. (C) Western blot of MDC1 in HCT116 cells at corresponding time points in (B).

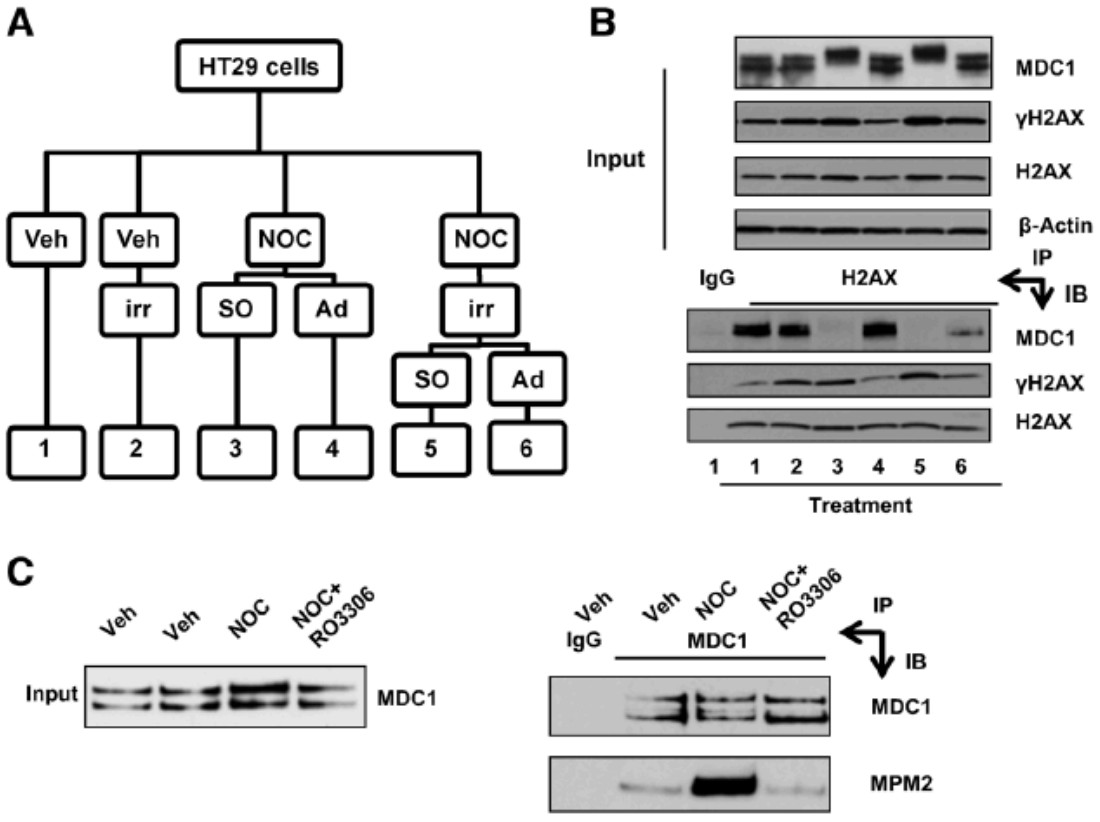


Figure 2. MDC1 exhibits decreased interaction with γH2AX in mitosis

(A) Schematic diagram of treatment of HT29 cells. Veh, vehicle; irr, γ-irradiation; NOC, nocodazole; SO, shake-off; Ad, adherent cells. (B) Coimmunoprecipitation of MDC1 and γH2AX in HT29 cells. Whole cell lysates were prepared from samples from (A). (C) Denaturing immunoprecipitation of MDC1. HCT116 cells were either treated with nocodazole alone (NOC) or with additional RO3306 for 1 h before harvest. IP, immunoprecipitation; IB, immunoblot.

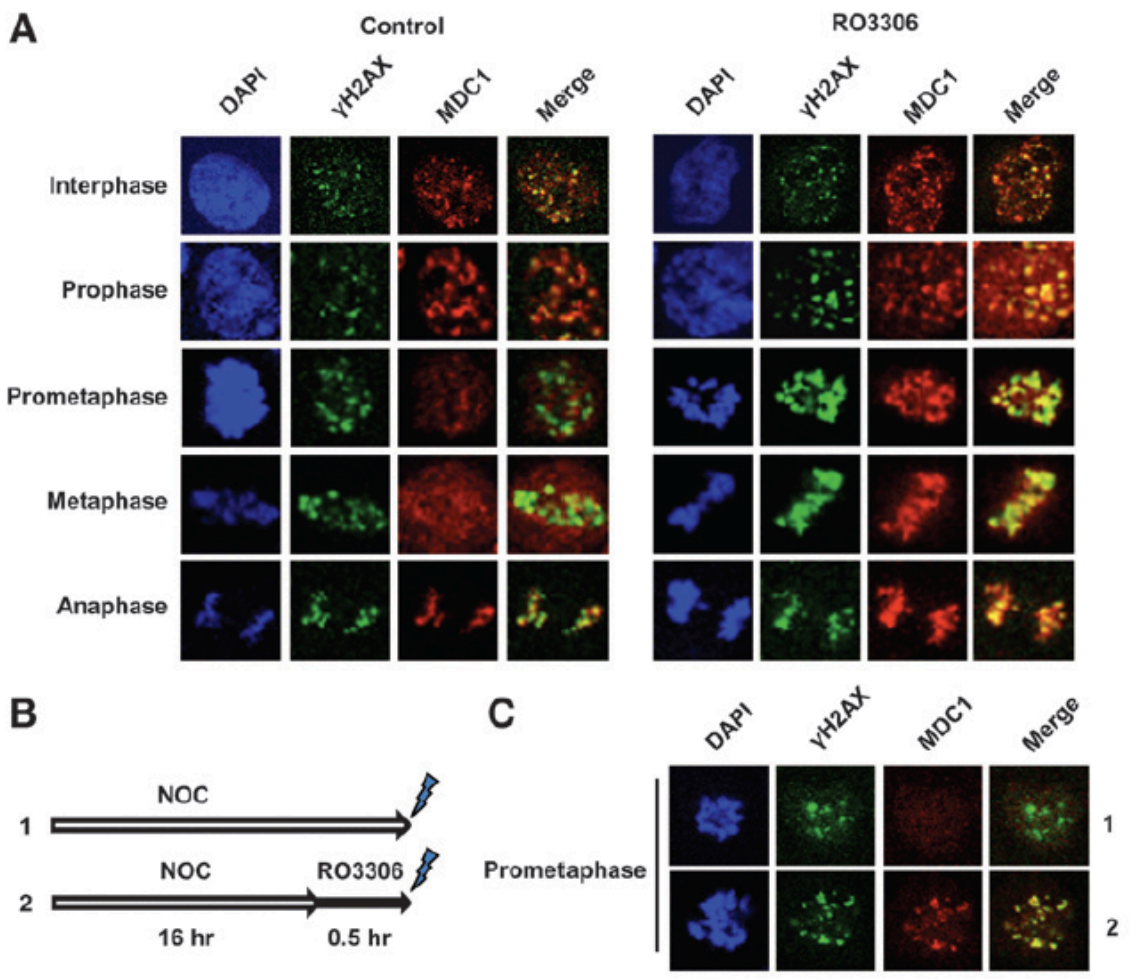


Figure 3. Inhibition of CDK1 activity increases MDC1-γH2AX colocalization in mitosis

(A) Immunofluorescent staining of MDC1 and γH2AX in HCT116 cells. HCT116 cells were pre-incubated with RO3306 or vehicle for 30 min, before subjected to 2 Gy γ-irradiation. (B) Schematic experimental procedure of (C). HCT116 cells were treated with nocodazole alone (NOC) or with additional RO3306 30 m before 2 Gy γ-irradiation. (C) Immunofluorescent staining of MDC1 and γH2AX in representative prometaphase cells.

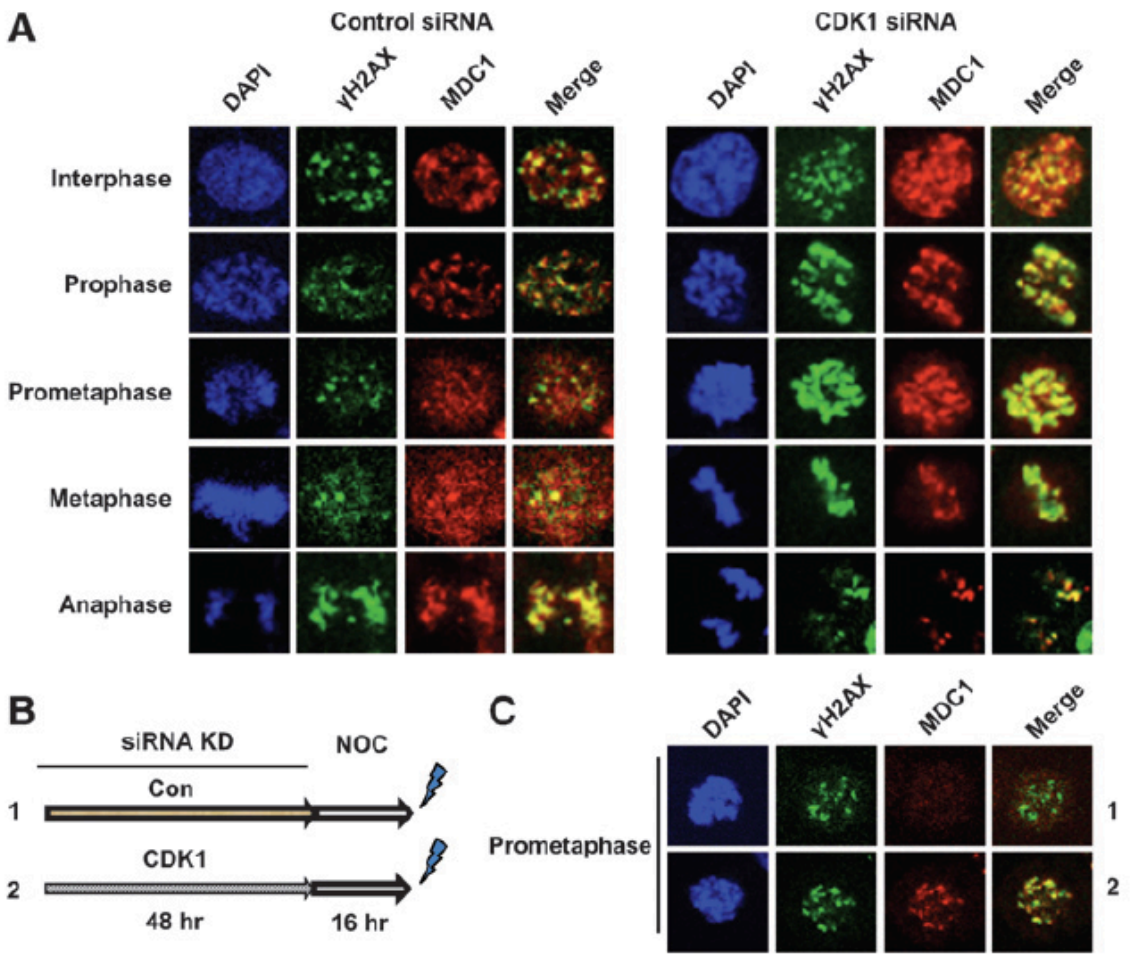


Figure 4. CDK1 downregulation increases MDC1-γH2AX colocalization during mitosis

(A) Immunofluorescent staining of MDC1 and γH2AX in HCT116 cells. Cells were transfected with non-specific control siRNA or siRNA targeting CDK1. Cells were irradiated with 2Gy γ-irradiation 48 h post transfection. (B) Schematic experimental procedure of (C). Following CDK1 knockdown, HCT116 cells were treated with nocodazole (NOC) for 16 h before 2 Gy γ-irradiation. (C) Immunofluorescent staining of MDC1 and γH2AX in representative prometaphase cells as treated in (B).

Supplemental Materials and Methods

Antibodies

The following antibodies were used: For western blotting and immunoprecipitation: MDC1 (rabbit, Abcam), γH2AX (mouse, Millipore), H2AX (rabbit, Bethyl Lab), β-actin and MPM2 (mouse, Millipore). For Immunofluorescent staining: MDC1 (Mouse, Abcam), γH2AX (rabbit, Abcam). Alexa Fluor® Dyes 488 and 568 secondary antibodies (Life Technologies). For flow cytometry: MPM2 (mouse, Millipore) and Alexa Fluor® Dyes 488 secondary antibody (Life Technologies).

FACS analysis

Cells were fixed in 70% ethanol/PBS at -20 °C overnight. Antibody incubation was 1 h at RT before Propidium iodide counter staining. Cell cycle profile data were acquired with a FACS Calibur (Becton Dickinson) and analyzed with FlowJo (Tree Star, Inc.).

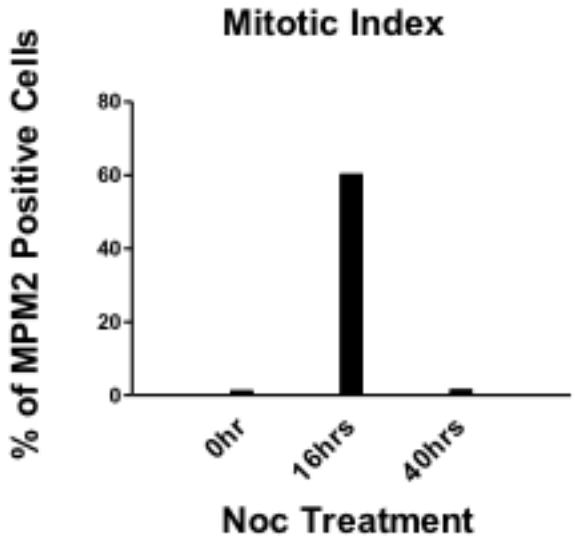
Detailed immunoprecipitation procedures

For co-mmunoprecipitation of γH2AX and MDC1, HT29 cells were grown for 24 h before nocodazole treatment for 16 h. HT 29 Cells were exposed to 10 Gy γ-radiation and allowed to recover for 30min. Mitotic cells were collected by mechanical mitotic shake-

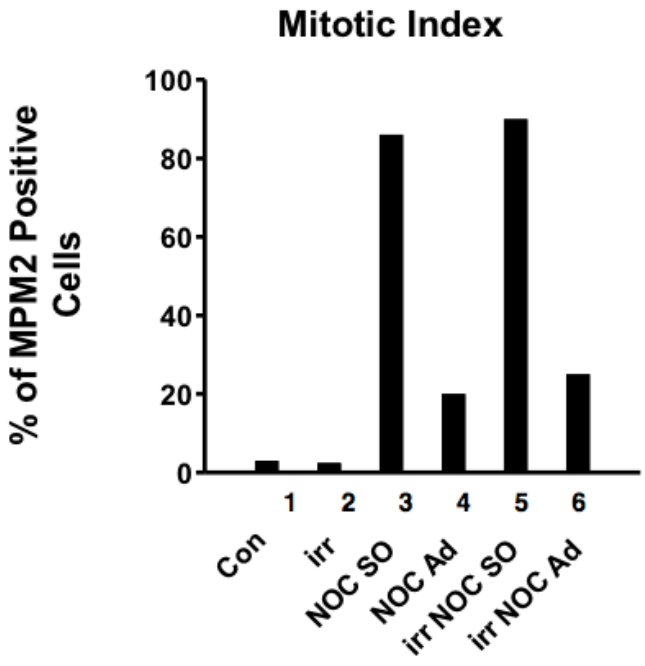
off, whereas attached cells and asynchronous cells were scraped off plates. Pre-IP lysates were prepared with Nuclear Complex Co-IP Kit (Active Motif). The only modification is to incubate cytoplasmic fraction with the supplied nuclease cocktail, supplemented with 1mM CaCl2. Nuclear fraction and cytoplasmic fraction of each sample were combined before IP. For denaturing MDC1 immunoprecipitation, HCT116 cells were seeded 24 h before 16hr nocodazole treatment. Whole cell extracts were prepared by boiling for 5 min. in denaturing lysis buffer (250mM NaCl, 50mM Tris, 5mM EDTA, 1% NP-40 and 1X protease inhibitor cocktail in dH2O, supplemented with 0.5% SDS, 5mM β-mercaptoethanol) were denatured by boiling in denaturing buffer. After boiling, cell lysates were diluted five times with lysis buffer (without SDS and β-mercaptoethanol) in order to quench excess SDS with NP-40. Then lysates were centrifuged at 14,000 g to pellet the mixed micelles. The supernatants then proceeded to immunoprecipitation. All immunocomplexes were pulled down by protein G magnetic beads (Cell Signaling).

Western blotting

Whole cell extracts were prepared by lysing cells with 2X Laemmli buffer. Proteins were separated on 4-15% SDS PAGE gel (Biorad) or 3-8% Tris-Acetate gel (Life Technologies) and transferred to nitrocellulose membrane by semi-dry transfer method.

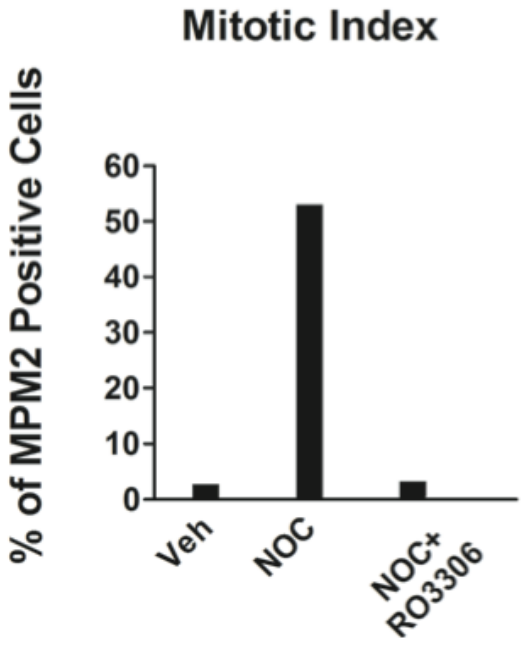


Supplemental Figure S1. Mitotic indices of HCT116 cells during nocodazole treatment. Mitotic index of each sample was determined by FACS analysis. Mitotic cells were determined by MPM2 reactivity.



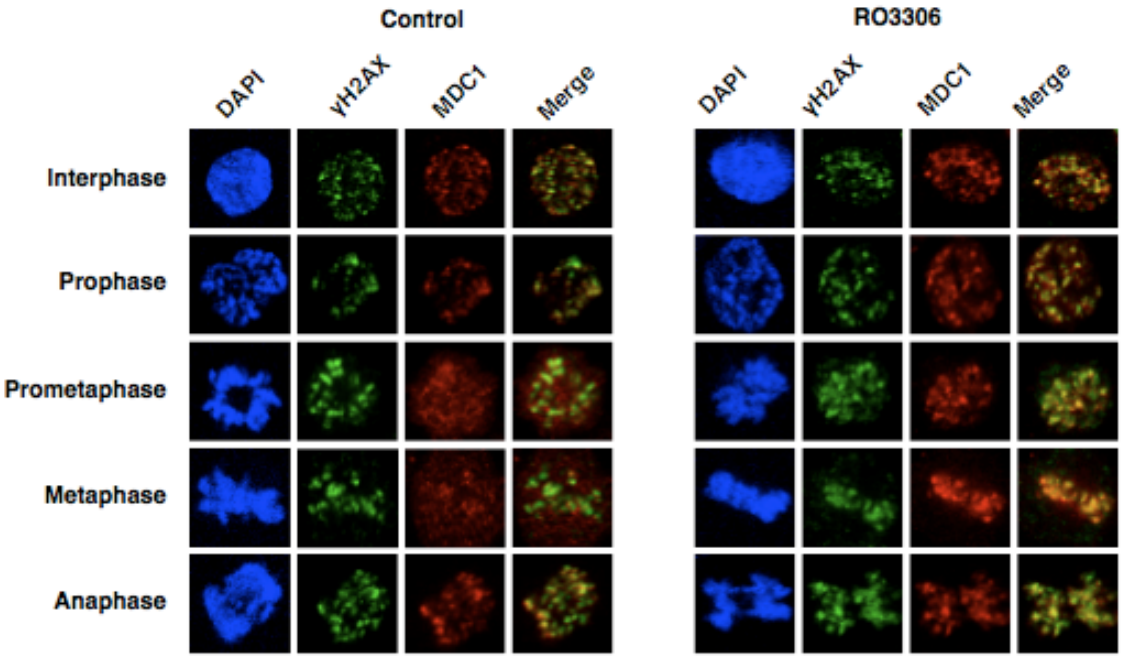
Supplemental Figure S2. Enrichment of mitotic cells by mitotic shake-off.

HT29 cells were treated as in Fig. 2A. Mitotic index of each sample was determined by FACS analysis. Mitotic cells were determined by MPM2 reactivity. Treatment Numbers correspond to Fig. 2A. Veh, vehicle; irr, 10 Gy γ-irradiation; NOC, nocodazole; SO, mitotic shake-off. Ad, adherent cells.



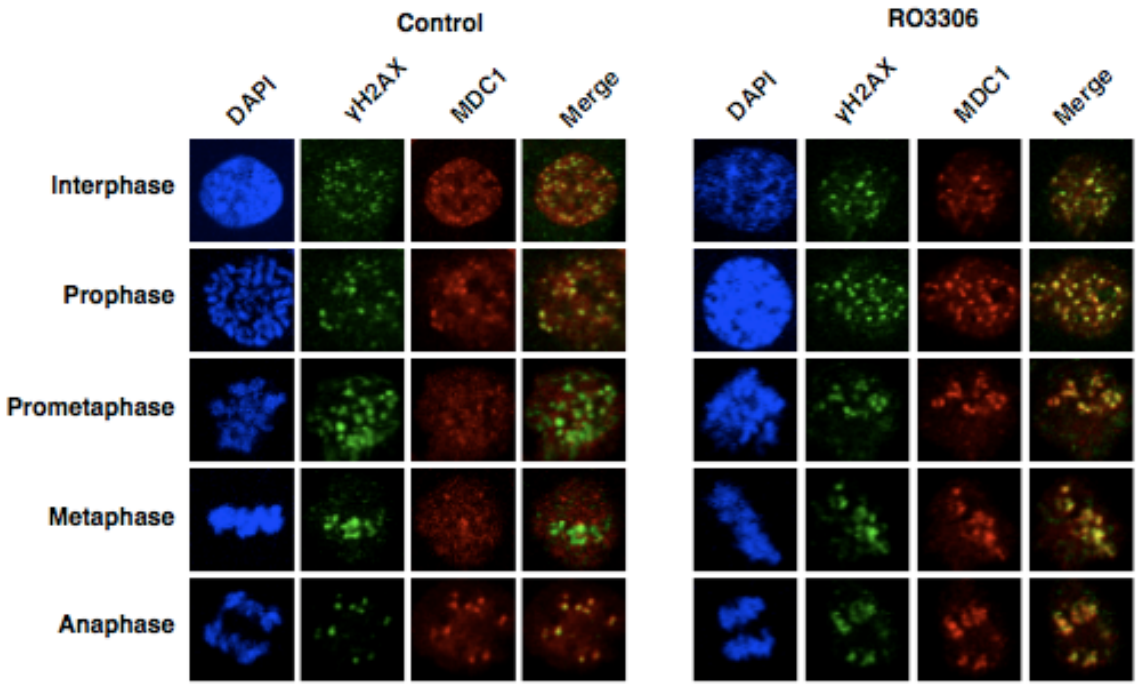
Supplemental Figure S3. Mitotic indices of cells.

Mitotic indices of HCT116 cells correspond to samples in Fig. 2C. Mitotic index of each sample was determined by FACS analysis. Mitotic cells were determined by MPM2 reactivity.

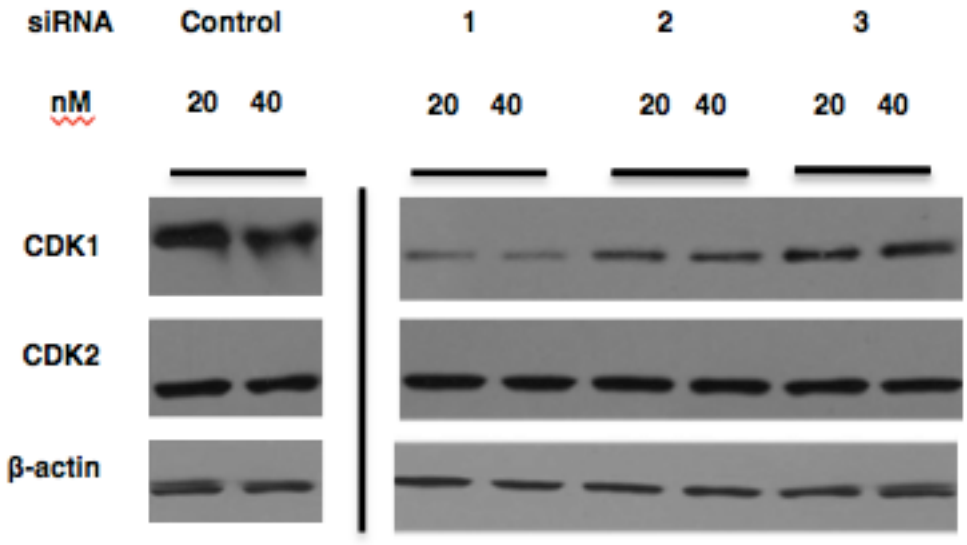


Supplemental Figure S4. Immunofluorescent staining of MDC1 and γH2AX in HCT116 cells.

Asynchronous HCT116 cells were pre-treated with RO3306 or vehicle control for 30 min before 10 Gy γ-irradiation.



Supplemental Figure S5. Immunofluorescent staining of MDC1 and γH2AX in HT29 cells. Asynchronous HT29 cells were pre-treated with RO3306 or vehicle control for 30 min. before 2 Gy γ-irradiation.



Supplemental Figure S6. siRNA knockdown of CDK1.

HCT116 cells were transfected with one of three oligonucleotides at either 20 or 40 nM for

48 h. Cells were then harvested and probed for CDK1, CDK2 and β-actin (loading control).

Chapter III

FBW7 Regulates Mediator of DNA damage checkpoint 1

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Abstract

Mediator of DNA damage checkpoint 1 (MDC1) is a large nuclear protein that plays important roles in DNA damage responses. Controlled degradation of MDC1 following DNA damage is crucial to proper DNA damage response. Currently, the mechanism regulating MDC1 under normal conditions is poorly understood. We report here that F-Box protein WD repeat domain-containing 7 (FBW7), a component of SCF^{FBW7} ubiquitin ligase and tumor suppressor promotes MDC1 turnover. We show that enforced expression of FBW7 downregulates MDC1. Conversely, depletion of endogenous FBW7 leads to the accumulation of MDC1 protein. Together, these data indicate FBW7 as a negative regulator of MDC1 level.

Introduction

DNA double-strand breaks (DSBs) can be exogenously generated by a variety of genotoxic agents, such as ionizing radiation and radiomimetic drugs (IR). They also arise from errors during normal DNA replication (110). Aberrant cellular response to DSBs often leads to genomic instability, which may ultimately contribute to the tumorigenesis (111, 112). To counter the deleterious consequences of DSBs, cells have developed a sophisticated signaling network to coordinate DNA damage repair and delay of cell cycle progression. Effective DSB response demands rapid recruitment and retention of DDR factors to damaged regions containing the broken DNA (111, 113), which is a highly conserved process (113). The major mediator protein in this progress is MDC1. By direct interaction with the damage marker γH2AX, MDC1 aggregates at the lesion sites and act as a scaffold protein to recruit and retain downstream DDR factors at the damaged chromatin sites (44). Once the repair foci is established, MDC1 is ubiquitinated and subsequently degraded by SUMO-directed E3 ligase RNF4 (84). This controlled degradation is vital to DNA damage signal transduction and timely recruitment of other DDR factors (84-86). However, RNF4 does not regulate MDC1 turnover in undamaged cells (84). Moreover, as a major repair mediator, MDC1 is shown to play an important role in cell survival decision through the regulation of p53 (114, 115). Consistently, MDC1 is cleaved by caspase 3 to inactivate the repair signaling pathway during apoptosis-induced DSBs.

The ubiquitin proteasome system is a major mechanism for protein turnover (116). Before degradation by 26S proteasome-mediated proteolysis, proteins are first covalently conjugated with ubiquitin moieties on specific lysine residues. The addition of activated ubiquitin conjugates requires the coordinated reactions of three enzymes, including ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin-protein ligase (E3) (117). E3 ligases are classified based on their subunit composition and are responsible for substrate specificity. SCF (complex of SKP1, CUL1 and F-box protein) complexes are ubiquitin ligases that bind to protein substrates and target them for ubiquitin proteasome degradation. F-box proteins are responsible for substrate recognition. FBW7 is an F-box protein that regulates key players in cell division and growth, including cyclin E, MYC, JUN and Notch (118-124). FBW7 is a tumor suppressor, and loss of FBW7 leads to chromosomal instability, probably due to the hyperactivation of its oncogenic substrates. FBW7 binds to its substrates via phosphorylated phospho-degron motifs, termed CPDs (Cdc4 phospho-degrons). CPD phosphorylation is highly regulated. Most FBW7 CPDs are phosphorylated by glycogen synthase kinase 3β (GSK3β) (125). GSK3β depends on a priming phosphorylation in CPD (+4 position) to bind and phosphorylate the substrate protein. Therefore, proteins containing CPDs could be potentially regulated by SCF^{FBW7} ubiquitin ligase. In this study, we show that FBW7 regulates MDC1 level in human colon cancer cells.

Materials and Methods

Cell culture and treatments

Wildtype HCT116 and HEK293T cell lines were obtained from American Type Culture Collection. HCT116 FBW7 null cells were obtained from Dr. Bert Vogelstein. Both HCT116 cell lines were maintained in McCoy's 5A medium, supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum. HEK293T cells were maintained in DMEM medium, supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum. γ-irradiation was conducted with a Cs-137 Gamma cell. Transfections of FBW7 siRNA (Life Technologies) were performed with Stealth Select siRNA by RNAiMax transfection (Life Technologies). Final concentration of siRNA oligonucleotide was 20 nM and subsequent analyses were carried out 48hrs post transfection. Plasmid transient transfections were performed with lipofectamine 2000 (Life Technologies), according to manufacturer's protocol.

Reagents and antibodies

Full-length HA-MDC1 and HA-MDC1-ΔPST were kindly provided by Dr. Michal Goldberg. Full-length pcDNA-FLAG-FBW7 was obtained from Dr. Michele Pagano. The following antibodies were used for western blotting: rabbit FBW7 (Bethyl), mouse HA (Millipore), rabbit MDC1 (Abcam), mouse β-actin (Sigma-Aldrich).

Cycloheximide (CHX) chase experiment

Cells were seeded 24 hrs before addition of cycloheximide (100μg/ml) to inhibit protein synthesis. Cells were then scraped off plates and lysed in RIPA buffer supplemented with phosphatase inhibitor and Protease inhibitor cocktail at different time points (0, 6, 12, and 24 hrs) on ice after cycloheximide treatment. Total cell lysates were analyzed by Western blotting. The amount of MDC1 at each time point was normalized with the original MDC1 level at time zero. The protein degradation rate is conveyed as half-life ($t_{1/2}$), the time for the degradation of 50% of the protein.

Results

Absence of PST domain stabilizes MDC1—Studies involving MDC1 deletion mutants reveal an interesting observation that ectopic expression of MDC1 lacking the central Proline-Serine-Threonine rich repeats (PST repeats) is significantly higher than the wildtype as well as other versions of truncated MDC1 proteins (69). To confirm this observation, we compared expression level of HA-tagged full-length MDC1 (HA-MDC1 WT) and a truncation mutant lacking PST region (HA-MDC1 ΔPST). HEK 293T cells were transfected with various amount of MDC1 plasmids and were later probed for exogenous MDC1 protein level using an HA antibody. As shown in Fig. 1A, HA-MDC1 ΔPST expressed at much higher level (lane 9), even when the initial plasmid amount is 1/20 of that of HA-MDC1 WT (lane 1). To test whether the enhanced expression efficiency stems from protein stabilization, we utilized a cycloheximide (CHX) assay to

compare the degradation rates of the two versions of MDC1. HEK 293T cells were transfected with either HA-MDC1 WT or HA-MDC1 ΔPST for 24hrs before CHX treatment. Cells were collected at different time points in the presence of the drug. As shown in western blot, mutant MDC1 degraded at a much slower rate than wildtype MDC1, when global protein synthesis was inhibited by CHX (Fig. 1B and C). These observations suggested that PST repeats might be the regulatory domain of MDC1 protein stability.

Since phosphorylation is the major regulatory mechanism of MDC1 function (47), we used prediction analysis (http://scansite3.mit.edu/) to examine potential kinases, that may target MDC1 for degradation via PST domain. Analysis revealed that the PST repeat region contained multiple putative phosphorylation sites for GSK3β, which may create binding sites for FBW7, the substrate-binding component of the SCF^{FBW7} E3 ligase (Fig. 1D). The potential caveat is that the deletion mutant might be stabilized due to disruption of the overall protein structure. However, this is unlikely the case, since absence of PST domain did not affect MDC1-mediated γH2AX and DDR factor foci formation (69).

FBW7 facilitates MDC1 turnover—Next, we tested whether enforced FBW7 expression could suppress the exogenous MDC1 level. We co-transfected HEK 293T cells with plasmids encoding FBW7 and HA-MDC1 WT. As shown in Fig. 2A and B, expression of FBW7 reduced exogenous MDC1 level. As a complementary approach, we further tested

the effect of FBW7 overexpression on endogenous MDC1 in HEK 293T cells. Consistently, FBW7 suppressed endogenous MDC1 levels (Fig. 2C and D). These data suggest that FBW7 promotes MDC1 steady state turnover.

Knockdown of FBW7 stabilizes MDC1—To further establish a role for FBW7 in MDC1 regulation, the effects of FBW7 knockdown on MDC1 were examined. HCT116 cells were transfected with either siRNA targeting FBW7 or nonspecific siRNA as a negative control. Cells were collected at 24 and 48 hrs post-transfection. Western blot analysis showed that MDC1 was enriched in cells with downregulated FBW7 at both time points (Fig. 3). These data suggest that FBW7 is required to maintain normal MDC1 level.

The absence of FBW7 resulted in an increase in endogenous MDC1—To further study the role of FBW7 in the regulation of MDC1 under physiological conditions, we utilized HCT116 FBW7 homozygous knockout cells (FBW7 null). Both wildtype and FBW7 null cells received 10 Gray (Gy) γ-irradiation and were collected at various time points afterwards (Fig. 4). In both cell lines, MDC1 level rapidly declined in response to γ-irradiation (Fig. 4A and B), consistent with previous reports (84, 126). However, compared to wildtype HCT116, FBW7 null cells maintained a higher basal level of MDC1 both under untreated (0 hr) and γ-irradiated conditions (Fig. 4 C). This observation confirms that FBW7 is involved in steady state level of MDC1 protein.

Discussion

Here we report that the MDC1 is negatively regulated by the FBW7. We show that (i) MDC1 level is downregulated by enforced expression of FBW7; (ii) MDC1 expression is stabilized in the absence of FBW7, both by transient and permanent depletion of the protein; (iii) the PST repeat might be the regulatory element in MDC1. These data indicate that FBW7 promotes MDC1 degradation via ubiquitin-proteasome pathway. MDC1 was shown to be removed from DSB sites and degraded following irradiation (84, 126). Because sustained MDC1 foci are deleterious to efficient repair, the rapid turnover of MDC1 allows downstream factors to timely accumulate at DSB sites and participate the repair signaling cascade. Although RNF4 is identified as the E3 ligase responsible for MDC1 degradation after DSB induction, it is not clear how MDC1 is regulated in undamaged cells. Our results suggest FBW7 as a potential regulator. However, modulation of FBW7 only induces partial change in MDC1 level, indicating potential redundant regulator(s) or other mechanisms, such as transcriptional control. So far, Specificity Protein 1 (Sp1) is shown to bind to MDC1 promoter and stimulate the transcriptional activation of MDC1 (127). To confirm that MDC1 turnover is mediated by FBW7, more work needs to be done to demonstrate that the SCF^{FBW7} E3 ligase is directly involved in MDC1 degradation. To avoid the off-target effects on other E3 ligase families, one can take advantage of the small molecule MLN4924, which specifically inhibits neddylation of the cullin subunit of SCFs, a process required for SCF complex activation (128-130). Furthermore, the MDC1-FBW7 interaction needs to be examined, since FBW7 is responsible for substrate recognition of SCF^{FBW7} complex. Lastly, it is

interesting to study the involvement of GSK3β in MDC1 downregulation, since phosphorylation by GSK3β creates FBW7 binding sites on most known SCF^{FBW7} substrates (131).

One could envision that upregulation of MDC1, through FBW7 deficiency, might provide cells, suffering from DSB damage, a survival advantage by favoring repair over apoptosis through regulation of p53 pathway. Hence, it is reasonable to speculate that MDC1 should be tightly regulated, partly by FBW7, even in unchallenged state.

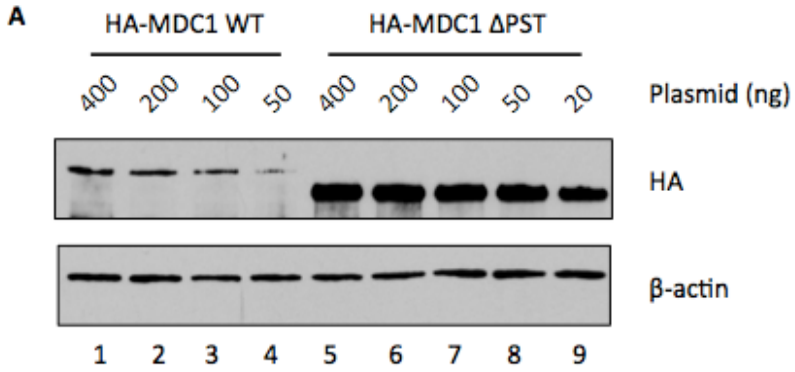

Figure 1. PST repeat regulates MDC1 protein stability.

A. PST repeat region regulates MDC1 level. HEK 293T cells were transfected with either wildtype MDC1 plasmid (HA-MDC1 WT) or truncated MDC1 lacking PST repeats (HA-MDC1 ΔPST), with the indicated plasmid amount per well in 12-well plates. Whole cell extracts were collected 24 hrs post-transfection and were immunoblotted with HA or β-actin antibodies. β-actin was used as a loading control.

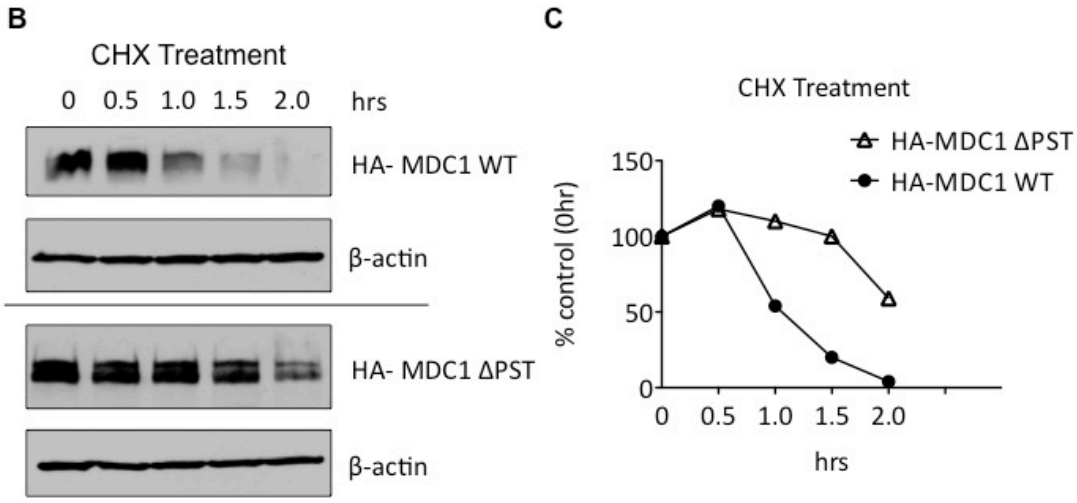

Figure 1. PST repeat regulates MDC1 protein stability

B. Absence of PST repeat region increases MDC1 stability. HEK 293T cells were transfected with the indicated plasmids 24 hrs before cycloheximide (CHX) treatment (100μg/ml). Whole cell lysates from different time points were immunoblotted with the HA or β-actin antibodies. β-actin was used as a loading control. C. HA-MDC1 ΔPST shows an increased half-life. Relative MDC1 protein amounts were quantified by densitometry (normalized to β-actin). Protein degradation was illustrated as declining protein amounts once CHX inhibited new protein synthesis. Levels of exogenous MDC1 at 0 hr were set at 100%.

D

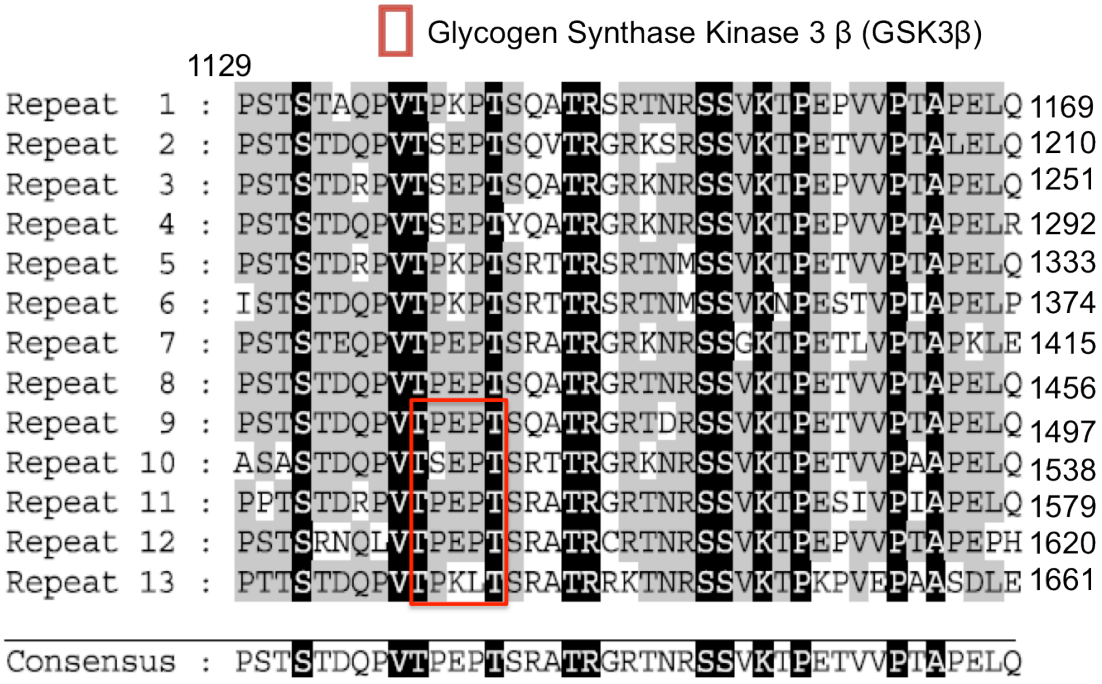

Figure 1. PST repeat regulates MDC1 protein stability

D. Alignments of repeats in the MDC1 PST region. The numbers indicate amino acid location in MDC1 protein. Predicted GSK3β phosphorylation sites are highlighted in the rectangle.

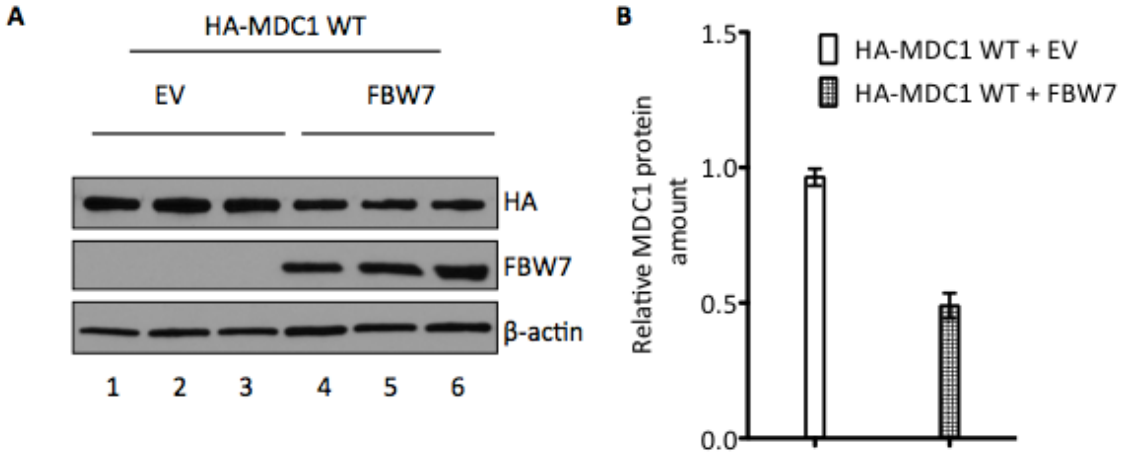

Figure 2. FBW7 downregulates MDC1.

A. Exogenous MDC1 is suppressed by FBW7. HEK 293T cells were co-transfected with HA-MDC1 WT (lane 1-3) and FBW7 plasmids (lane 4-6) in triplicate. Empty vector (EV) was used as a blank control for FBW7 plasmid. Whole cell extracts were collected 24 hrs post transfection and were immunoblotted with HA, FBW7 or β-actin antibody. β-actin was used as a loading control. B. Densitometry quantification of exogenous MDC1 protein, normalized to β-actin level. Error bar represents SD of the triplicate samples.

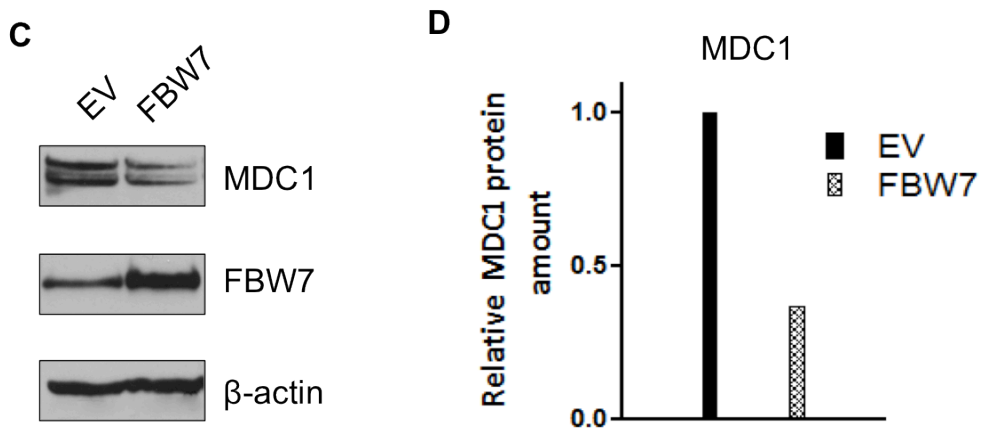

Figure 2. FBW7 downregulates MDC1.

C. Overexpression of FBW7 decreases endogenous MDC1 level. HEK293T cells were transfected with FBW7 plasmid or equal amount of empty vector (EV) for 24 hrs before harvest. Whole cell extracts were immunoblotted with MDC1, FBW7 or β-actin antibody. β-actin was used as a loading control. D. Quantification of endogenous MDC1, normalized to β-actin level.

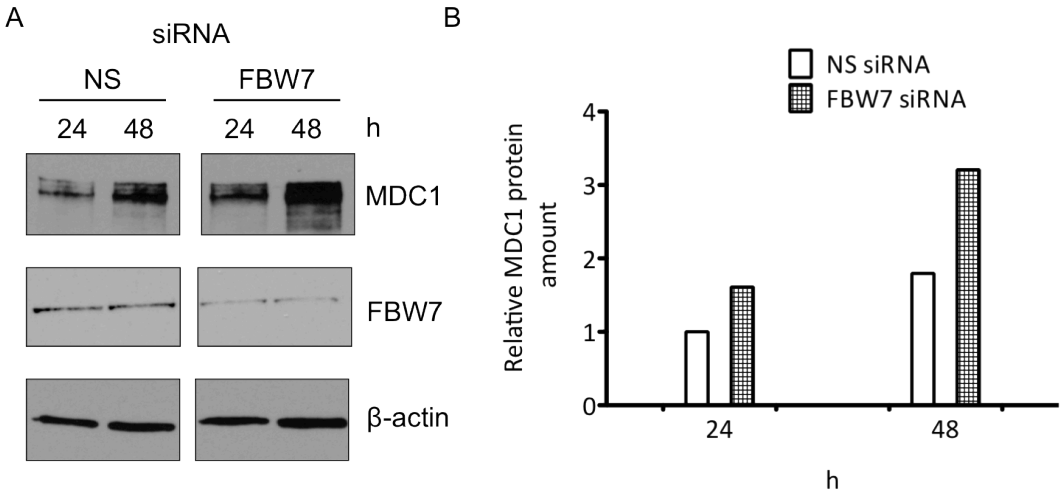

Figure 3. Knockdown of FBW7 increases MDC1 level.

A. siRNA knockdown of FBW7. HCT116 cells were transfected with either FBW7 specific siRNA (FBW7) or non-specific siRNA (NS) at 20nM final concentration. Cells were collected at 24 and 48 hrs post transfection. Whole cell lysates were immunoblotted with MDC1, FBW7 or β-actin antibody. β-actin was used as a loading control. B. Quantification of MDC1, normalized to β-actin level. MDC1 level in cells transfected with NS siRNA for 24 hrs was set as 1.

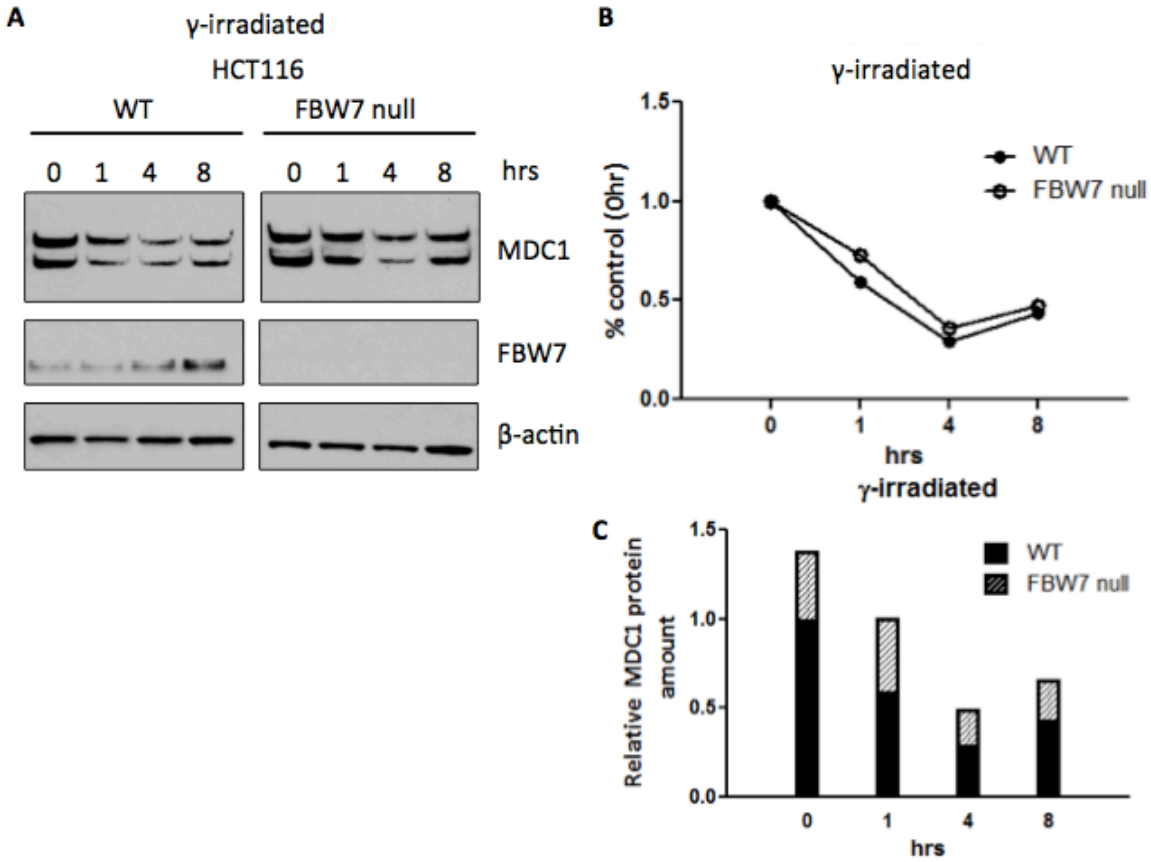

Figure 4. Absence of FBW7 increases basal MDC1 level.

A. MDC1 level in response to γ-irradiation. Both HCT116 wildtype (WT) and FBW7 null cells received 10 Gy γ-irradiation. Cells were collected at indicated time points afterwards. Whole cell lysates were immunoblotted with MDC1, FBW7 or β-actin antibody. β-actin was used as a loading control. FBW7 immunoblotting was used to confirm the absence of FBW7 in null cells. B. MDC1 downregulation following γ-irradiation is independent of FBW7 status. Quantification of MDC1 normalized to β-actin level is shown in panel A. The amount of MDC1 in each untreated HCT116 line (0 hr) was set as 100%. C. FBW7 null cells maintain higher MDC1 level, irrespective of γ-irradiation. Quantification of MDC1, normalized to β-actin level is shown in panel A. The amount of MDC1 in untreated HCT116 WT cells was set as 1.

Chapter IV

Summary and Future direction

Among all types of DNA damage, DSBs are the most cytotoxic type of lesions. Efficient and faithful repair are required to ensure genomic stability. DDR in interphase cells has been the research focus for the past decade or so. However, the DDR during the most vulnerable cell cycle phase, mitosis, is largely unexplored. In addition, the functional activity of DDR specifically during mitosis has not been well characterized. The outdated perception that mitosis lacks DNA damage checkpoint is being challenged by recent discoveries of partial DDR during this stage. The current model describes a phenomenon in which mitotic DSBs are marked by γH2AX foci, however, downstream repair is postponed until cells finish metaphase-anaphase transition (132). The mechanism and functional significance are being explored. In this dissertation work, I sought to investigate the regulator(s) of mitotic DDR by focusing on the dynamics of MDC1-γH2AX interaction.

I provide evidence from both biochemical and morphological approaches to show that MDC1- γH2AX interaction is attenuated by CDK1 during mitosis, in contrast to interphase when CDK1 remains inactive, due to the absence of activating partner Cyclin B (Chapter IV, Fig. 1). Future studies are needed to identify the MDC1 domain(s)

targeted by CDK1 phosphorylation and whether such phosphorylation events are responsible for reduced MDC1- γH2AX affinity. Another interesting observation is the presence of γH2AX foci, though MDC1 recruitment to DSB sites is downregulated during mitosis. Since current immunofluorescent staining data are unable to examine γH2AX range and density at DSB sites, it remains unclear whether mitotic DNA damage induces γH2AX foci formation to the similar level as in interphase cells. Alternatively, such efficient γH2AX formation can result from a lack of dephosphorylation of γH2AX. Indeed, Wip1, a major phosphatase to dephosphorylate multiple DDR factors, including γH2AX, is inactivated by phosphorylation and proteolysis during mitosis, whereas ectopic expression of Wip1 reduces γH2AX level in mitotic cells (133). Therefore, downregulation of phosphatases maybe decrease the necessary threshold of MDC1 for sufficient γH2AX formation.

Why do cells in mitosis lack efficient DDR? One explanation is that the highly condensed mitotic chromosomes prevent the assembly of DDR signaling complexes, which are required for substantial chromatin remolding process. However, several lines of evidence challenge this model. It is well established now that DNA damage can induce robust γH2AX foci formation during mitosis, indicating that relevant DDR factors such as MRN, pATM and MDC1 can access lesion sites in compacted chromosomes. Furthermore, during meiosis, cells deliberately generate DSBs in condensed chromosomes, for the sake of genetic diversity of offspring. These endogenous DNA

DSBs do initiate a full DDR signaling cascade for efficient homologous recombination repair (134). Therefore, inactivation of full DDR in mitotic cells is unlikely due to the compact chromosome structure.

Then why do cells prioritize mitotic progression over timely repair? There could be a few explanations. First, if a DNA damage checkpoint were to become fully activated during mitosis, DSBs encountered during mitotic progression could result in inactivation of CDK1/Cyclin B and result in premature mitotic exit. Maintenance of high CDK1 activity, rather than Cyclin B propels mitotic progression. Inhibiting CDK1 activity with small molecule inhibitor RO3306 could override activated SAC and results in generation of aneuploidy and polyploidy in daughter cells (108). Such events have recently shown to increase tumorigenic potential. Indeed, checkpoint effector CHK2 remains catalytically inactive during mitotic DNA damage further strengthens this view (100). Hence it can be expected that cellular mechanisms exist to prevent inappropriate CDK1 inactivation during mitotic DDR. Indeed, DNA damage during mitosis had previously been shown to be unable to slow down mitotic progression.

Second, there is a finite time for mitotic completion. Spontaneously prolonged mitosis is characteristic of human cancer cells. Previously, our lab has reported the novel finding that induced mitotic arrest results in an elevation of DSBs marked by γH2AX staining and confirmed by morphological investigation of mitotic chromosomes (1). If mitotic DSBs were to delay mitotic progression, as they do in other phases of cell cycle, one would

expect a rise of chromosomal abnormality similar to that observed in mitotic arrest.

Finally, several DDR factors have been shown to regulate normal mitosis in the absence of DNA damage. For example, phosphorylated DNA-PKcs colocalize with centrosomes during mitosis. Downregulation of DNA-PKcs, either by knockdown or inhibitor, leads to chromosome misalignment and delayed mitosis (135). MDC1 has also been shown to regulate normal metaphase to anaphase transition, probably by contributing to activation of APC complex, which in turn targets Cyclin B for degradation. Interestingly, MDC1 utilizes the same domain BRCT to interact with γH2AX. Therefore, it is conceivable that CDK1 activity directs MDC1, as well as other DDR factors, to their role in normal mitotic progression.

In addition, we provided preliminary results, suggesting FBW7 as a negative regulator of MDC1. MDC1, as a master organizer of DDR factors at DSB sites, needs to be degraded for efficient repair following DSB induction. A SUMO-directed E3 ligase RNF4 has recently emerged as novel regulator in DDR *in vivo.* Interestingly, RNF4 also targets MDC1 for degradation in damaged cells, which can at least partially explain the defective DDR in absence of RNF4, since MDC1 foci disassembly is a prerequisite for downstream DDR factor IRIF formation at late stage DDR.

As an important mediator of DDR, MDC1 possesses inherent anti-apoptotic activity, since repair and apoptosis are both viable solutions to eliminate DSB-induced mutations from multicellular organisms. On the one hand, MDC1 was found to promote inhibitory phosphorylation of p53 and the stabilization of p53-specific E3 ubiquitin protein ligase homolog (MDM2), an inhibitor of p53 (115, 136). On the other hand, MDC1 was inactivated by caspase 3 cleavage during apoptotic DSB, as a mechanism to inhibit DNA damage repair pathway (137). These data may explain why MDC1, as a potential tumor suppressor, also needs to be tightly regulated.

While most studies focus on MDC1's role in eliminating DSBs, two research groups presented MDC1 as a potential therapeutic target in cancer treatment. MDC1 was shown to be upreguated both in mRNA and protein levels in human cervical tumors, whereas MDC1 knockdown induced radiosensitivy, cell cycle arrest and apoptosis in several cervical cancer cell lines (138). In the same study, HeLa cells, depleted of MDC1, also demonstrated lower tumor growth potential in mouse xenograft model . Mechanistically, MDC1 knockdown was shown to impair Rad51 foci formation and decrease its protein stability in breast cancer cells. The resulting defects in Rad51-mediated HR repair rendered cells hypersensitive to γ-irradiation and DNA-crosslinking agents, both inducing DSBs (139). Such an oncogenic role of MDC1 could be partly explained with its anti-apoptotic effects. MDC1 is vital for NHEJ, an efficient but error-prone repair mechanism (29). It is possible that hyperactivation of NHEJ could lead to

mutations induced by DSBs. Indeed, inhibition of DNA ligase IV, which seals DSBs in NHEJ repair, induced cytotoxicity in various cancer cell lines as well as mouse models (140).

In summary, I have provided new insights into the regulations of MDC1, both in the context tof mitotic DNA damage and in the undamaged state. Better understanding of the MDC1-mediated repair mechanisms can contribute to improved efficacy and specificity of cancer therapy, as many DDR inhibitors are in clinical use or undergoing development. Chemotherapeutic strategies targeting MDC1 could block downstream repair ability of cancer cells to restore broken chromatin. Such accumulation of DNA damage would propel cancer cells to initiate suicide pathways and be eliminated from the human body.

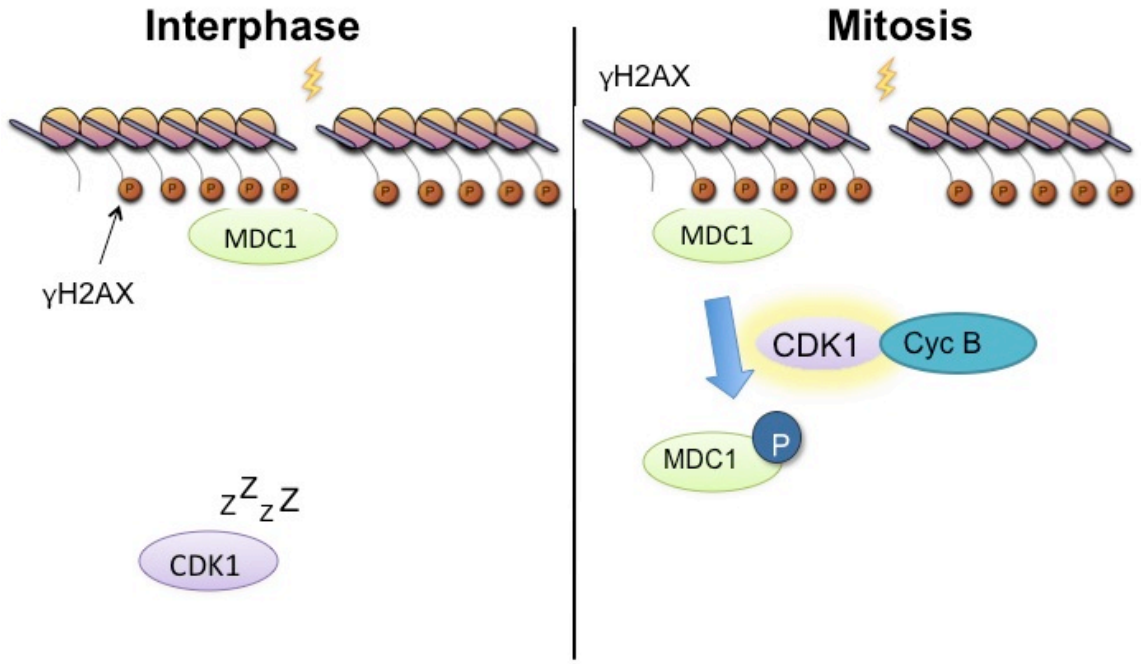

Figure 1. Working model of MDC1- γH2AX interaction by CDK1 regulation.

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