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Cell Cycle News & Views

Assaying DNA double-strand break induction and repair as fast as a speeding comet

Comment on: Weingeist DM, et al. Cell Cycle 2013; 12:907–15; PMID:23422001; http://dx.doi.org/10.4161/cc.23880

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Most cancer treatments exploit the hypersensitivity of rapidly dividing tumor cells to DNA damage, largely reflecting problems with replicating damaged DNA templates. Many cancer chemotherapeutics directly damage DNA, and most types of DNA damage block replication forks. Other classes of chemotherapeutics include antimetabolites that reduce nucleotide pools and starve DNA polymerases or directly inhibit DNA polymerases, causing fork stalling. Blocked or stalled replication forks are initially stabilized by DNA damage response (DDR) proteins, including checkpoint and DNA repair proteins.[1] Forks that fail to restart in a timely manner may regress to a "chicken foot" structure, which is subject to cleavage, causing fork collapse to double-strand breaks (DSBs).[2] When replication forks encounter single-strand breaks (SSBs) and gaps (which can arise during repair of single-strand damage) this can result in direct fork collapse to DSBs. About half of cancer patients are treated with ionizing radiation, which directly induces DSBs, as well as base damage and SSBs that can be converted to DSBs during DNA replication. Thus, the common thread in all of these therapeutic strategies is DSB induction (**Fig. 1**). DSBs are highly cytotoxic, which explains their efficacy in cancer therapy and the intense effort to elucidate mechanisms of DSB induction and repair.

Several assays have been developed to measure DSB induction and repair. The induction of one or a few DSB at defined loci by the rare-cutting endonucleases I-SceI and I-PpoI, and their repair, can be measured with PCR assays using primers that flank the DSB.[3] Immunofluorescence microscopy is frequently used to detect phosphorylated histone H2AX (γ-H2AX) foci, which appear adjacent to DSBs within 30 min of DSB induction, and their disappearance is taken as evidence of repair.[4] γ-H2AX can also be detected by western blot, which provides an estimate of global DSB load in a population of cells. For more than 20 y, pulse field gel electrophoresis has been used to measure the fraction of broken DNA released from wells into the gel, providing a direct measure of DSBs in genomic DNA that is quantitative and reproducible. The comet assay is a related gel electrophoresis technique, in which DNA migrates out of individual cells embedded in agar on a microscope slide, producing DNA "tails" that extend from the body of the cell in a characteristic comet shape. Comet tail length (measured visually) and "tail moment" (product of tail length and the fraction of DNA in the tail determined by analysis of pixel intensities) are proportional to the number of DSBs; however, reproducible scoring of tail lengths or moments has proven difficult.[5]

Each of the DSB assays above has its strengths and weaknesses, but none are particularly well-suited to high-throughput analysis. Enter the Engelward lab, which, in collaboration with engineers from the Bhatia lab, modified the comet assay to a 96-well format in which each of the 96 "macrowells" is subdivided into microfabricated "microwells," ranging from 25–45 μm in diameter that each hold one to several cells.[6] In a study by lead authors Weingeist and Ge in the March 15, 2013 issue of *Cell Cycle*,[7] Engelward and colleagues at MIT and Harvard then demonstrated that this platform is very well-suited to high-throughput analysis of DSB induction and repair. The "CometChips" allow analysis of up to 96 different experimental conditions on a single gel, and because cells are arrayed, each comet can be scored using an automated image capture system, which greatly increases assay speed and reproducibility. How important is a reliable, high-throughput assay that directly measures DSB induction and repair? The DDR in general, and DSB repair in particular, are major determinants of cell survival and cell death and, thus, cancer treatment efficacy. The DDR is mediated by an incredibly complex network of proteins that includes, for example,

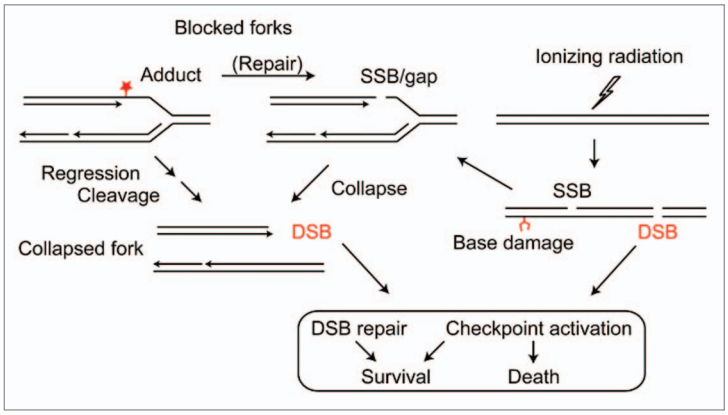

Figure 1. Replication forks blocked by DNA adducts may regress and be cleaved causing fork collapse to a DSB. Fork collapse can also occur when forks stall when DNA polymerase is inhibited or starved for nucleotides (not shown). SSBs/gaps can cause direct fork collapse. Ionizing radiation induces DSBs directly and indirectly through SSBs and base damage. DSBs activate checkpoint and DSB repair pathways that enhance cell survival. However, when a cell suffers too much damage, checkpoints can trigger cell death by apoptosis.

the ATM and ATR kinases, which are activated by DSBs and have at least 900 known targets on 700 different proteins.[8] Therefore, the ability to rapidly test responses of tumor and normal cells to the combined effects of DNA damaging agents and large chemical libraries of potential DDR inhibitors is very important indeed.

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Death becomes her: FBH1, DNA damage and apoptosis

Comment on: Jeong YT, et al. Cell Cycle 2013; 12:1128–32; PMID:23466708; http://dx.doi.org/10.4161/cc.24165

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The process of DNA replication is fraught with danger: there are nicks and harmful modifications to the DNA template strand that can impede fork progression, alterations and imbalances in the dNTP pool can slow down fork progression and a lack of communication between leading and lagging strand replication can thwart the entire process. When replication forks stall, the first step involves a restart process, which tries, in the most effective but least drastic way, to get replication back on track. If the blocks to replication persist, and homologous recombination does not reassemble the fork, then replication can restart by repairing DNA breaks through homologous recombination and using new origins to continue replication.1 However, when the damage to the template strand is particularly severe, the cell instead makes DNA breaks through an active process that enhances the DNA damage response and targets the cell for apoptosis. The key players in DNA break formation are the 3′–5′ helicase FBH12-4 and the nuclease MUS81.5

As expected from such a scenario, disruption of FBH1 or MUS81 in cells leads to resistance to replication stress and reduced formation of DNA breaks. Why would cells have pathways that willfully damage DNA and then target the cell for apoptosis? An answer to this comes from a recent study[6] that showed that oncogenic stress, resulting in continued replication stress, is counteracted by FBH1 action. In this most altruistic behavior, apoptosis destroys the individual cell but preserves the tissue or organism. Thus it would be expected that mutations in FBH1, while providing resistance to DNA damage, would ultimately be harmful, as they would prevent very damaged cells from undergoing apoptosis and, instead, would promote propagation of cells with grossly damaged and rearranged genomes (**Fig. 1**, see opposite page). In a survey of 19 melanoma cells lines, Jeong et al.[6] found that 55% had deletions of FBH1. Moreover, the FBH1 locus was deleted in a large fraction of melanomas in the NCBI-GEO database. Melanocytes are under constant exposure to UV damage. Hence FBH1 would be needed to destroy highly damaged cells, and loss of FBH1 seems to promote transformation to melanomas. Other tissues highly susceptible to UV damage, including lung and lung cancer cell lines, are also more often deleted for FBH1 than other tissue cancer cell lines. In **Figure 1**, we suggest that both translesion polymerase Polη and FBH1 allow either error-free bypass of UV damage or destruction of DNA with multiple damage sites. In the absence of FBH1, we suggest that translesion polymerase Polη is not sufficient for replication bypass of all the UV damage sites, and hence error-prone translesion polymerases are used, resulting in increased mutagenesis.

The screening results of the melanoma cells lines are strongly suggestive, but only correlative, of the role that FBH1 helicase plays in preventing the growth of cells with highly damaged genomes and cell transformation. To further establish the connection between FBH1 and prevention of transformation, the authors induced transformation by UV irradiation in FBH1-depleted primary human melanocytes. Without UV exposure, the cells did not show any phenotypes related to transformation, but the FBH1-depleted cells showed a rapid increase in transformation promoted by UV irradiation, demonstrating the protective action of FBH1. Thus, treating melanomas with agents that cause replication stress may be counterproductive and potentially harmful if those tumors have reduced FBH1 levels or mutations that inactivate FBH1. It is possible that replication in the presence of UV damage and in the absence of FBH1 leads to mutagenic bypass or processing, leading to mutations and genome rearrangements.[7] These studies also point out one marker that could be easily assessed prior to treatment. As the goal of chemotherapy is to induce apoptosis to kill tumor cells, the genetic makeup, including the FBH1 status of these cells must be taken into consideration to insure that appropriate treatment options are chosen.

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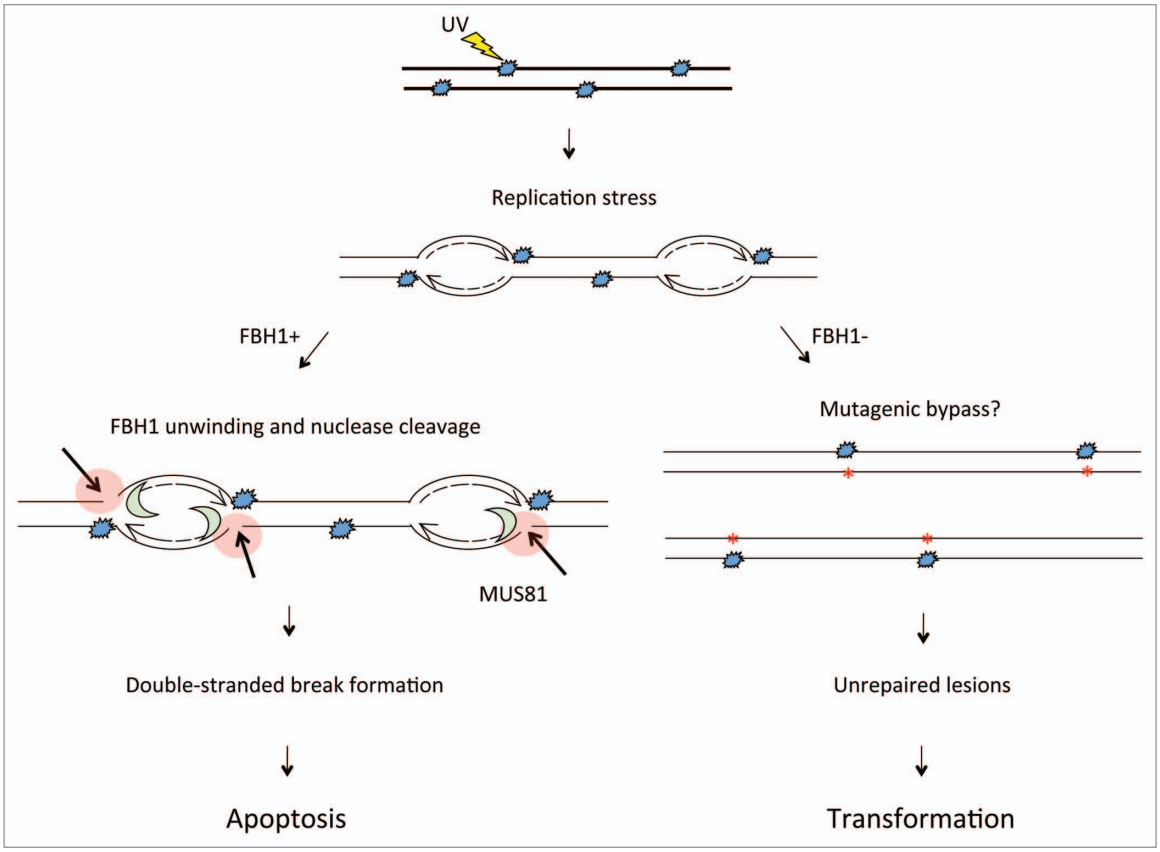

Figure 1. UV radiation causes lesions in DNA that block replication machinery, resulting in fork stalling. The cell employs a mechanism whereby 3′–5′ helicase FBH1 (green) unwinds lagging strand DNA, creating a substrate for a nuclease, such as MUS81, that cleaves (red circles) and thus promotes the formation of double-stranded breaks. These double-stranded breaks activate the DNA damage response, and if the DNA damage is extensive enough, this will eventually result in apoptosis of the cell. When FBH1 is absent or depleted, cells continue to replicate damaged DNA, possibly through mutagenic pathways, leading to increased genome instability that can result in cellular transformation and cancer.

Fine-tuning the p53 response to DNA damage: A new piece in the puzzle

Comment on: Moumen A, et al. Cell Cycle 2013; 12:698–704;
PMID:23343766; http://dx.doi.org/10.4161/cc.23592

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The tumor suppressor p53[1] is a master regulator of cell cycle checkpoint responses to DNA damage whose activity is tightly controlled. It functions as a transcription factor for genes regulating cell cycle progression and apoptotic cell death. Following DNA damage, p53 is stabilized and its transcriptional activity is markedly stimulated.

Among the many factors that can modulate p53 activity, the RNA-binding protein hnRNP K (heterogeneous ribonucleoprotein K) was characterized as a key player in the p53-dependent response to DNA damage in human cells, acting as a transcriptional coactivator for p53.[2] hnRNP K and p53 are indeed co-recruited to the promoters of p53-responsive genes and cooperate to elicit their activation. Interestingly, similar to p53, hnRNP K levels are regulated by the E3 ubiquitin ligase HDM2, which targets hnRNP K for ubiquitylation and proteasomal degradation.[2] DNA damage triggers the dissociation of the hnRNP K-HDM2 complex, leading to hnRNP K stabilization. Because these events are dependent on the DNA damage checkpoint kinases ATM (ataxia telangiectasia mutated) and ATR (ATM- and Rad3-related),[2] it was tempting to speculate that hnRNP K function in the DDR (DNA damage response) could be regulated by phosphorylation.

This issue has been explored in a recent study published in *Cell Cycle,* where Moumen et al. showed that a phosphorylated form of hnRNP K can be detected with a phospho-ATM/ATR substrate antibody upon IR (ionizing radiation)-induced damage in human cells.[3] Given that hnRNP K levels are elevated in an ATM-dependent manner in response to IR,[2] the authors tested the possibility that this key upstream DNA damage checkpoint kinase could target hnRNP K. Using an ATM-specific inhibitor and siRNA-mediated depletion of the ATM kinase, they demonstrate that hnRNP K is phosphorylated in an ATM-dependent manner after IR. They also identify four S/T-Q

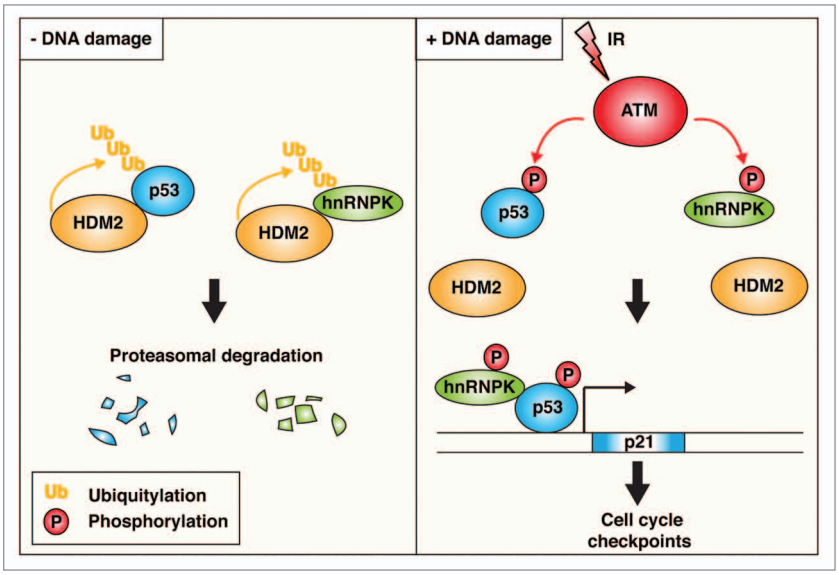

Figure 1. Control of hnRNP K by phosphorylation: Integrating DNA damage signals to fine-tune p53-dependent transcriptional responses.

ATM consensus target motifs in the hnRNP K sequence (S121, T174, T390, T440) that can serve as ATM phosphorylation sites. Similar phosphorylation events may be mediated by ATR following UV irradiation, because hnRNP K is stabilized in an ATR-dependent manner in this context.[2]

Next, Moumen et al. examined the functional relevance of such phosphorylation of hnRNP K in the DDR. Taking advantage of a phospho-deficient mutant where the four S/T sites are mutated to alanines, they established that phosphorylation of hnRNP K is critical for its dissociation from HDM2 and subsequent stabilization in response to IR. It is not yet clear whether the ATM-dependent phosphorylation of hnRNP K directly inhibits its interaction with HDM2, because how hnRNP K interacts with HDM2 is currently unknown. Importantly, cells expressing the phospho-deficient hnRNP K protein also display impaired recruitment of p53 to its target gene *p21*^{Waf1} and defective stimulation of p53 transcriptional activity. Together, these findings support a model where ATM-mediated phosphorylation protects hnRNP K from proteasomal degradation in response to damage, allowing it function as a coactivator for p53 (**Fig. 1**). Identifying phosphatases able to reverse hnRNP K phosphorylation would help define how this response is switched off after damage.

Remarkably, such phospho-dependent control of hnRNP K levels in response to DNA damage mirrors the upregulation of p53, whose phosphorylation by ATM also leads to its stabilization upon dissociation from HDM2. This striking parallel between two proteins collaborating in the DDR reveals the tight control that ATM exerts on p53 transcriptional activity. By achieving the same goal via two converging pathways, ATM imposes a double lock on cell cycle checkpoint responses (**Fig. 1**).

These observations should be considered in light of other DNA damage-dependent modifications on hnRNP K that control p53-dependent transcription, including sumoylation and methylation.[4-6] It will be of major interest to identify potential cross-talk and/or interference between these modifications to shed light on how they collectively contribute to the fine-tuning of hnRNP K activity in response to damage. It would also be important to evaluate whether ATM-dependent phosphorylation effects other functions of hnRNP K in RNA metabolism, including its ability to repress p53-target genes via an interaction with the p53-induced large intergenic noncoding RNA lincRNA-p21.[7] Finally, the critical role of ATM phospho-target sites in regulating hnRNP K protein levels opens up the possibility that mutations of these residues could be instrumental in driving the upregulation of hnRNP K frequently observed in human tumors.

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The dimeric Mcm8–9 complex of *Xenopus laevis* likely has a conserved function for resistance to DNA damage

Comment on: Gambus A, et al. Cell Cycle 2013; 12:1225–32; PMID:23518502; http://dx.doi.org/10.4161/cc.24310

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The hexameric Mcm2–7 complex, which is composed of the evolutionarily related Mcm2 to Mcm7 subunits, forms the core of the eukaryotic replicative helicase (**Fig. 1**). In the late M to G_1 phase, Mcm2–7 is recruited to replication origins with the assistance of at least three essential replication factors, ORC, Cdc6 and Cdt1, to form the pre-replicative complex (pre-RC). In the S phase, Cdc45 and GINS associate with Mcm2–7 via the action of two kinases, DDK and S-CDK, and other replication factors to assemble the active Cdc45-Mcm-GINS helicase, which translocates away from origins to unwind double-stranded DNA at replication forks.[1] Mcm2–7 appears to be

the key factor for the control of DNA replication, as most known regulatory mechanisms are dedicated to Mcm2–7, rather than to DNA polymerases.

Mcm8 and Mcm9 are evolutionarily related to the other Mcm proteins, all of which contain the Walker A and B motifs for ATP hydrolysis within the MCM family domain, as well as the zinc- and arginine-finger motifs (**Fig. 1**). A phylogenetic analysis has suggested that the last common ancestor of eukaryotes had the *MCM8* and *MCM9* genes.[2] These two genes are conserved in many eukaryotic species but seem to have been lost together in yeast and *C. elegans,* suggesting the co-evolution of Mcm8 and Mcm9. Drosophila species are an exception, as they have only the *MCM8* homolog, termed *REC*.[2] As can be imagined, based on the role of Mcm2–7 in DNA replication, early studies suggested independent and controversial roles for Mcm8 and Mcm9 in DNA replication. In Xenopus egg extracts, Mcm8 was shown to be involved in elongation of the replication fork.[3] Conversely, in the same system, Mcm9 was reported to interact with Cdt1 for the loading of Mcm2–7, i.e., pre-RC formation.[4] Recently, knockout mice and chicken DT40 cells lacking the *MCM8* or *MCM9* gene were successfully generated, clearly indicating that the two proteins are not essential for DNA replication in these organisms.[5-7] Moreover, consistent with the notion that the *MCM8* and *MCM9* genes have co-evolved in most eukaryotes, Mcm8 and Mcm9 form a complex and are required for tolerance to DNA damage, suggesting that the Mcm8–9 complex plays a role in DNA repair.[6,7]

In the April 15, 2013 issue of *Cell Cycle*, Gambus and Blow re-examined the role of Mcm8 and Mcm9 in DNA replication in Xenopus egg extracts to address the discrepancies observed in previous reports. As is the case with humans, mice and chickens,[6,7] the authors demonstrated that Xenopus Mcm8 and Mcm9 form a stable complex; however, neither of them associates with Cdt1 and Mcm2–7. Using glycerol gradient and size-exclusion experiments, the Mcm8–9 complex was found to be a dimer, unlike the previous suggestion of the formation of a hexamer in DT40 cells.[7] During the replication of sperm chromatin DNA, Mcm8 and Mcm9 associate with chromatin from the late S to G_2 phase, suggesting that they are not essential for DNA replication. In fact, depletion of Mcm9 from egg extracts, which eventually co-depletes Mcm8, has a marginal effect on normal DNA replication. Finally, the authors showed that various types of DNA damage induce association of Mcm8–9 to chromatin, which is consistent with the observation that Mcm8 and Mcm9 are required for resistance to DNA damage.[6,7]

The most important future question regarding this issue is: "What is the role of the Mcm8–9 complex in resistance to DNA damage?" Taking into account the fact that the knockout mice and DT40 cells lacking *MCM8* or *MCM9* exhibit a severe defect in gametogenesis and homologous recombination (HR), respectively,[5-7] it is likely that the Mcm8–9 complex has a conserved function in HR repair. Another question is: "Does the Mcm8–9 complex function as a helicase?" If so, is the dimeric Mcm8–9 complex converted to a hexameric complex similar to Mcm2–7, and are there any accessory factors, such as Cdc45 and GINS in the case of Mcm2–7, that are required for its helicase activity? The characterization of Mcm8 and Mcm9 might shed light on a new mechanism of HR repair. Future analyses are warranted.

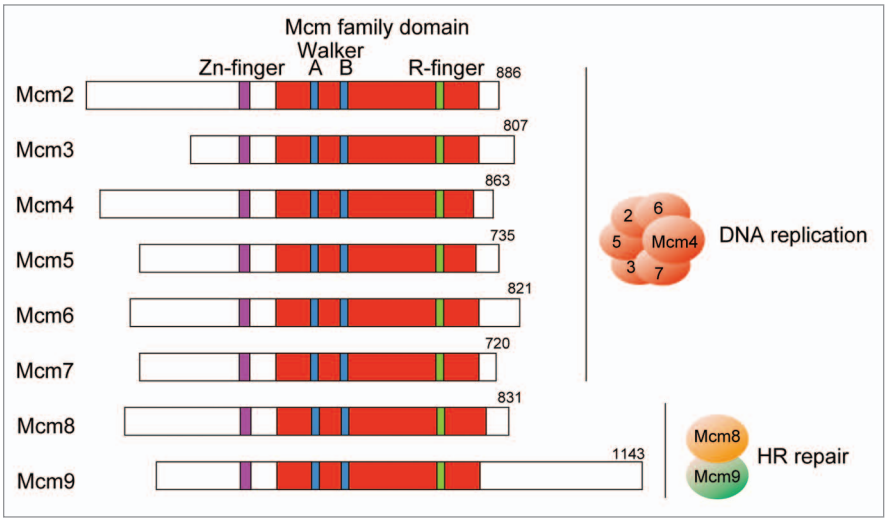

Figure 1. Schematic illustration of the Mcm family proteins of *Xenopus laevis*. The Walker A and B motifs within the Mcm family domain, and the zinc (Zn)- and arginine (R)-finger motifs are indicated. Mcm2 to Mcm7 form a hexameric complex that is essential for DNA replication. Mcm8 and Mcm9 form a dimer that is likely to be involved in HR repair.

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