

Mrc1/Claspin: a new role for regulation of origin firing

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Abstract Mrc1 and its vertebrate homologue Claspin serve as a mediator for replication stress checkpoint signaling, receiving the signal from Mec1/Rad3/ATR sensor kinase and transmitting it to the effector Rad53/Cds1/Chk1 kinase. They are likely to be a part of the replisome and facilitate the S-phase progression by promoting replication fork progression. Recent reports on Mrc1/Claspin indicate their new role in regulating the replication initiation through interaction with Cdc7, a key conserved serine–threonine kinase that triggers firing at each replication origin. Mrc1/Claspin has a specific domain that specifically interacts with Cdc7, and this domain is involved also in intramolecular interaction with its N-terminal segment. Mechanisms for novel regulation of origin firing and its timing through recruitment of Cdc7 to Mrc1/Claspin will be discussed.

Keywords Mrc1 · Claspin · Cdc7 kinase · Replication timing · Origin firing · Intramolecular interaction

Mrc1/Claspin: an integral replication fork factor that facilitates and monitors replication fork movement

Once cells commit to DNA replication, the process needs to be completed until the entire genome is replicated. Failure

to do so will result in immediate generation of genomic instability that involves various lesions on the chromosomes (Masai et al. 2010; Muñoz and Méndez 2016). However, the chromosomes are destined to encounter various obstacles during the course of DNA replication. This so-called “replication stress” is generated by various DNA damages including chemical modification of bases and nucleosides as well as DNA strand breaks, unusual DNA structures, unscheduled presence of DNA binding proteins along the path of replication, lack of nucleotide precursors for DNA replication, and head-on encounter with transcription. Cells have developed strategies to cope with various replication stresses to facilitate the completion of genome replication, along with other cellular protection systems (Gadaleta et al. 2016; Palou et al. 2016).

The replication checkpoint pathway plays a central role in this process (Errico and Costanzo 2012). The conserved Mec1/Rad3/ATR (sensor kinase)-Mrc1/Claspin-Rad53/Cds1/Chk1 (effector kinase) pathway is activated and sends cell cycle block signals that transiently inhibit progression of S and G2/M phases to permit the removal of any obstacles causing the stress. Mrc1 was originally discovered in fission yeast as a factor required for activation of Cds1 (Tanaka and Russell 2001), and Claspin was discovered as a factor binding to Chk1 in *Xenopus* extracts (Kumagai and Dunphy 2000) and was found to be an ortholog of Mrc1. Subsequent studies indicated that Mrc1/Claspin plays an essential role as a mediator that transmits the replication stress signal from the sensor kinase to the effector kinase (Kumagai and Dunphy 2003; Christiano et al. 2003; Hae et al. 2006; Lindsey-Boltz et al. 2009; Lee et al. 2003, 2005; Sar et al. 2004).

On the other hand, yeast Mrc1 was found to be present in the replication progression complex that was isolated by immunoprecipitation using an antibody targeting Cdc45

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(Gambus et al. 2006). It was further shown that Mrc1 was required for efficient progression of S phase both in yeast and mammalian cells (Szyjka et al. 2005; Lin et al. 2004). More recently, Mrc1 was shown to directly stimulate replication fork progression in the in vitro reconstituted replication assay system (Yeeles et al. 2017). This indicates a crucial role of Mrc1/Claspin as an integral factor for the replisome. It facilitates fork progression through interaction with helicase components in conjunction with Tof1/Swi1/Timeless-Csm3/Swi3/Tipin complex and monitors the fork progression through interaction with sensor and effector kinases to transduce replication stress signal.

One notable feature of mutants in replication checkpoint factors is the loss of suppression of late-firing origins (Crabbé et al. 2010). This is also true for Mrc1/Claspin and $\Delta mrc1$ deregulates late-firing origins in the presence of HU or MMS. This reflects the checkpoint-dependent function of Mrc1, since late origin firing is observed also in a checkpoint-specific mutant of *mrc1* (Zhao et al. 2003; Xu et al. 2006). On the other hand, it was reported that origin firing is enhanced at some early-firing origins in $\Delta mrc1$ cells (Hayano et al. 2011). Interestingly, this deregulation is checkpoint-independent, and is not observed in the checkpoint-specific mutant. In budding yeast, phenomena called “scaling” has been known (Koren et al. 2010). This is exemplified by the observation that prolonging S phase results in delayed firing of origins while maintaining the relative order of origin firing time (Alvino et al. 2007). This regulation was abrogated in $\Delta mrc1$ and was later shown to be independent of its checkpoint function (Gispan et al. 2014).

Mrc1/Claspin and Cdc7: a novel link of Mrc1/Claspin to initiation

Cdc7 is a conserved serine/threonine kinase that plays an important role in firing of replication origins as well as in replication checkpoint (Masai et al. 2010; Masai and Arai 2002; Matsumoto et al. 2010). Its crucial target for initiation of DNA replication is Mcm, and its phosphorylation by Cdc7 triggers association of Sld3-Cdc45 with pre-replicative complex (pre-RC) and formation of CMG helicase, an active replicative helicase complex (Deegan et al. 2016). Previous studies indicated close physical and functional interactions between Cdc7 and Mrc1/Claspin in both yeast and mammalian cells (Kim et al. 2008; Shimmoto et al. 2009; Rainey et al. 2013), and Mrc1/Claspin was shown to be a bona fide substrate of Cdc7, although its physiological significance has not been known.

Recent studies on Mrc1 in fission yeast revealed a part of the mechanism on how Mrc1, in conjunction with Cdc7, may regulate origin activation in a checkpoint-independent

manner. Studies on Claspin also showed functional importance of its interaction with Cdc7 and indicated similar mechanisms for regulation of origin firing in mammalian cells. These studies strongly indicate evolutionally conserved, novel roles of Mrc1/Claspin in regulation of initiation of DNA replication. In the followings, we will discuss how Mrc1/Claspin-Cdc7 interaction is involved in regulation of origin firing.

Checkpoint mutations bypass Cdc7 function for DNA replication

Cdc7 kinase, originally discovered in budding yeast, is essential for growth under normal growth condition. Unexpectedly, we discovered that loss of replication checkpoint function in $\Delta cds1$ or $\Delta mrc1$ enabled $\Delta hsk1$ (Cdc7 homologue of fission yeast) cells to grow (Hayano et al. 2011; Matsumoto et al. 2011). We reasoned that increased replication potential in these mutants led to bypass of Cdc7 function for initiation of DNA replication. We further discovered that the *hsk1*⁺ function can be bypassed at a higher growth temperature, where late origin deregulation is observed (Matsumoto et al. 2011). These results indicate that Cdc7 function is dispensable for DNA replication under certain genetic or environmental backgrounds.

mrc1-3A (a *mrc1* mutant specifically defective in checkpoint function due to the loss of Rad3-mediated phosphorylation sites) was also found to bypass the requirement for Cdc7 for growth. However, the efficiency of bypass was lower than that by $\Delta mrc1$, suggesting the presence of a checkpoint-independent mechanism for *hsk1* bypass (Hayano et al. 2011). The *hsk1* bypass appears to be brought about by conditions that alter the cellular replication potential, which would lead to activation of dormant or weak origins. In fact, we were able to identify *rif1* as a major factor that determines the timing of late/dormant origin firing during normal growth by searching for a novel factor that can restore growth of $\Delta hsk1$ cells (Hayano et al. 2012).

HBS (Hsk1 bypass segment) required for checkpoint-independent bypass of Hsk1

We previously reported that Mrc1 binds selectively to early-firing origins before the action of Hsk1 kinase, suggesting a possibility that Mrc1 regulates early-firing origins as well. We also noted that the firing timing and efficiency of weak early-firing origins are stimulated in $\Delta mrc1$ cells. Interestingly, this effect was not observed in *mrc1-3A* mutant, suggesting that it may be regulated by the checkpoint-independent function of Mrc1 (Hayano et al. 2011).

We have used the ability of *mrc1* mutants to bypass the requirement of *hsk1* for growth as a read out to delineate the checkpoint-independent function of *mrc1*. We have identified HBS (Hsk1 bypass segment) on Mrc1 the deletion of which results in bypass of *hsk1* function for growth. *mrc1* Δ HBS is resistant to HU, and thus, it is checkpoint proficient. Bypass of *hsk1* by *mrc1* Δ HBS is less efficient than by Δ *mrc1*. However, interestingly, the combination of *hsk1-3A* and *mrc1* Δ HBS restored the efficient bypass, suggesting that these two pathways function independently (Matsumoto et al. 2017).

HBS is involved in checkpoint-independent regulation of early origin firing and required for binding to Hsk1 kinase

In *mrc1* Δ HBS, weak early-firing origins are precociously activated as in Δ *mrc1*. However, in this mutant, late/dormant origins are not activated in the presence of HU, consistent with its resistance to HU. Thus, HBS is specifically involved in checkpoint-independent regulation of early-firing origins. We previously reported that Mrc1 interacts with Hsk1 and is phosphorylated by this kinase (Shimmoto et al. 2009). We showed that HBS is required for interaction of Mrc1 with Hsk1. HBS alone is not sufficient for binding to Hsk1, and NTHBS (N-terminal-Target-of-HBS) is required for Hsk1 binding as well. HBS is required for efficient phosphorylation of Mrc1 by Hsk1 both in vivo and in vitro, and thus, it is likely that HBS functions as a recruiter of Hsk1 kinase for efficient execution of initiation at early-firing origins.

Intramolecular interaction and Hsk1 regulate conversion between the “break-on” and “break-off” conformations of Mrc1

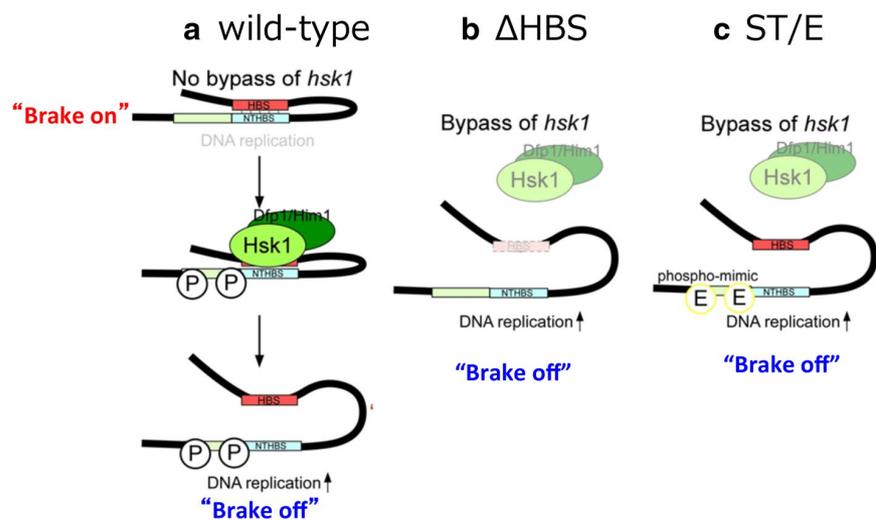
Further analyses of Mrc1 showed that HBS interacts with NTHBS. This interaction is regulated by Hsk1, since substitution of all the serines/threonines with glutamic acid in the aa 238–395 led to loss of this intramolecular interaction as well as acquisition of bypass ability of Δ *hsk1*. The replacement with glutamic acid presumably generated phosphomimic state of the Mrc1. Indeed, these residues are the targets of Hsk1-mediated phosphorylation in vitro.

On the basis of these results, we have proposed that Hsk1 exists in two distinct conformations; “break-on” and “break-off” states (Matsumoto et al. 2017; Fig. 1). Before initiation, Mrc1, selectively bound at the early-firing origins (Hayano et al. 2011), is in the “break-on” conformation through intramolecular interaction between HBS and NTHBS. At the onset of S phase, Hsk1 kinase, recruited to NTHBS-HBS, phosphorylates aa 238–395, converting Mrc1 to the “break-off” conformation through loss of intramolecular interaction (Fig. 1a). The deletion of HBS (Fig. 1b) or ST/E mutation of NTHBS (Fig. 1c) disrupts the intramolecular interaction, forcing the shift to the “break-off” conformation.

Regulation of origin firing timing in fission yeast

The genetic dissection of bypass of Hsk1 function for DNA replication led to identification of novel checkpoint-independent regulation of early-origin firing by Mrc1 as well as to a new factor, Rif1, that plays a crucial role in checkpoint-independent regulation of late/dormant origins. These findings led to general view on how origin firing timing is

Fig. 1 Potential mechanism of origin regulation by Mrc1 in fission yeast. Three modes of release of Mrc1-mediated “brake” on replication initiation are described (Matsumoto et al. 2017). See text for detail



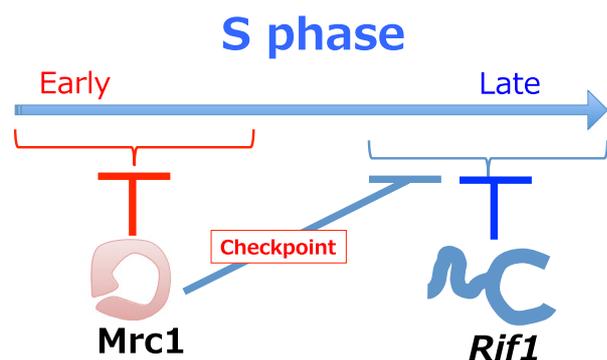


Fig. 2 Two major pathways that regulate the replication timing in fission yeast. Mrc1 (checkpoint-independent function) and Rif1 suppress origin activation in early and late S phases, respectively. Checkpoint-dependent function of Mrc1 suppresses the late origin firing in the presence of replication stress

regulated in fission yeast (Fig. 2). We propose that Mrc1 and Rif1 are the dual regulators of origin firing program during normal growth that suppress early- and late-firing origins, respectively. In the presence of replication stress, on the other hand, the checkpoint-dependent suppression of late/dormant origins by Mrc1 and other checkpoint regulators would operate.

Mammalian Claspin also recruits Cdc7 and is regulated by intramolecular interaction

It is of interest whether the mode of regulation of Mrc1 by Cdc7 is conserved in higher eukaryotes as well. In fact, it is well established that Claspin interacts with Cdc7 and is efficiently phosphorylated by this kinase (Kim et al. 2008; Rainey et al. 2013). We have identified a segment near the C-terminus of Claspin that can bind to Cdc7. The segment, designated AP for acidic patch, is rich in acidic residues and is essential for the functions of Claspin (Yang et al. 2016). AP interacts with a N-terminal segment of Claspin and this interaction is abrogated by Cdc7-mediated phosphorylation of the N-terminal segment. DE/A mutant of AP (in which all the acidic residues in AP were replaced by alanine) is non-functional and is not able to recruit Cdc7 for phosphorylation of essential substrate, Mcm. Intramolecular interaction masks DNA and PCNA binding activity of Claspin which resides in its N-terminal segment. Claspin undergoes conformation change upon phosphorylation by Cdc7, resulting in enhanced DNA and PCNA bindings (Yang et al. 2016; Fig. 3).

In normal cells, Cdc7-mediated phosphorylation of Mcm is significantly reduced by Claspin depletion, resulting in defect in DNA replication. On the other hand, Mcm phosphorylation is not affected by Claspin knockdown in

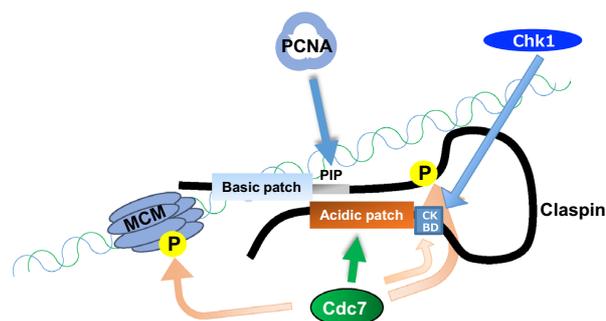


Fig. 3 Model for novel regulation of initiation by Claspin. Cdc7 is recruited to AP (acidic patch) of Claspin, in which AP makes intramolecular interaction with N-terminal segment. Recruited Cdc7 facilitates phosphorylation of Mcm as well as that of Claspin itself, disrupting intermolecular interaction and increasing its DNA and PCNA bindings (Yang et al. 2016). Chk1 binding to Claspin may also be stimulated by Cdc7-mediated phosphorylation (Yang et al. unpublished data)

cancer cells, such as HeLa or U2OS cells. This is probably due to overexpression of Cdc7 in cancer cells, in which the Claspin step is bypassed. We speculate that Claspin provides another layer of regulation over initiation of DNA replication in normal cells by regulating the access of Cdc7 to its crucial substrates.

Cdc7 is an acidophilic kinase, generally favoring the target residues surrounded by acidic amino acids. The presence of recruiters or a docking site for Cdc7 has been reported for some instances. In budding yeast, it was reported that the N-terminal docking sequence on Mcm4 facilitates the Cdc7-mediated phosphorylation of Mcms (Sheu and Stillman 2006). Tof1 and Csm3 (Tim-Tipin in higher eukaryotes) recruit Cdc7 to the replisome during the course of premeiotic DNA replication in budding yeast, enabling it to phosphorylate a key substrate for DSB formation (Murakami and Keeney 2014). The recruiter would probably increase the substrate specificity and efficiency of Cdc7-mediated phosphorylation.

Intramolecular interaction as a mean to regulate protein functions

In spite of the divergence of the primary sequences between fission yeast Mrc1 and human Claspin, there seems to be strong conservation in their structures and the mode of regulation through intramolecular interaction. Regulation of protein functions through intramolecular interactions appears to be a common strategy to modulate protein functions.

It was reported that TopBP1 is regulated by intramolecular interaction (Liu et al. 2014), which is disrupted by

acetylation, causing its BRCT to be recognized by other phosphorylated proteins (Rad9 or Treslin). Intramolecular interaction of the ring-shaped PCNA domain Rad9 with its C-terminal tail was also reported to regulate its binding to DNA and TopBP1 (Takeishi et al. 2015). We believe that there will be increasing instances in which proteins change their conformation through intramolecular interactions in response to various cellular stimuli.

Summary and outstanding questions

Mrc1/Claspin has been regarded as a mediator of replication stress checkpoint and was also shown to be a part of the replisome and to promote the replication fork progression. Indeed, it was recently reported that Mrc1 and Tof1/Csm3 (Tim-Tipin in mammals) complex stimulates the fork rate in an *in vitro* reconstituted replication system (Yeeles et al. 2017). In this short review, we show that Mrc1/Claspin regulates initiation through interaction with Cdc7 and through conformational transition induced by dynamic intramolecular interactions. The new findings implicate Mrc1/Claspin in initiation, elongation, and monitoring steps of DNA replication.

There are still a number of questions regarding the mechanisms of how Mrc1/Claspin regulates early-firing origins. (1) How is Mrc1 recruited selectively to early-firing origins? Mrc1 was reported to interact with C-terminal segment of Mcm6 in budding yeast (Komata et al. 2009). However, interaction with Mcm6 alone would not explain the specificity for early-firing pre-RC. Does it recognize some chromatin feature or modification of pre-RC components specific to early-firing origins? (2) How does Mrc1 send the brake signal to the firing process? How is it related to its “brake-on” conformation? (3) How is the brake released and how is it related to the “brake-off” conformation? (4) What is the structural basis for conformational change induced by Cdc7-mediated phosphorylation? (5) How does the conformation of Mrc1/Claspin change during the cell cycle or in response to replication stress in the cells? (6) What is the role of Cdc7 in regulation of replication checkpoint? Is it related to its interaction with Claspin? (7) Why is Claspin dispensable for Cdc7-mediated phosphorylation of Mcm in cancer cells? Is it related to the apparent overproduction of Cdc7 in cancer cells?

Mrc1/Claspin interacts with many replication factors as well as with other signal transducers. Further analyses of its functions and structure will continue to provide us with unexpected surprises.

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