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RESEARCH ARTICLE

Checkpoint-Independent Regulation of Origin Firing by Mrc1 through Interaction with Hsk1 Kinase

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ABSTRACT Mrc1 is a conserved checkpoint mediator protein that transduces the replication stress signal to the downstream effector kinase. The loss of *mrc1* checkpoint activity results in the aberrant activation of late/dormant origins in the presence of hydroxyurea. Mrc1 was also suggested to regulate orders of early origin firing in a checkpoint-independent manner, but its mechanism was unknown. Here we identify HBS (Hsk1 bypass segment) on Mrc1. An Δ*HBS* mutant does not activate late/dormant origin firing in the presence of hydroxyurea but causes the precocious and enhanced activation of weak early-firing origins during normal S-phase progression and bypasses the requirement for Hsk1 for growth. This may be caused by the disruption of intramolecular binding between HBS and NTHBS (N-terminal target of HBS). Hsk1 binds to Mrc1 through HBS and phosphorylates a segment adjacent to NTHBS, disrupting the intramolecular interaction. We propose that Mrc1 exerts a "brake" on initiation (through intramolecular interactions) and that this brake can be released (upon the loss of intramolecular interactions) by either the Hsk1-mediated phosphorylation of Mrc1 or the deletion of HBS (or a phosphomimic mutation of putative Hsk1 target serine/threonine), which can bypass the function of Hsk1 for growth. The brake mechanism may explain the checkpoint-independent regulation of early origin firing in fission yeast.

KEYWORDS DNA replication timing, Mrc1, Hsk1, intramolecular interaction, replication checkpoint

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In eukaryotic cells, DNA replication is initiated from a number of sites (replication origins) (1, 2). In fission yeast, more than 1,000 prereplicative complexes (pre-RCs) are generated on chromosomes during G_1 phase, and only a subset of them are used for initiation (3–9). The choice of origins to be fired and the determination of the timing of their activation are influenced by many factors, including chromatin structures, local histone modifications, transcription factors, and even physiological conditions such as temperature (7, 10–17).

The DNA replication checkpoint (intra-S checkpoint) operates to delay the activation of late-firing/dormant origins in response to an inhibition of DNA synthesis. The conserved Mec1-Mrc1-Rad53/Rad3-Mrc1-Cds1/ATR-Claspin-Chk1 pathway plays a major role in mediating the replication checkpoint signal to the downstream pathway. Mrc1/Claspin plays a major role as a mediator for the activation of this pathway. We previously reported that Hsk1 is required for the activation of the replication checkpoint, probably through the phosphorylation of Mrc1 (18). On the other hand, Mrc1 was also shown to be required for the scaling of DNA replication timing in budding yeast. In most mutants that extend the S phase, the activation timings of most origins are delayed in proportion to the duration of S phase. In contrast, early-firing origins fire early, even in the extended S phase, in Δ*MRC1* cells (19). Importantly, this requirement of Mrc1 for the scaling of origin activation could still be mediated by a checkpoint-

deficient mutant of *mrc1*, suggesting that Mrc1 regulates early origin firing in a checkpoint-independent manner (20). However, the mechanisms by which Mrc1 regulates early-firing origins are unknown.

Hsk1 is the fission yeast homologue of Cdc7 kinase (21), known to be essential for the initiation of DNA replication. We previously reported that a deletion of *mrc1* can bypass the requirement of Hsk1 kinase for the initiation of DNA replication. We further found that a checkpoint-specific mutant of *mrc1* as well as Δ*cds1* (*cds1* encodes an effector kinase for replication checkpoint signaling) could also bypass Hsk1 (11). Growth of Δ*hsk1* cells was restored even by increasing the growth temperature. Thus, we concluded that Hsk1 kinase is important for initiation in fission yeast cells but not absolutely essential and that its requirement can be circumvented by factors that would change the replication potential.

We reported previously that Mrc1 selectively binds to early-firing origins and that precocious activation of early-firing origins occurs in Δ*mrc1* cells but not in a checkpoint-deficient mutant of *mrc1* (22). These observations have suggested a checkpoint-independent function of Mrc1 that may operate specifically in the regulation of replication timing of early-firing origins. However, the mechanisms of Mrc1-mediated regulation of early-firing origins have been unclear. We have taken advantage of the above-described Hsk1 bypass assays to identify the segments on Mrc1 that may exert checkpoint-independent regulation of origin firing.

We have identified a 98-amino-acid (aa) segment, HBS (Hsk1 bypass segment), near the C terminus of Mrc1 that is required for the checkpoint-independent bypass of Hsk1 functions. In an Δ*HBS* mutant, precocious and more efficient firing of early replication origins is observed. HBS interacts with an N-terminal segment (NTHBS [N-terminal target of HBS]). Hsk1 kinase, interacting with Mrc1 through HBS and NTHBS, phosphorylates Mrc1 at a segment adjacent to NTHBS. The HBS-NTHBS interaction is reduced by this phosphorylation. We propose that Mrc1 undergoes an interconversion between a "brake-on" form (HBS-NTHBS interacting; incapable of bypass) and a "brake-off" form (HBS-NTHBS dissociated; capable of bypass) and that the conversion from the former to the latter is stimulated by Hsk1, resulting in the firing of early-firing origins. Thus, Mrc1 plays novel and crucial roles in the regulation of early-firing origins by its ability to recruit Hsk1 kinase through HBS, in addition to its well-known role in regulating late/dormant origins in a checkpoint-dependent manner.

RESULTS

Identification of HBS on Mrc1 that is involved in checkpoint-independent rescue of Δ*hsk1* cells. We previously reported that an Δ*mrc1* mutation can rescue the lethality of the Δ*hsk1* mutant (11). We further showed that *mrc1-3A* (S604AT645AT653A, specifically defective in checkpoint function [23, 24]), as well as Δ*cds1*, can rescue the Δ*hsk1* mutant. Late/dormant origins are fired in the presence of hydroxyurea (HU) in these checkpoint mutants, leading us to conclude that the checkpoint defect can bypass the requirement of *hsk1* for growth, probably due to a general derepression of the replication potential.

We noticed that the Δ*mrc1* mutation always rescued the Δ*hsk1* mutant more efficiently than did *mrc1-3A* or Δ*cds1* (11). Therefore, we speculated that there may be checkpoint-dependent and -independent pathways for the suppression of the *hsk1* mutation. This suggests the presence of a checkpoint-independent regulation of origin activation by the Mrc1 protein. Indeed, we discovered that the firing efficiency of weak early-firing origins was improved and occurred earlier in the synchronous S-phase progression of Δ*mrc1* cells in the absence of HU (22). Interestingly, this stimulation was not observed for *mrc1-3A* (22). Furthermore, previous reports suggest the presence of checkpoint-independent scaling of origin firing mediated by Mrc1 (19, 20). These results point to the possibility that Mrc1 plays an unknown but novel role in the regulation of early origin firing. Analyses of the checkpoint-independent bypass of *hsk1* would provide us with an excellent opportunity to dissect Mrc1 for this novel and important function.

We previously showed that Mrc1(157–879) but not Mrc1(157–781) inhibited bypass growth (no growth at 30°C, a nonpermissive temperature for an *hsk1* temperature-sensitive [*hsk1*^{ts}] allele, *hsk1-89*) when expressed ectopically in *hsk1-89* Δ*mrc1* cells (11). Since both mutants are resistant to HU, this suggested that aa 782 to 879 play a role in the checkpoint-independent bypass of *hsk1* (11). In order to more precisely characterize the checkpoint-independent pathway for *hsk1* bypass, we constructed strains in which the genomic *mrc1*$^{+}$ gene was replaced by the *mrc1* mutant lacking aa 782 to 879. *mrc1*Δ(*782–879*) cells are resistant to HU to an extent comparable to that of wild-type (WT) *mrc1*$^{+}$, but the *mrc1*Δ(*782–879*) mutation rescues the growth of *hsk1-89* cells at 30°C (Fig. 1A and C), confirming that aa 782 to 879 play a role in the checkpoint-independent bypass of *hsk1*. We previously reported the presence of a putative phosphodegron sequence within the deleted segment (aa 860 to 865) (25). Therefore, we constructed another internal deletion mutant, *mrc1*Δ(*782–846*), which retains the phosphodegron sequence (Fig. 1B and D). This mutant is resistant to HU and rescued *hsk1-89* as efficiently as *mrc1*Δ(*782–879*) (Fig. 1C and D). Therefore, the phosphodegron sequence itself is not responsible for the rescue of *hsk1-89*. We designated the region spanning aa 782 to 879 HBS.

Checkpoint-dependent and -independent pathways function additively for the rescue of *hsk1*^{ts}. We then showed that the *mrc1*Δ*HBS* mutation could rescue the growth of Δ*hsk1* cells (Fig. 1E). Neither *mrc1-3A* (specifically defective in the replication checkpoint) nor *mrc1*Δ*HBS* (proficient in the checkpoint and HU resistant) (Fig. 1F) restored the growth of Δ*hsk1* cells as efficiently as Δ*mrc1* did (Fig. 1E). Therefore, we examined whether the combination of two mutations may restore the efficiency of the bypass. The [*mrc1*(*3A*+Δ*HBS*)] double mutant rescued *hsk1-89* more efficiently than did each of the single mutants, indicating that checkpoint-dependent and -independent pathways independently contribute to the bypass (Fig. 1F).

Δ*HBS* mutations result in elevated initiation at weak early-firing origins. Analysis by a BLAST search of the amino acid sequence of HBS revealed two conserved segments (Fig. 1B), 6 or 7 acidic amino acids toward the N terminus of HBS and the KAF (lysine-alanine-phenylalanine) motif near the middle of HBS. The *KAF/AAA* mutant was resistant to HU to an extent similar to that exhibited by wild-type Mrc1 (data not shown) but rescued *hsk1-89* albeit to a lesser extent than *mrc1*Δ*HBS* (Fig. 2A).

We then examined whether the deletion of HBS or a *KAF/AAA* mutation affects the firing of early-firing origins using two-dimensional (2D) gel electrophoresis. We synchronized the cell cycle by first arresting cells in M phase with *nda3-KM311* at 20°C and releasing the cells into the cell cycle at 30°C. Fluorescence-activated cell sorter (FACS) analyses indicate that cell cycle progression was very similar between different mutants (Fig. 2B). In the wild type, only a strong Y arc (representing passive replication) was detected at *ars1*, a weak early-firing origin, indicating that *ars1* is largely replicated passively from neighboring origins (Fig. 2C). In the Δ*mrc1* mutant, bubble arc (representing initiation) formation was significantly increased at *ars1*, as shown previously (22). In *mrc1*Δ*HBS* and *mrc1-KAF/AAA* cells, a strong bubble arc also appeared at 20 to 30 min, which is indicative of efficient initiation at *ars1* in these mutants (Fig. 2C).

At *ars2004*, a strong early-firing origin, a bubble arc was detected 20 min after release in wild-type cells, and the relative strength of the bubble arc (compared to the Y arc) was not significantly affected by *mrc1* mutations (Fig. 2D). These results indicate that HBS is required for the checkpoint-independent suppression of subsets of early-firing origins.

Chromatin immunoprecipitation (ChIP) analyses and *in vitro* binding assays showed that Mrc1Δ*HBS* and Mrc1-*KAF/AAA* bind to an early-firing origin, excluding the possibility that a loss of origin binding is responsible for elevated initiation in the *mrc1*Δ*HBS* and *mrc1-KAF/AAA* mutants (Fig. 2E; see also Fig. 8C and D).

The *mrc1*Δ*HBS* mutant does not facilitate the firing of late/dormant origins in the presence of HU. Since the *mrc1*Δ*HBS* mutant is proficient for the replication checkpoint (resistant to HU) (Fig. 1A and F), the results described above suggest the

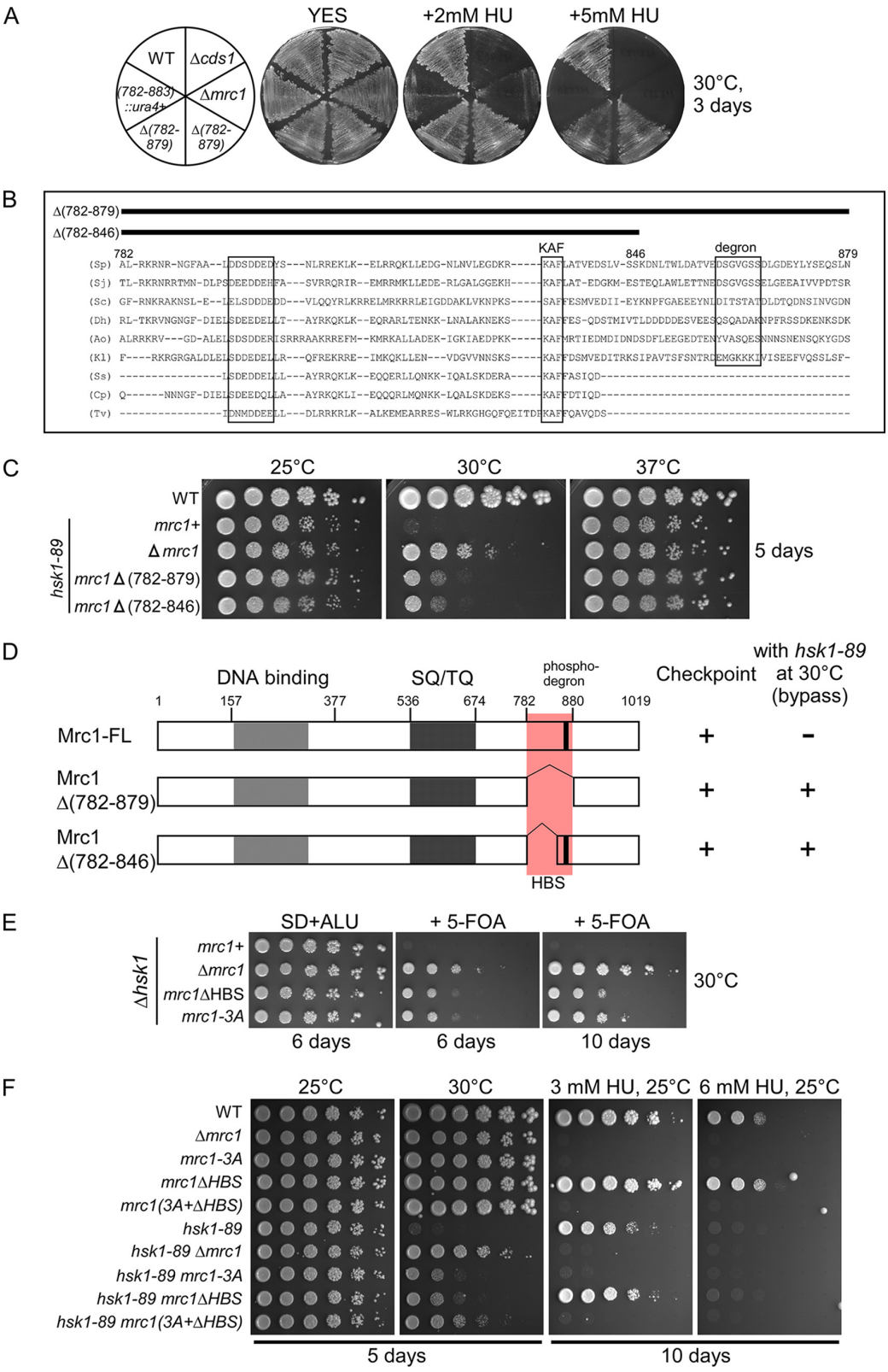

FIG 1 *mrc1ΔHBS* mutants are resistant to HU but can rescue the growth of *hsk1-89*. (A) WT (YM71), Δ*mrc1* (MS252), Δ*cds1* (NI453), *mrc1*Δ(*782–883*)::*ura4*$^+$ (MS560), and two independent *mrc1*Δ(*782–879*) isolates (MS563 and MS564) were streaked onto YES agar containing 0, 2, or 5 mM HU as indicated and incubated at 30°C for 3 days. (B) Three conserved segments within Mrc1(782–879)

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possibility that HBS specifically regulates early-firing origins but not late/dormant origins. We tested this by examining bromodeoxyuridine (BrdU) incorporation at a late/dormant origin (Chr2_2580) in a synchronously released culture in the presence of HU. We found that BrdU is incorporated at this late/dormant origin in *mrc1-3A* mutant cells but not in *mrc1ΔHBS* mutant cells (Fig. 2F), which supports the above-mentioned possibility. Genome-wide analyses of BrdU incorporation into *mrc1ΔHBS* cells in the presence of HU also confirmed that late/dormant origins are not activated in *mrc1ΔHBS* cells (Fig. 2G). These results indicate that HBS is not involved in the regulation of late/dormant origins in the presence of replication stress, consistent with the checkpoint-proficient nature of the Δ*HBS* mutant. These results also show that we were able to separate the domain(s) for checkpoint-independent functions from those involved in checkpoint-dependent functions (which is the SQ/TQ domain) (Fig. 1D).

Involvement of the N-terminal segment of Mrc1 in checkpoint-independent bypass of *hsk1* and its interaction with HBS. To explore the mechanisms of bypass of Hsk1 function and deregulation of early-firing origins in the *mrc1ΔHBS* mutant, we addressed whether mutations/deletions of other segments of Mrc1 could induce similar effects on Mrc1. We introduced a serial deletion from the N terminus of Mrc1 and examined its function in the checkpoint response as well as in the rescue of *hsk1-89*. Mrc1(225–879) was HU resistant and could not bypass *hsk1*. A further 152-aa deletion [Mrc1(377–879)] led to the bypass of *hsk1* but did not significantly affect HU sensitivity (Fig. 3A and B). These results indicate that the 152-aa (residues 225 to 376) segment, in addition to HBS, is also involved in the checkpoint-independent bypass of *hsk1*.

N-terminal truncations beyond aa 377 resulted in a stepwise loss of the checkpoint (increased sensitivity to HU), and those extending into the SQ/TQ segment (beyond aa 536) result in a complete loss of the checkpoint (sensitive to HU) and suppression of *hsk1-89*. The reason why Mrc1(536–879), which is checkpoint deficient, is unable to suppress *hsk1-89* is not clear. It may be dominantly inhibiting growth in this particular genetic background.

These results also suggest the functional and/or physical interactions between C-terminal HBS and the N-terminal segment. Therefore, we examined the physical interaction between the C-terminal (aa 781 to 1019) and the N-terminal segments of Mrc1. Myc-tagged Mrc1(781–1019) was expressed alone or coexpressed with Flag-tagged full-length Mrc1 (Mrc1-degSSAA), Mrc1(1–781), Mrc1(225–781), Mrc1(396–781), or Mrc1(536–781) in Δ*mrc1* cells. Immunoprecipitation with anti-Flag antibody showed that Mrc1(781–1019) was coimmunoprecipitated with Mrc1-degSSAA, Mrc1(1–781), Mrc1(225–781), or Mrc1(396–781) (Fig. 3C, lanes 8 to 11) but was not coimmunoprecipitated with Mrc1(536–781) or in the absence of the Flag-tagged polypeptide (Fig. 3C, lanes 7 and 12). We expressed and purified the Mrc1ΔHBS, Mrc1(1–781), Mrc1(225–781),

FIG 1 Legend (Continued)
are boxed. They are stretches of acidic residues, the KAF (lysine-alanine-phenylalanine) motif and the phosphodegron previously shown to be phosphorylated by Hsk1 kinase and to trigger the proteasome-mediated degradation of Mrc1. Sp, *Schizosaccharomyces pombe* (aa 782 to 879 of Mrc1); Sj, *Schizosaccharomyces japonicus* (aa 703 to 802 of Mrc1); Sc, *Saccharomyces cerevisiae* (aa 792 to 892 of Mrc1); Dh, *Debaryomyces hansenii* CBS767 (aa 957 to 1057 of DEHA2E14190p); Ac, *Arthroderma otae* CBS 113480 (aa 939 to 1040 of a hypothetical protein); Kl, *Kluyveromyces lactis* NRRL Y-1140 (aa 693 to 788 of a hypothetical protein); Ss, *Scheffersomyces stipitis* CBS 6054 (aa 884 to 931 of a hypothetical protein); Cp, *Candida parapsilosis* (aa 890 to 946 of a hypothetical protein); Tv, *Trypanosoma vivax* Y486 (aa 27 to 80 of a putative ATP binding protein-like protein). (C) Fivefold serial dilutions of exponentially growing WT, *hsk1-89* (KO147), *hsk1-89* Δ*mrc1* (MS317), *hsk1-89 mrc1*Δ(*782–879*) (MS567), and *hsk1-89 mrc1*Δ(*782–846*) (MS571) cells were spotted onto YES agar and incubated for 5 days at 25, 30, or 37°C. The *hsk1-89* mutant grows at 37°C, since high temperature bypasses the requirement of Hsk1 kinase for growth (11). (D) Schematic representation of structures and phenotypes of the *mrc1*Δ(*782–879*) and *mrc1*Δ(*782–846*) mutants. The checkpoint was evaluated by HU sensitivity. Δ*HBS* mutants are resistant to HU but can bypass an *hsk1* mutation. (E) The Δ*HBS* mutant can restore the growth of the Δ*hsk1* mutant. Fivefold serial dilutions of Δ*hsk1* cells with the indicated *mrc1* mutations carrying pREP2*hsk1*wt (*ura4*$^+$ marker) were spotted onto SD agar plates containing Ade, Leu, and Ura (ALU) with or without 5-FOA (1 mg/ml). Plates were incubated at 30°C for 6 or 10 days. (F) Fivefold serial dilutions of exponentially growing WT, Δ*mrc1*, *mrc1-3A* (KT1398), *mrc1*Δ(*782–879*) (MS565), *mrc1*[*3A*+Δ(*782–879*)] (MS663), *hsk1-89*, *hsk1-89* Δ*mrc1*, *hsk1-89 mrc1-3A* (HM258), *hsk1-89 mrc1*Δ(*782–879*), and *hsk1-89 mrc1*[*3A*+Δ(*782-879*)] (MS667) cells were spotted onto YES agar plates containing 0, 3, or 6 mM HU. Plates without HU were incubated for 5 days at 25 or 30°C. Plates with HU were incubated for 10 days at 25°C. The Δ*HBS* and *mrc1-3A* mutants bypass *hsk1* less efficiently than does the Δ*mrc1* mutant, and the combination of the two mutations resulted in more efficient bypass, suggesting that the two mutations bypass *hsk1* in independent pathways.

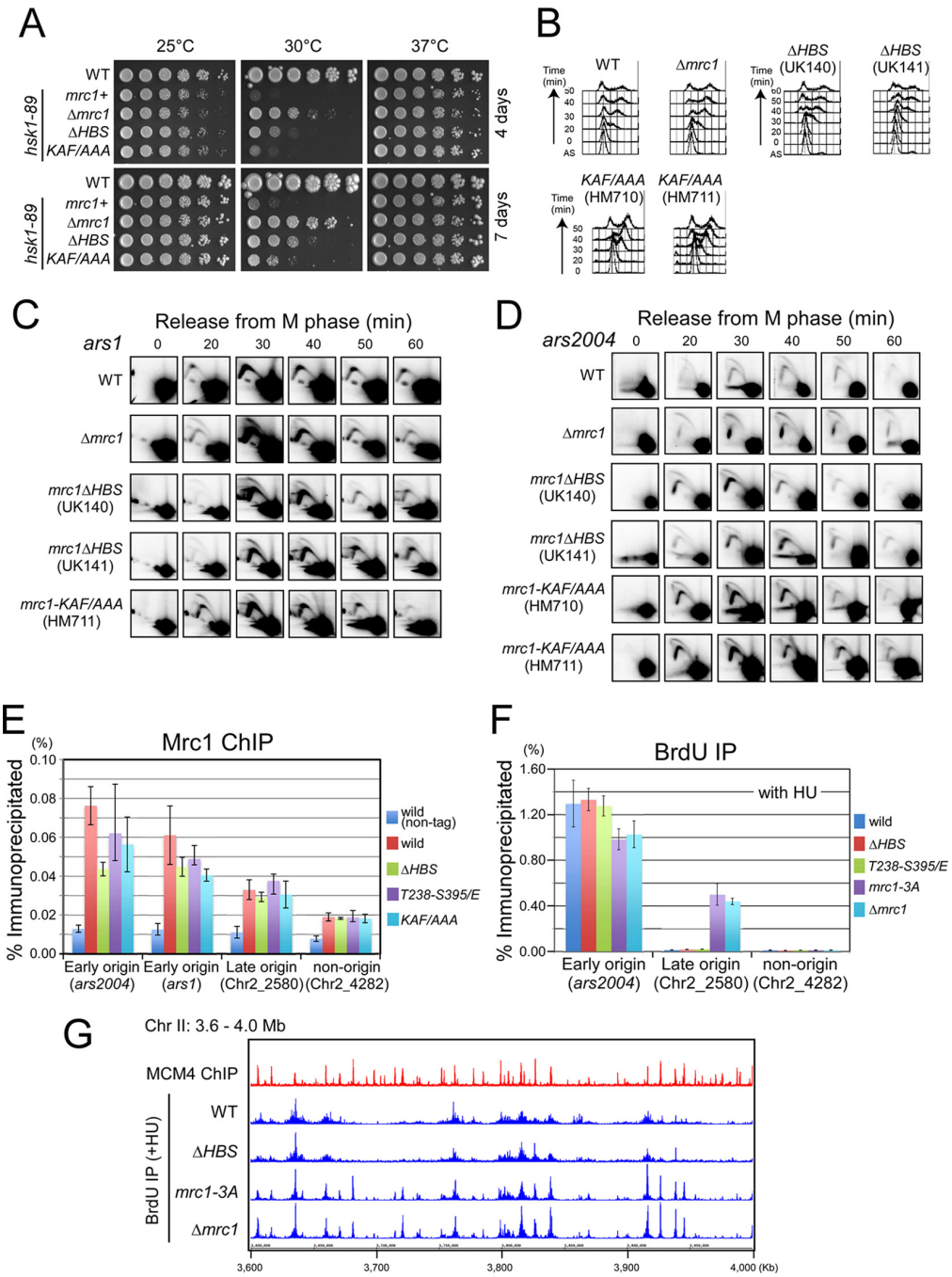

FIG 2 Early-firing origins but not late-firing origins are affected in HBS mutants of *mrc1*. (A) Fivefold serial dilutions of exponentially growing WT, *hsk1-89*, *hsk1-89* Δ*mrc1*, *hsk1-89 mrc1*Δ*HBS*, and *hsk1-89 mrc1-KAF/AAA* cells were spotted onto YES agar and incubated for 4 days or 7 days at 25, 30, or 37°C. (B) Strains with the indicated *mrc1* genotypes were arrested at M phase (by *nda3-KM311*) and released into the cell cycle at 30°C. Cells were harvested at the indicated time points, and DNA contents of the cells were analyzed by FACS analysis. (C and D) 2D electrophoresis analysis of replication intermediates prepared from the cells in panel B at two early-firing origins, *ars1* (weak origin) (C) and *ars2004* (strong origin) (D). Data for WT and Δ*mrc1* cells at *ars2004* were described in a previous study (see Fig. 5 in reference 11), which was conducted in the same series of experiments shown here. In *mrc1* mutants, fork movement appears to be slowed down within the EcoT22I fragment examined (for *ars2004*), causing an accumulation of a radioactive signal in the last part of the Y arc. This also causes the incoming fork outside this fragment to collide with the outbound replication fork, leading to the appearance of a cone signal. (E) Strains with the indicated *mrc1* genotypes were arrested at M phase (by *nda3-KM311*) and released. Twenty minutes after release, the cells were fixed and harvested. Mrc1 was chromatin immunoprecipitated, and the IP efficiency (relative to the input genomic DNA) was assessed by quantitative real-time PCR (means ± standard errors of the means from three independent experiments). (F) Strains with the indicated *mrc1* genotypes were arrested at M phase (by *nda3-KM311*) and released into the

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Mrc1(377–781), Mrc1(396–781), and Mrc1(536–781) polypeptides from 239T cells and examined their interactions with Mrc1(782–879) *in vitro*. Consistent with the *in vivo* results, the HBS polypeptide was coimmunoprecipitated with Flag-tagged Mrc1ΔHBS, Mrc1(1–781), Mrc1(225–781), Mrc1(377–781), and Mrc1(396–781), while it was not coimmunoprecipitated with Mrc1(536–781) very efficiently (Fig. 3D). These results establish that HBS of Mrc1 directly interacts with the N-terminal segment of Mrc1 (designated NTHBS), most likely through intramolecular interactions. An intermolecular interaction of HBS and NTHBS is possible but unlikely, since Flag-tagged Mrc1 and Myc-tagged Mrc1 coexpressed in fission yeast cells did not coimmunoprecipitate (Fig. 3E). The results described above also indicate that aa 396 to 535 are an essential segment of NTHBS for binding to HBS. Mrc1(377–879), but not Mrc1(225–879), is able to bypass Hsk1 (Fig. 3), although the HBS-NTHBS interaction is likely to be maintained at aa 377 to 879. These results suggest that aa 225 to 376, in addition to HBS, are required for checkpoint-independent origin regulation by stabilizing the brake-on conformation.

Expression of HBS in *mrc1ΔHBS* cells leads to growth inhibition of *hsk1*. Next, the question was whether the HBS-NTHBS interaction is functionally related to the bypass of *hsk1*. We first postulated that Mrc1ΔHBS is a brake-off form that is able to bypass *hsk1*, whereas the presence of HBS, through its interaction with NTHBS, converts Mrc1 to a brake-on form that is not able to bypass *hsk1*. Thus, we next examined whether the addition of the C-terminal polypeptide containing HBS in *trans* can convert the brake-off form to the brake-on form. To test this model, we expressed full-length Mrc1 or Mrc1 C-terminal polypeptides (aa 781 to 879, 781 to 1019, and 880 to 1019) in *hsk1-89 mrc1ΔHBS* cells, and the effect on growth at 30°C was examined by a colony-forming assay (Fig. 4A). Growth suppression indicates the conversion of the brake-off form into the brake-on form. The expression of full-length Mrc1 suppressed the bypass, resulting in almost zero relative CFU at 30°C, as expected (Mrc1FL) (Fig. 4B). Whereas Mrc1(880–1019) lacking HBS showed only a slight reduction in the number of CFU compared to the vector, a notable reduction of the number of CFU was observed with Mrc1(781–879) and Mrc1(781–1019) (Fig. 4B, bottom). In contrast, the expression of the same C-terminal polypeptides in *hsk1-89* Δ*mrc1* cells showed only a subtle effect on CFU (Fig. 4C, top). In addition to the reduction of CFU, sizes of colonies appearing in these assays were substantially different. Small colonies appeared in cells expressing Mrc1(781–879) and Mrc1(781–1019) compared to those appearing in cells expressing Mrc1(880–1019) (Fig. 4C). These results support the idea that the Mrc1 HBS-NTHBS interaction converts the brake-off form into the brake-on form, although we cannot exclude the possibility that the titration of different factors by expressed polypeptides plays a role in these assays. Growth inhibition was more potent with Mrc1(781–1019) than with Mrc1(781–879), suggesting that, in addition to HBS, the C-terminal 140 amino acids may also contribute to the intramolecular interaction.

Binding of Mrc1 to Hsk1 requires both HBS and NTHBS. We previously reported that Mrc1 interacts with Hsk1 kinase (25) and that it is efficiently phosphorylated *in vitro* by this kinase (18). To determine the Mrc1 segment required for the interaction with Hsk1, we first examined the interaction of Mrc1(157–781) and Mrc1(157–879) with Hsk1, since the former but not the latter can suppress *hsk1-89* (11). Coimmunoprecipitation assays show that Mrc1(157–879) binds to Hsk1 but that Mrc1(157–781) does not,

FIG 2 Legend (Continued)
cell cycle in the presence of 25 mM HU and 200 μg/ml BrdU. Sixty minutes after release, cells were fixed, genomic DNA was extracted, and BrdU-incorporating DNA was immunoprecipitated. The IP efficiency (relative to the input genomic DNA) was assessed by quantitative real-time PCR (means ± standard errors of the means from three independent experiments). *ars2004* is the early-firing origin, and *Chr2_2580* is a late/dormant origin that fires in the presence of HU in checkpoint-deficient cells but not in wild-type cells. Chr2_4282 is a nonorigin sequence. A late/dormant origin is fired in the presence of HU in *mrc1-3A* (checkpoint defective) but not in *mrc1ΔHBS* and *mrc1*(*T238-S395/E*) cells. (G) ChIP-seq data (vertical axes show pileup-aligned read distributions) of the Mcm4 binding site (red) and incorporation of BrdU (blue) in HU-treated wild-type, *mrc1ΔHBS*, *mrc1-3A*, and Δ*mrc1* cells at around the Mb 3.6 to 4.0 coordinate on chromosome II. BrdU patterns are identical between the wild type and the Δ*HBS* mutant, whereas late/dormant origins are fired in the *mrc1-3A* and Δ*mrc1* mutants.

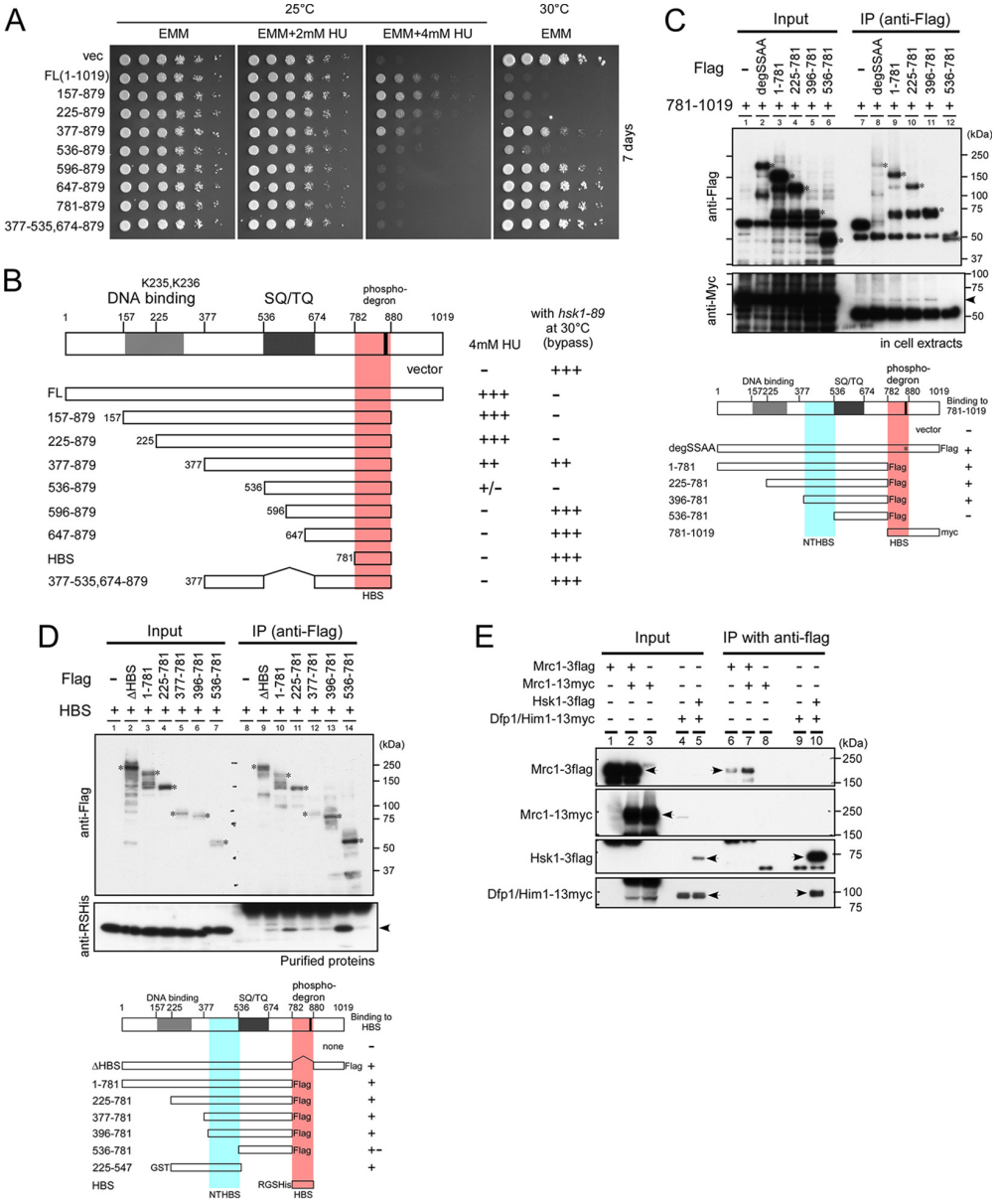

FIG 3 Physical interaction between the Mrc1 N-terminal segment and the C-terminal segment containing HBS is involved in the bypass of *hsk1*. (A) Fivefold serial dilutions of exponentially growing *hsk1-89* Δ*mrc1* cells harboring pREP41 (vector [vec]), pREP41mrc1-3Flag (FL), or pREP41-3Flag-based plasmids expressing the indicated Mrc1 segments were spotted onto EMM containing 0, 2, or 4 mM HU and incubated for 7 days at 25 or 30°C, as indicated. (B) Summary of phenotypes of Mrc1 derivatives analyzed in panel A. +, ++, and +++ indicate the efficiency of growth. HBS is shown in red. (C) Cell extracts were prepared from Δ*mrc1* cells expressing Mrc1(781–1019)-13Myc and the indicated Flag-tagged Mrc1 segments. Immunoprecipitation was conducted with anti-Flag antibody, and coimmunoprecipitation of the C-terminal polypeptide (aa 781 to 1019) was examined by Western analyses using anti-cMyc antibody (bottom). The Flag-tagged Mrc1 segments expressed were Mrc1-degSSAA (aa 1 to 1019) (lanes 2 and 8), Mrc1(1–781) (lanes 3 and 9), Mrc1(225–781) (lanes 4 and 10), Mrc1(396–781) (lanes 5 and 11), and Mrc1(536–781) (lanes 6 and 12). Lanes 1 and 7, empty vector pREP41. The transferred membrane was first blotted with anti-cMyc antibody and then blotted with anti-Flag antibody. (D) Immunoprecipitation with purified proteins. RGS-His-tagged Mrc1(782–879) expressed and purified from *E. coli* was mixed with Flag-tagged Mrc1ΔHBS, Mrc1(1–781), Mrc1(225–781), Mrc1(377–781), Mrc1(396–781), or Mrc1(536–781) expressed and purified from mammalian cells, and immunoprecipitation was conducted with anti-Flag antibody. The transferred membrane was blotted first with anti-RGS-His antibody and then with anti-Flag antibody. (E) Cell extracts were prepared from Δ*mrc1* cells expressing Mrc1-13Myc and/or Mrc1-3Flag, immunoprecipitation was conducted with anti-Flag antibody, and coimmunoprecipitation of Mrc1-13Myc was examined by Western analyses using anti-cMyc antibody as described above for panel C (lanes 6 to 8). Cell extracts from *him1/dfp1-13myc* and *him1/dfp1-13myc hsk1-3Flag* cells were used as a control for coimmunoprecipitation (lanes 9 and 10). Him1/Dfp1 is coimmunoprecipitated with Hsk1. In panels C and D, Flag-tagged Mrc1 polypeptides and HBS-containing polypeptides are indicated by asterisks and arrowheads, respectively.

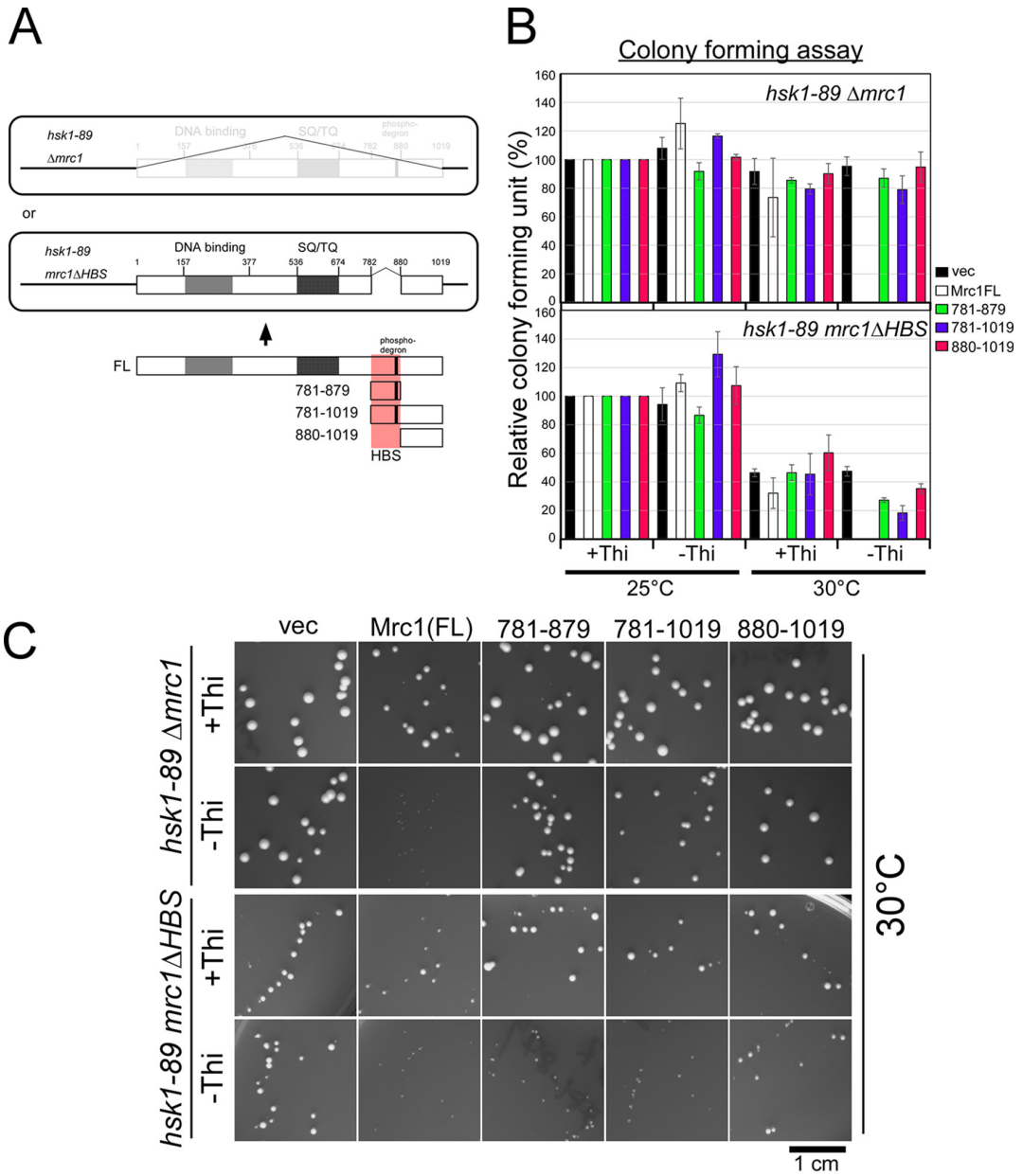

FIG 4 C-terminal polypeptides containing HBS inhibit growth of *hsk1 mrc1ΔHBS* cells. (A) Schematic representation of the full-length Mrc1 or Mrc1-C polypeptides that were expressed in *hsk1-89 Δmrc1* or *hsk1-89 mrc1ΔHBS* cells. (B) *hsk1-89 Δmrc1* (top) or *hsk1-89 mrc1ΔHBS* (bottom) cells harboring various plasmids (expressing the full-length polypeptide or the polypeptide spanning aa 781 to 879, 781 to 1019, or 880 to 1019) were spread onto EMM with or without thiamine and incubated at 25 or 30°C. Colonies were counted after 9 or 10 days. The number of colonies was normalized to that on plates (with thiamine) that were incubated at 25°C for each strain, and the values are expressed as relative CFU (percent). Error bars indicate the standard errors of the means between three independent experiments. (C) Colonies appearing after a 7-day incubation at 30°C. Plasmids used were pREP41 (vector), pREP41mrc1(FL)-3Flag, pREP41mrc1(781-879)-3Flag, pREP41mrc1(781-1019)-3Flag, and pREP41mrc1(880-1019)-3Flag. The sizes of the colonies of *hsk1-89 mrc1ΔHBS* cells carrying pREP41mrc1(781-879)-3Flag or pREP41mrc1(781-1019)-3Flag (in the absence of thiamine) are much smaller than those carrying pREP41mrc1(880-1019)-3Flag or those of *hsk1-89 Δmrc1* cells.

suggesting that HBS (aa 782 to 879) is required for binding to Hsk1 (Fig. 5A). This was confirmed by showing that the internal deletion of aa 782 to 879 led to a loss of Hsk1 binding (Fig. 5B). However, further deletion analyses of Mrc1 indicate that HBS is not sufficient for Hsk1 binding (Fig. 5C to E and data not shown). Mrc1(377–879) retains the ability to bind to Hsk1, but the deletion of another 159 amino acids resulted in

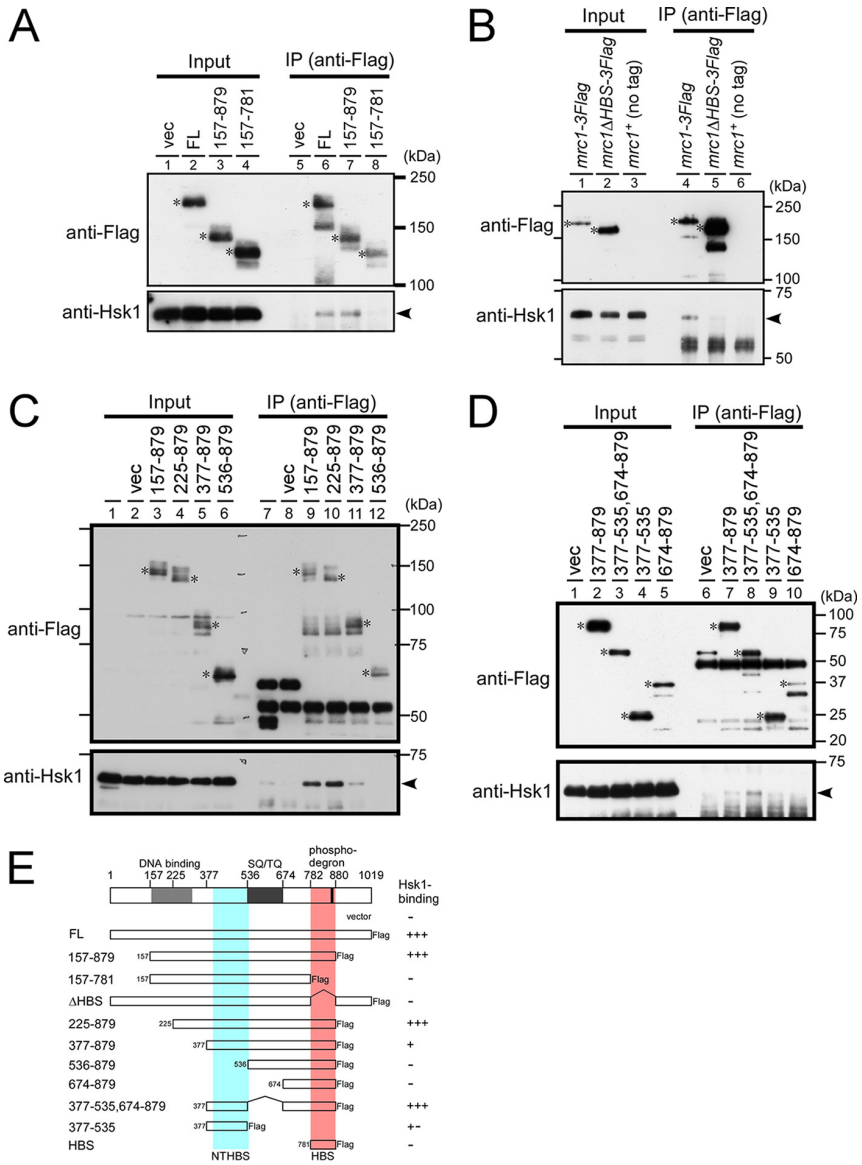

FIG 5 Interaction of Hsk1 with Mrc1 requires HBS and NTHBS. (A, C, and D) Various polypeptides of Mrc1 fused to a Flag tag at the C terminus were expressed in WT cells (YM71). (B) *mrc1-3Flag*, *mrc1ΔHBS-3Flag*, and *mrc1*$^{+}$ (no tag) cells. Immunoprecipitates were prepared from whole-cell extracts with anti-Flag antibody and analyzed by Western blotting with anti-Flag or anti-Hsk1 antibody. "377–535, 674–879" in panel D indicates the fusion of the two segments. Inputs were 10% (A and B) or 5% (C and D) of the extracts used for immunoprecipitation. Flag-tagged Mrc1 segments and Hsk1 are labeled by asterisks and arrowheads, respectively. (E) Summary of the results. HBS and NTHBS (aa 377 to 535) are required for efficient binding to Hsk1.

a loss of Hsk1 binding. Interestingly, a fusion of the two Mrc1 segments spanning aa 377 to 535 and aa 674 to 879 containing HBS binds to Hsk1 with high efficiency (Fig. 5D and E).

Hsk1-mediated phosphorylation of Mrc1 inhibits the HBS-NTHBS interaction and converts it to a brake-off form. The results presented above suggest that the HBS-NTHBS intramolecular interaction regulates the activity of Mrc1. This interaction is mediated by the C-terminal segment containing HBS and the central segment containing NTHBS (aa 396 to 535), through which Hsk1 kinase can bind to Mrc1. Questions arise as to how the HBS-NTHBS interaction is regulated by Hsk1 kinase. The Mrc1 protein was

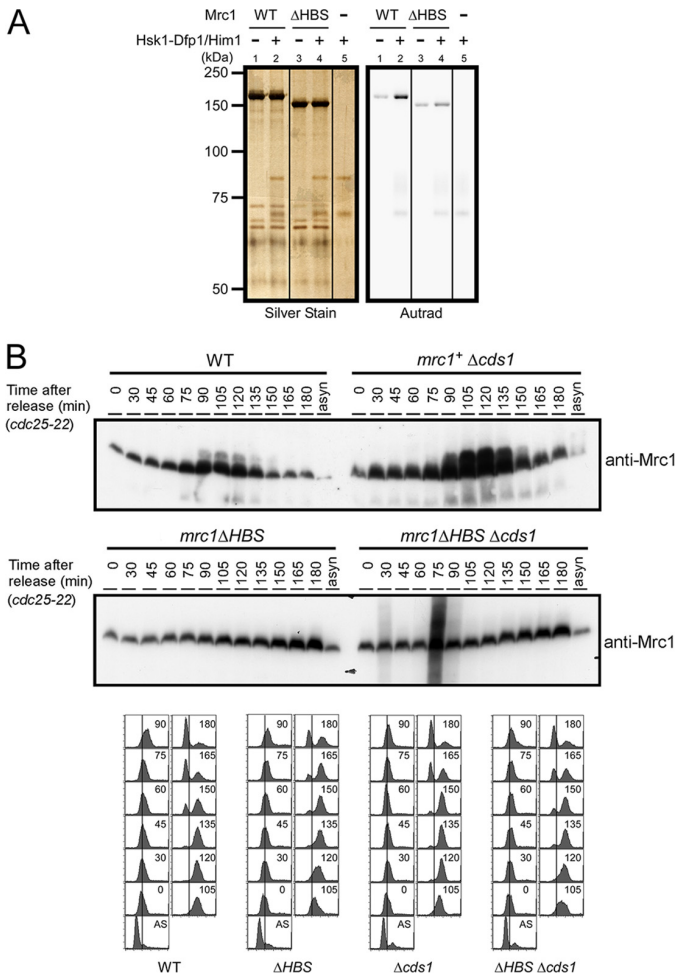

FIG 6 HBS is required for robust phosphorylation of Mrc1 *in vitro* and *in vivo*. (A) *In vitro* phosphorylation of the WT and the ΔHBS mutant of Mrc1 by the Hsk1-Dfp1/Him1 kinase complex. The wild-type and ΔHBS Mrc1 proteins expressed and purified from mammalian cells were phosphorylated *in vitro* by the Hsk1-Dfp1/Him1 kinase complex purified from insect cells under the conditions described in Materials and Methods. Proteins were run on 8% SDS-PAGE gels, stained with silver (left), dried, and autoradiographed (right). (B) *mrc1*$^{+}$, Δ*cds1*, *mrc1ΔHBS*, and *mrc1ΔHBS* Δ*cds1* cells were arrested at M phase (by *cdc25-22*) and released into the cell cycle. At the indicated times after release, aliquots of cells were harvested, and whole-cell extracts were analyzed by Western blotting using anti-Mrc1 antibody. A mobility shift of Mrc1, caused by phosphorylation, is observed during S phase in wild-type cells but not in Δ*HBS* mutant cells. The extent of phosphorylation of wild-type Mrc1 increases in the Δ*cds1* mutant (23), but a mobility shift of Δ*HBS* is not observed, even in the Δ*cds1* mutant. Bottom panels show FACS profiles for each cell. The rates of cell cycle progression were similar among the four cell types.

vigorously phosphorylated by Hsk1-Dfp1/Him1 kinase *in vitro* (18), whereas Mrc1Δ*HBS* was only poorly phosphorylated (Fig. 6A). During unperturbed growth, Mrc1 is phosphorylated during S phase (as indicated by a specific mobility shift), and this phosphorylation is dependent on HBS (Fig. 6B). These results indicate that the recruitment of Hsk1 to Mrc1 through HBS is required for efficient Hsk1-mediated phosphorylation of Mrc1.

The fission yeast Mrc1 sequence possesses many serine/threonine residues surrounded by acidic residues (glutamic acid or aspartic acid), which may be favored by Cdc7/Hsk1 kinase (26, 27). We speculate that the Hsk1-mediated phosphorylation of Mrc1 may somehow disrupt the intramolecular HBS-NTHBS interaction and that the protein is converted to the brake-off form. If this is the case, a phosphorylation mimic mutation of the key serine/threonine residues of Mrc1 could reduce the HBS-NTHBS

interaction, resulting in conversion to a brake-off form and suppressing the *hsk1* mutant. Although HBS is a prime candidate for phosphorylation, an *HBS*(*A*) or *HBS*(*E*) mutant, in which all the serine and threonine residues in HBS (except for the three serines in the phosphodegron) were converted to alanines or glutamic acids, respectively, showed no detectable rescue of *hsk1-89*, and the mutant proteins exhibited an Hsk1-depndent mobility shift (data not shown), leading us to conclude that the major target sequences of Hsk1 may lie somewhere else in the Mrc1 polypeptide. Therefore, we selected four different segments of the Mrc1 N-terminal region (aa 76 to 199, aa 238 to 395, aa 409 to 537, or aa 541 to 754) and replaced all the serine/threonine residues with glutamic acid in each segment separately. All the ST/E mutants showed normal growth at 25°C, showing that they function properly during the unperturbed S phase. In the presence of HU, the S76-S199/E, T238-S395/E (SE), and S409-S537/E mutants were resistant to HU, whereas the S541-S754/E mutant was significantly more sensitive than the wild type (Fig. 7A). aa 541 to 754 overlap the SQ/TQ-rich segment, which is a target of Rad3. The ST/E substitutions in the S541-S754/E mutant do not include SQ/TQ residues, but the glutamic acid substitutions near the Rad3 target sites may cause an adverse effect on checkpoint responses. The Δ*HBS* mutant was significantly more resistant to HU in the presence of thiamine. This is probably due to a higher level of the protein caused by a loss of the degron signal present in the deleted HBS (Fig. 8B).

Among the four mutants, only the T238-S395/E mutant supported the growth of *hsk-89* cells at 30°C (Fig. 7A). We constructed a strain in which the genomic *mrc1*$^{+}$ gene was replaced by this *mrc1*(*T238-S395/E*) mutant and confirmed that the T238-S395/E mutant rescues the growth of Δ*hsk1* cells (Fig. 7B). Late origin firing in the presence of HU was completely suppressed in the T238-S395/E mutant (Fig. 2F), indicating that the DNA replication checkpoint (intra-S checkpoint) is intact in this mutant. Thus, it is likely that the T238-S395/E mutant is defective in the checkpoint-independent suppression of early origin firing.

These results also suggest that the polypeptide spanning aa 238 to 395 contains key serine/threonine residues whose phosphorylation may induce the dissociation of HBS and NTHBS of Mrc1. Consistent with this speculation, the SE mutant polypeptide of Mrc1(224–547) interacted poorly with HBS (aa 782 to 879) *in vitro* compared to the wild type or the T238-S395/A (SA) mutant of Mrc1(224–547) (Fig. 7C). DNA binding activity was not affected in the T238-S395/E mutant, as indicated by ChIP analyses (Fig. 2E) and *in vitro* DNA binding assays (Fig. 8C and D). The polypeptide spanning aa 225 to 547 was phosphorylated by Hsk1 *in vitro*, and this phosphorylation was largely eliminated with the T238-S395/A mutant version of the same polypeptide (Fig. 8E and F), indicating that the mutated serine/threonine residues constitute targets of Hsk1 kinase. These *in vitro* and *in vivo* results support the idea that Mrc1 changes the conformation through the NTHBS-HBS intramolecular interaction and that this interaction is regulated by Hsk1 kinase. Crucial roles of phosphorylation at aa 238 to 395 are consistent with the requirement of aa 225 to 376 for Mrc1 to adopt a brake-on form. The loss of HBS or of aa 225 to 376 or phosphomimic mutations in the latter segment convert Mrc1 to a brake-off form in the absence of Hsk1, which would result in the precocious activation of early-firing origins and the bypass of Hsk1.

DISCUSSION

In addition to its well-known role in the DNA replication checkpoint as an adaptor protein, Mrc1 has been known to have checkpoint-independent functions. It has been implicated in efficient replication fork progression (28–30). It was also reported that the checkpoint-independent function of Mrc1 is involved in the scaling of origin activation in slowed S-phase progression (19, 20). We previously reported the possibility that Mrc1 regulates the firing of some early-firing origins in a checkpoint-independent manner (22). However, the precise mechanisms of the checkpoint-independent functions of Mrc1 in origin regulation have been unclear.

The new results reported here provide novel insights into and mechanisms of origin firing regulation during unperturbed growth and present strong evidence for a central

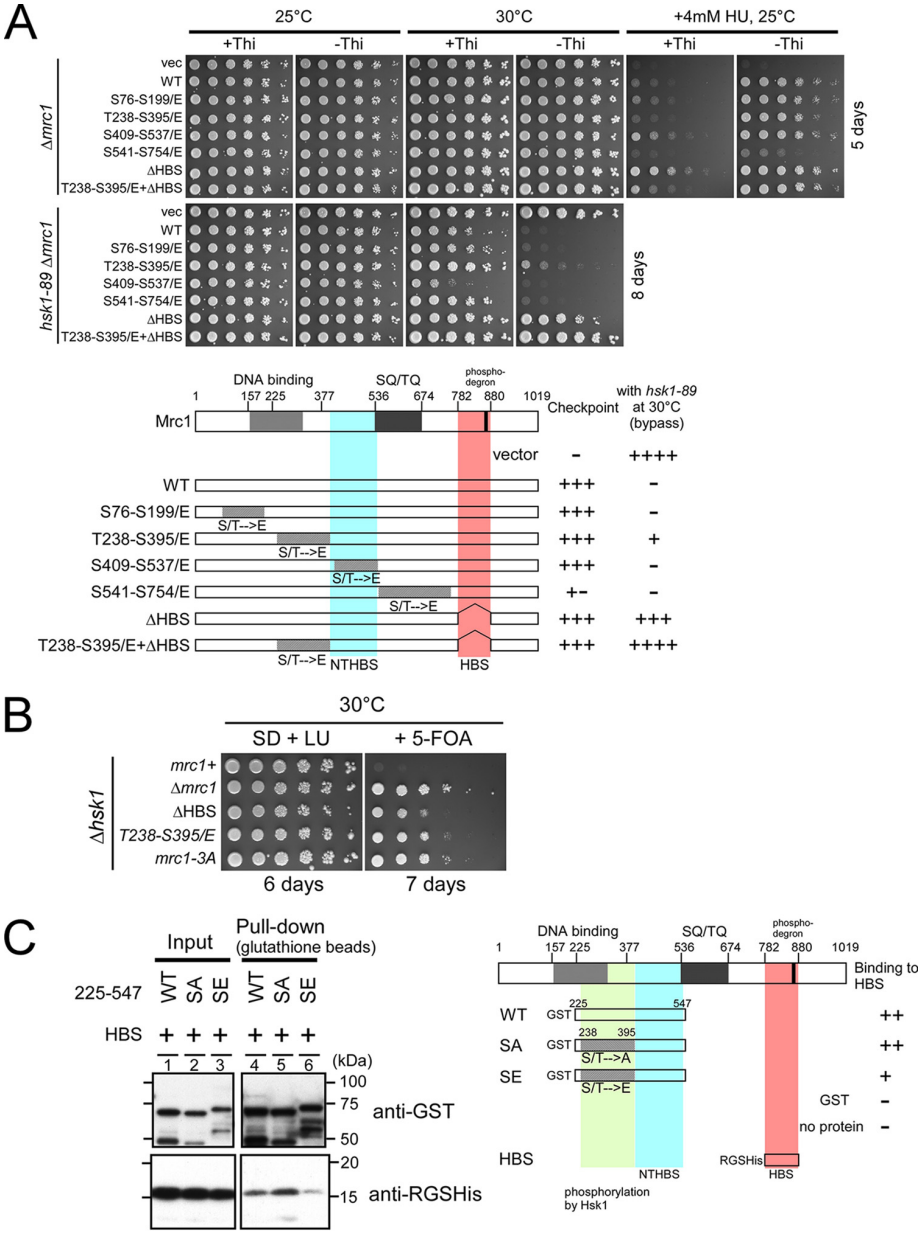

FIG 7 A phosphorylation mimic mutant of NTHBS bypasses *hsk1* and exhibits a reduced interaction with HBS. (A) Fivefold serial dilutions of exponentially growing Δ*mrc1* (top) and *hsk1-89* Δ*mrc1* (bottom) cells harboring plasmids expressing wild-type Mrc1 or various mutant forms of Mrc1, as shown, were spotted onto EMM with or without thiamine containing 0 or 4 mM HU and incubated at 25 or 30°C for 5 or 8 days. (B) Fivefold serial dilutions of Δ*hsk1* cells with the indicated *mrc1* mutations on the chromosome carrying pREP2*hsk1*wt (*ura4*$^+$ marker) were spotted onto SD agar plates containing Leu and Ura (LU) with or without 5-FOA (1 mg/ml). Plates were incubated at 30°C for 6 or 7 days. (C) Purified RGS-His-tagged Mrc1(782–879) (HBS) was mixed with GST or GST-tagged Mrc1(225–547) (N-terminal segment [WT and SA and SE mutants]), pulled down with glutathione beads, and analyzed by Western blotting using anti-GST or anti-RGS-His antibody. SA, T238-S395/A; SE, T238-S395/E. Red, blue, and green segments represent HBS, NTHBS, and the Hsk1 target region containing the serine/threonine residues important for the checkpoint-independent function of Mrc1, respectively.

role of Mrc1-Cdc7 in this regulation. While we previously reported that Rif1 plays a major role in regulating late/dormant origin firing (7, 14), the regulation of early origin firing has been elusive. The findings in this report shed light on this unanswered question and bear general significance in understanding the complete pictures of origin regulation in eukaryotic cells.

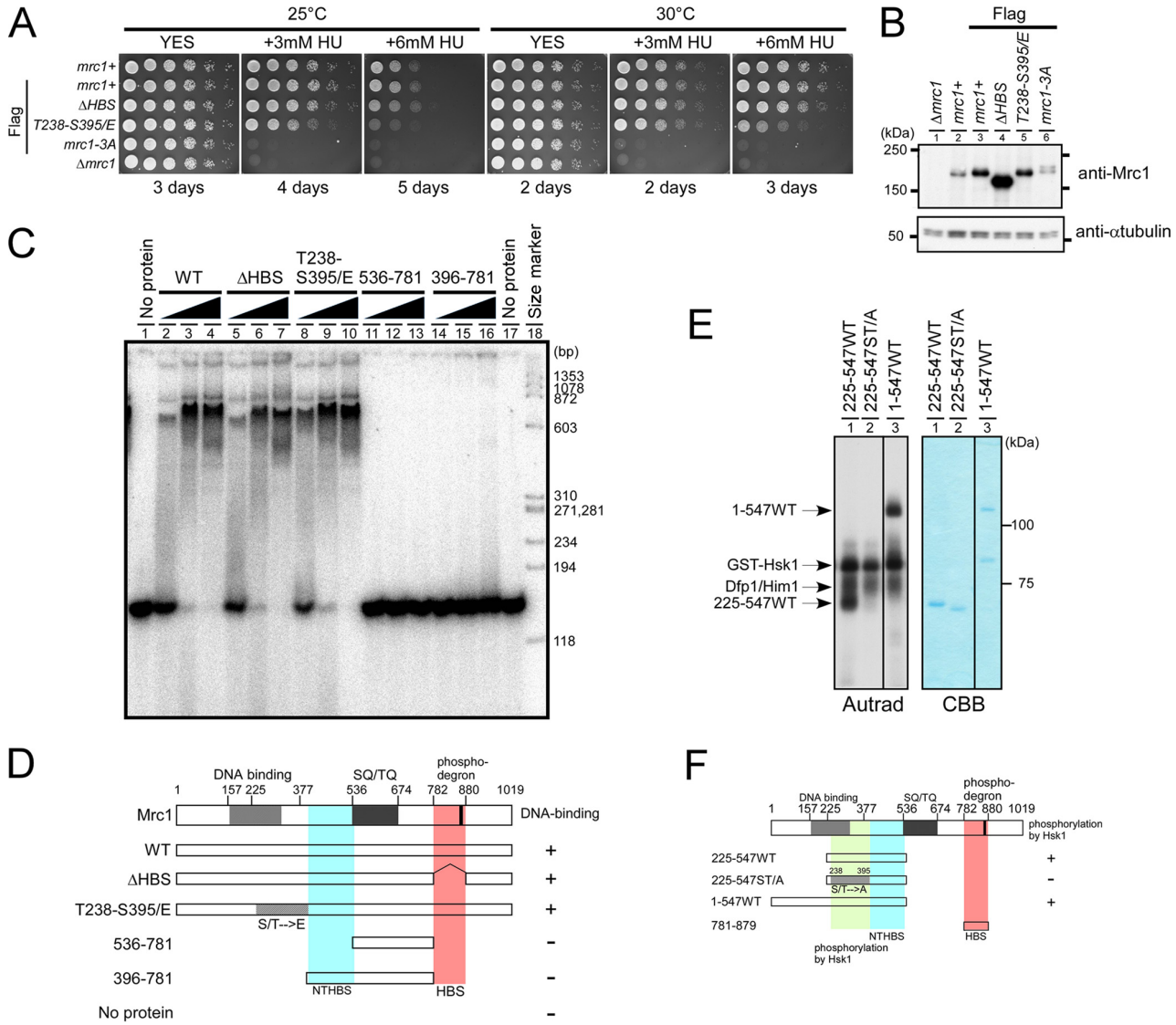

FIG 8 *mrc1*ΔHBS and ST/E mutants are resistant to HU and bind to DNA, and the ST/A mutant is not phosphorylated by Hsk1 kinase. (A) Fivefold serial dilutions of exponentially growing cells with the indicated *mrc1* genotypes were spotted onto YES agar containing 0, 3, or 6 mM HU. Plates were incubated at 25 or 30°C for 2 to 5 days. (B) Whole-cell extracts were prepared from exponentially growing cells used for the experiment shown in panel A and analyzed by Western blotting using anti-Mrc1 or anti-α-tubulin antibody. (C) The wild type and the ΔHBS and T238-S395/E mutants of Mrc1, N-terminally fused with RGS-His, were expressed and purified from *E. coli* cells. Mrc1 polypeptides (aa 536 to 781 and aa 396 to 781), N-terminally fused with 6×His and C-terminally fused with 3×Flag, were expressed and purified from 293T cells. Gel shift assays were conducted with 2 nM ^{32}P-labeled Y-fork DNA and increasing amounts of each protein (45, 90, and 180 nM), as described previously (42). Lanes 1 and 17, no protein added. Samples were run on an 8% polyacrylamide gel (29:1) containing 10% glycerol (1× Tris-borate-EDTA [TBE]). (D) Schematic drawing of the mutant Mrc1 proteins used for the gel shift assay depicted in panel C and summary of the results. (E) The wild-type and ST/A versions of the polypeptide (aa 225 to 547) and the wild-type polypeptide (aa 1 to 547), N-terminally fused with 6×His and C-terminally fused with 3×Flag, were expressed and purified from 293T cells and used as the substrates for *in vitro* kinase assays with the GST-Hsk1–Him1 kinase complex expressed and purified from insect cells. The reaction mix also contained an RGS-His-HBS polypeptide (aa 781 to 879) to facilitate the recruitment of Hsk1 kinase. Samples were analyzed on 7.5% (29:1) SDS-PAGE gels, stained with Coomassie brilliant blue (CBB) (right), dried, and autoradiographed (Autrad) (left). RGS-His-HBS ran off the gel. (F) Schematic drawing of the mutant Mrc1 proteins used for the kinase assays in panel E. For panels D and F, red, blue, and green segments are described in the Fig. 7 legend.

HBS, a segment essential for checkpoint-independent regulation of early-firing origins. We reported previously that Δ*mrc1* as well as other mutations and physiological conditions can bypass the requirement of Hsk1 for DNA replication (7, 11) and suggested a possibility that this reflects the altered regulation of origin firing. On the basis of analyses of both Δ*mrc1* and *mrc1-3A* (checkpoint-specific) mutants, we suggested the presence of a checkpoint-independent bypass pathway. Here we have uti-

lized the *hsk1* bypass system as a readout for altered origin regulation and have identified a segment, designated HBS, on Mrc1, the deletion of which leads to the checkpoint-independent rescue of Δ*hsk1*. In HBS mutants, early-firing origins are precociously fired during the normal progression of S phase, while late/dormant origins are not activated in the presence of HU (Fig. 2). In contrast, precocious firing of early-firing origins is not observed in an *mrc1* checkpoint mutant (22). Checkpoint-dependent and -independent pathways function additively for the bypass of *hsk1* (Fig. 1F), indicating that they are two separate pathways (nonepistatic).

Intramolecular interactions may regulate the function of Mrc1. We discovered that HBS (located near the C terminus) physically interacts with the N-terminal segment (NTHBS) of Mrc1. Since an intermolecular interaction of Mrc1 was not observed *in vivo* (Fig. 3E) and the purified Mrc1 protein behaves as a monomer (data not shown), this interaction appears to occur in an intramolecular manner and regulates the function of Mrc1. Exogenously expressed HBS can antagonize the bypass mediated by *mrc1ΔHBS* (Fig. 4). This is probably through the interaction of HBS with NTHBS. We postulated that Mrc1 interchanges between brake-on (e.g., full-length protein) and brake-off (e.g., Δ*HBS* mutant) forms through the intramolecular interaction. A loss of the interaction would convert the former to the latter (Fig. 9A).

Hsk1-mediated phosphorylation regulates the conversion between the brake-on and brake-off forms. The Δ*HBS* mutant bypasses the requirement for Hsk1 kinase. Mrc1 is a substrate of Hsk1, and thus, the simplest interpretation is that the Hsk1-mediated phosphorylation of Mrc1 converts it to the brake-off form and that the Δ*HBS* mutant adopts a conformation that mimics the brake-off form without phosphorylation by Hsk1. HBS is also required for Mrc1 to interact with Hsk1 and to be efficiently phosphorylated. Thus, it could be inferred that the Hsk1-mediated phosphorylation of the N-terminal segment may regulate the interaction between HBS and NTHBS. Consistent with this prediction, glutamic acid substitutions of serine/threonine residues in a segment (aa 238 to 395) resulted in a reduced interaction of the polypeptide spanning aa 225 to 547 with HBS (Fig. 7C). The same mutation on full-length Mrc1 converted it to a brake-off form, permitting the bypass of *hsk1* (Fig. 7B). A glutathione *S*-transferase (GST)-fused polypeptide containing aa 225 to 547 of Mrc1 is phosphorylated *in vitro* by Hsk1 kinase, and this phosphorylation is largely lost by an SA mutation at aa 238 to 395 (Fig. 8E and F). These results support the idea that Hsk1-mediated phosphorylation of Mrc1 regulates the conversion from the brake-on to the brake-off conformation. Interestingly, the combination of the T238-S395/E and ΔHBS mutations resulted in an enhanced rescue of *hsk1-89* compared to the single mutation alone (Fig. 7A). Similar additive effects on bypass were observed with the Δ(*225–376*) and Δ*HBS* mutants (data not shown). These results indicate that the T238-S395 segment may play additional roles in regulating the Mrc1 function besides modulating the NTHBS-HBS interaction.

Recruitment of Cdc7 kinase to pre-RCs through Mrc1 may be important for ordered firing of origins. We also show here the possibility that Mrc1 plays a crucial role in recruiting Cdc7/Hsk1 kinase to the early-firing origins that have not been fired and in the ordered firing of early-firing origins. During the normal course of DNA replication, Hsk1 may be recruited to Mrc1, which is preferentially located on early-firing origins (22), phosphorylates its targets on Mrc1, and turns it into a brake-off form.

Mrc1 was reported previously to be required for the scaling of origin activation in budding yeast, and this role of Mrc1 was shown to be checkpoint independent (19, 20). We speculate that mechanisms involving the Mrc1-Hsk1 interaction may impose similar regulation of origin firing in fission yeast as well, since the Δ*HBS* mutant exhibits precocious firing of selective early-firing origins.

Unresolved questions are how Mrc1 is recruited preferentially to early-firing origins, how the brake-on form of Mrc1 prevents initiation from prematurely occurring at some early-firing origins, and what is the nature of the molecular transition from the brake-on to the brake-off form. Further biochemical studies on wild-type and mutant Mrc1

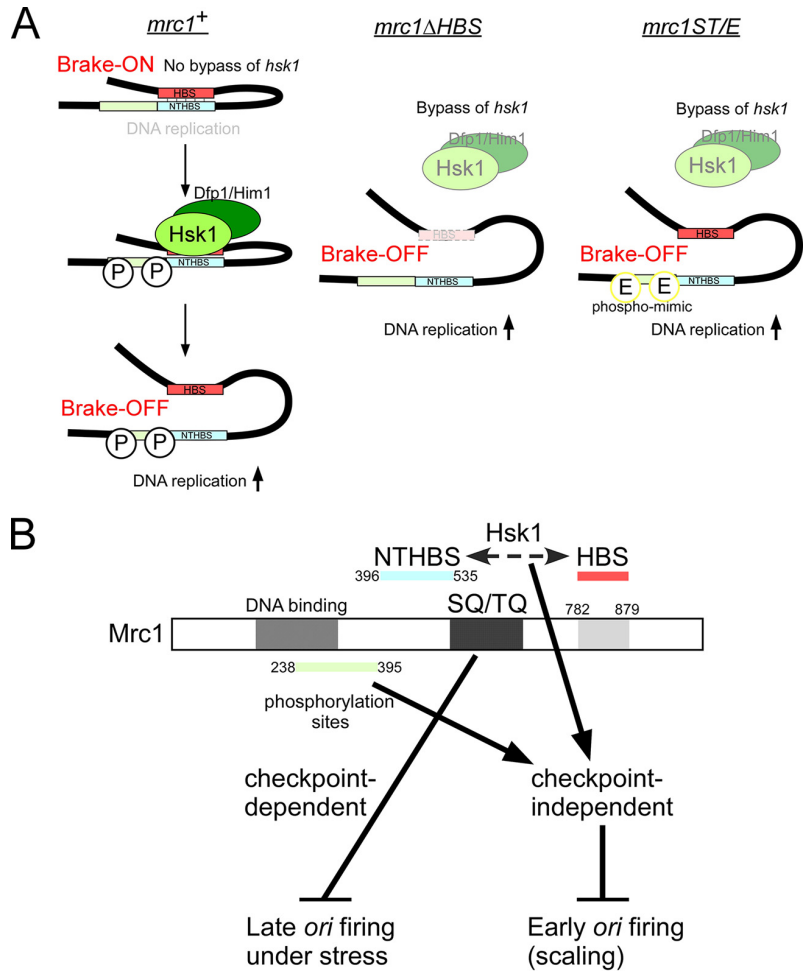

FIG 9 Summary and model of regulation of the Mrc1 protein through intramolecular interactions and Hsk1 kinase. (A) Model. Under *mrc1*$^{+}$ conditions, an intramolecular interaction between HBS (red) and NTHBS (blue) induces a brake-on conformation in wild-type Mrc1 that binds to early-firing origins and is transiently inhibitory for firing. Hsk1 is recruited to Mrc1 on chromatin through HBS and NTHBS and phosphorylates residues in the segment (green) adjacent to NTHBS. Phosphorylated Mrc1 is converted to a brake-off conformation, which is permissive for firing of the early origins. Under *mrc1ΔHBS* conditions, the lack of the HBS-NTHBS interaction constitutively induces the brake-off conformation, and precocious firing of some early-firing origins is induced. Under *mrc1ST/E* conditions, a phosphorylation mimic mutation (SE) in the key Ser/Thr residues of the segment adjacent to NTHBS (aa 238 to 395) reduces the HBS-NTHBS interaction, resulting in the brake-off conformation. Mrc1 in the constitutively brake-off conformation (*mrc1ΔHBS* and *mrc1ST/E*) can bypass the requirement of Hsk1 for DNA replication. (B) Mrc1 suppresses initiation of DNA replication in two independent pathways in fission yeast. It inhibits late origin firing in the presence of replication stress through the Rad3-dependent phosphorylation of SQ/TQ motifs. The HBS/NTHBS-mediated regulation of firing of early-firing origins may play a major role in the scaling of origin firing or in the determination of the DNA replication program during unperturbed S phase. HBS (aa 782 to 879) interacts with NTHBS (aa 396 to 535), and both segments are required for the interaction with Hsk1, which phosphorylates aa 238 to 395 (green). This phosphorylation as well as the intramolecular interaction between HBS and NTHBS could contribute to the checkpoint-independent regulation of early origin firing. The abrogation of either inhibitory pathway can lead to the bypass of Hsk1 function for DNA replication.

proteins using recently-developed *in vitro* replication systems (31–33) would facilitate the elucidation of detailed mechanisms of origin activation processes and their regulation.

Mrc1 is a central regulator of origin firing under normal and stressed conditions. Important roles of Mrc1 in the suppression of late/dormant origins through the checkpoint pathway are well established. Here we report a novel checkpoint-

independent pathway that regulates early-firing origins. The checkpoint pathway does not affect early-firing origins (22). Similarly, the checkpoint-independent pathway does not affect the firing of late/dormant origins (Fig. 2F and G). Thus, the two pathways are independent. Indeed, the two pathways also function additively in the bypass of *hsk1* (Fig. 1F). Mrc1, with these two crucial functions, can be placed at the center of origin firing regulation: one regulating late/dormant origins through its well-established checkpoint function and the other regulating early-firing origins through the checkpoint-independent mechanism that is discussed in this report (Fig. 9B). It is of interest whether this dual regulation of the origin firing program is also conserved in higher eukaryotes. This likely may be the case, since we recently reported that Claspin, the mammalian homologue of Mrc1, can regulate origin firing by recruiting Cdc7 kinase and through an intramolecular interaction that controls its DNA and PCNA binding activities (34).

MATERIALS AND METHODS

Strains and culture conditions. Methods and media for culturing of *Schizosaccharomyces pombe* were described previously (35, 36). All the strains used in this study are listed in Table S1 in the supplemental material. All epitope-tagged strains were made by the integration of a 3×Flag-, 5×Flag-, or 13×Myc-tagged gene fragment into the original chromosome loci. All the tags for expression in yeast were located at the C terminus. Five micrograms per milliliter of thiamine was added to Edinburgh minimal medium (EMM) to suppress transcription from the nmt1 promoter on pREP expression plasmids. In order to introduce mutations into HBS, a genomic DNA sequence corresponding to aa 782 to 883 of Mrc1 was first replaced with the *ura4*$^+$ gene, generating an *mrc1*Δ(*782–883*)::*ura4*$^+$ strain (MS560). MS560 was unexpectedly found to be HU sensitive, possibly because truncated Mrc1 accidentally contains an additional 31 amino acids, SKLSYKSHWLYVCICVKKVCIDYLIYSAFFL, after Gly-781 and before the appearance of a termination codon. The *ura4*$^+$ gene of MS560 was then replaced with mutated Mrc1 DNA fragments containing mutations in HBS, and transformed cells were selected on synthetic dextrose minimal medium (SD) plates containing 0.1 mg/ml 5-fluoroorotic acid (FOA) (Wako). The presence of mutations in the strains generated were confirmed by PCR and sequencing. For cell cycle synchronization, *nda3-KM311* cells were arrested at M phase by incubation for 5 h at 20°C and were released at 30°C in yeast extract with supplements (YES) containing 0 or 200 μg/ml BrdU with or without 25 μM HU. BrdU incorporation and immunoprecipitation (IP) analyses were performed as described previously (37).

Two-dimensional gel electrophoresis. 2D gel electrophoresis was performed as described previously (22). DNA in agarose plugs was digested with BglII or EcoT22I for the detection of *ars1* or *ars2004*, respectively. Sequences of the primers used for probes are as follows: 5′-GGTTGCCCTGCAGGAAATTG-3′ (sense) and 5′-TTCTTTGTGGGGCTAGCCTGTA-3′ (antisense) for *ars1* BglII and 5′-AAAGTGCGTGCATGGCT TTAGG-3′ (sense) and 5′-TGAGAGAGTACAGTCAAGCGTAGAG-3′ (antisense) for *ars2004* HaeIII.

Chromatin immunoprecipitation and real-time PCR. A total of 1.0 × 10^9 cells were cross-linked with 1% formaldehyde for 15 min at 30°C and prepared for ChIP as previously described (37). Briefly, cross-linked cell lysates solubilized by multibead shocker (Yasui Kikai Co.) and sonication (Branson) were incubated with anti-Flag antibody (M2; Sigma)-conjugated protein G Dynabeads (Dynal) for 4 h at 4°C. The beads were washed several times, and the coprecipitated materials were eluted with elution buffer (50 mM Tris-HCl [pH 7.6], 10 mM EDTA, and 1% SDS) for 20 min at 68°C. The eluates were incubated at 68°C overnight to reverse cross-links and then treated with RNase A and proteinase K. DNA was precipitated with ethanol and further purified by using a QIAquick PCR purification kit (Qiagen). Quantitative PCR was performed by using SYBR Premix Ex *Taq* (TaKaRa Bio) on a LightCycler 480 instrument (Roche). The immunoprecipitation efficiency is presented as a percentage of the input chromatin. The primer sets used are listed in Table S2 in the supplemental material.

Next-generation sequencing (NGS) and ChIP-seq. DNA from the input and the immunoprecipitated materials was fragmented to an average size of ~150 bp by ultrasonication (Covaris). The fragmented DNAs were end repaired, ligated to sequencing adaptors, and amplified according to the protocol of the NEBNext ChIP-Seq Library Prep master mix set and NEBNext Multiplex Oligos for Illumina (New England BioLabs). The amplified DNA (around 275 bp) was sequenced on an Illumina MiSeq instrument to generate single reads of 100 bp. The generated ChIP or input sequences were aligned to the *S. pombe* genomic reference sequence provided by PomBase by Bowtie 1.0.0 using default settings (38). Peaks were called with model-based analysis of ChIP sequencing (ChIP-seq) (MACS2.0.10) by using the following parameters (39): macs2 callpeak -t ChIP.sam -c Input.sam -f SAM -g 1.4e107 -n result_file -B -q 0.01. The pileup graphs were loaded into the Affymetrix Integrated Genome Browser (IGB 8.0) (40). We adopted a criterion (fold enrichment of >3.0) to select candidate peaks because we used the MACS2 option "-nomodel" to generate the result file. MCM4 ChIP-seq data were obtained from *nda3-KM311 hsk1-89* cells pregrown at 25°C, arrested at M phase at 20°C for 8 h, and released into the cell cycle at 30°C (nonpermissive for *hsk1-89*) for 1 h to bring the cells to the G_1/S boundary.

Antibodies. Mouse anti-Flag M2 monoclonal antibody (Sigma), rabbit anti-Hsk1 antibody (21), mouse anti-cMyc (A-14) monoclonal antibody (Santa Cruz), mouse anti-GST (B-14) monoclonal antibody (Santa Cruz), and mouse anti-RGS-His monoclonal antibody (Qiagen) were used for Western blotting. Rabbit anti-Mrc1 antibody was raised against a GST fusion of Mrc1(1–180) and used for Western blotting.

Purification of Mrc1 fragments. The open reading frame of full-length Mrc1, Mrc1Δ*HBS*, or various Mrc1 segments (spanning positions 1 to 781, 225 to 781, 377 to 781, 396 to 781, or 536 to 781) was cloned at the BamHI site of the mammalian expression vector ver.3-4, resulting in the generation of N-terminally 6×His- and C-terminally 3×Flag-tagged polypeptides (41). The expression plasmid was transfected into 293T cells, and Mrc1, Mrc1Δ*HBS*, or each Mrc1 deletion protein was purified by consecutive steps with anti-Flag antibody and nickel affinity columns. GST-fused Mrc1(225–547) (WT or SA or SE mutant) was purified from *Escherichia coli* BL21(DE3) cells harboring a pGEX-2T-derived vector expressing each polypeptide by using glutathione-Sepharose 4B (GE Healthcare). Mrc1(674–879) and Mrc1(782–879) were purified from BL21(DE3) cells carrying pT7-7/QE30 expressing N-terminally RGS-His-tagged Mrc1(674–879) and Mrc1(782–879), respectively, by using Ni-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen). Full-length Mrc1, Mrc1Δ*HBS*, and Mrc1(T238-S395/E) were also expressed on the pT7-7/QE30 expression vector as RGS-His-tagged polypeptides and were purified from BL21(DE3) cells for use in gel shift assays.

Accession number(s). All the ChIP-seq data sets have been deposited in the Gene Expression Omnibus (GEO) database under accession number GSE93178.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/MCB.00355-16.

TEXT S1, PDF file, 0.04 MB.

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S.M., Y.K., and H.M. conceived of and designed experiments. S.M. constructed strains and performed and analyzed all *S. pombe* experiments. Y.K. performed ChIP-quantitative PCR and ChIP-seq. M.S. and H.M. performed biochemical experiments. M.H. performed two-dimensional gel electrophoresis and ChIP-quantitative PCR. K.U. aided in the initial phase of this project with the construction of strains and biochemical analysis. R.F., N.K., and H.M. constructed some plasmids and purified Mrc1 proteins. S.M. and H.M. wrote the paper.

We declare that we have no conflict of interest.

REFERENCES

1. Masai H, Matsumoto S, You Z, Yoshizawa-Sugata N, Oda M. 2010. Eukaryotic chromosome DNA replication: where, when, and how? Annu Rev Biochem 79:89–130. https://doi.org/10.1146/annurev.biochem.052308.103205.
2. Mechali M, Yoshida K, Coulombe P, Pasero P. 2013. Genetic and epigenetic determinants of DNA replication origins, position and activation. Curr Opin Genet Dev 23:124–131. https://doi.org/10.1016/j.gde.2013.02.010.
3. Heichinger C, Penkett CJ, Bahler J, Nurse P. 2006. Genome-wide characterization of fission yeast DNA replication origins. EMBO J 25:5171–5179. https://doi.org/10.1038/sj.emboj.7601390.
4. Feng W, Collingwood D, Boeck ME, Fox LA, Alvino GM, Fangman WL, Raghuraman MK, Brewer BJ. 2006. Genomic mapping of single-stranded DNA in hydroxyurea-challenged yeasts identifies origins of replication. Nat Cell Biol 8:148–155. https://doi.org/10.1038/ncb1358.
5. Patel PK, Arcangioli B, Baker SP, Bensimon A, Rhind N. 2006. DNA replication origins fire stochastically in fission yeast. Mol Biol Cell 17:308–316.
6. Hayashi M, Katou Y, Itoh T, Tazumi A, Yamada Y, Takahashi T, Nakagawa T, Shirahige K, Masukata H. 2007. Genome-wide localization of pre-RC sites and identification of replication origins in fission yeast. EMBO J 26:1327–1339. https://doi.org/10.1038/sj.emboj.7601585.
7. Hayano M, Kanoh Y, Matsumoto S, Renard-Guillet C, Shirahige K, Masai H. 2012. Rif1 is a global regulator of timing of replication origin firing in fission yeast. Genes Dev 26:137–150. https://doi.org/10.1101/gad.178491.111.
8. Daigaku Y, Keszthelyi A, Muller CA, Miyabe I, Brooks T, Retkute R, Hubank M, Nieduszynski CA, Carr AM. 2015. A global profile of replicative polymerase usage. Nat Struct Mol Biol 22:192–198. https://doi.org/10.1038/nsmb.2962.
9. Kaykov A, Nurse P. 2015. The spatial and temporal organization of origin firing during the S-phase of fission yeast. Genome Res 25:391–401. https://doi.org/10.1101/gr.180372.114.
10. Aparicio OM. 2013. Location, location, location: it's all in the timing for replication origins. Genes Dev 27:117–128. https://doi.org/10.1101/gad.209999.112.
11. Matsumoto S, Hayano M, Kanoh Y, Masai H. 2011. Multiple pathways can bypass the essential role of fission yeast Hsk1 kinase in DNA replication initiation. J Cell Biol 195:387–401. https://doi.org/10.1083/jcb.201107025.
12. Knott SR, Peace JM, Ostrow AZ, Gan Y, Rex AE, Viggiani CJ, Tavare S, Aparicio OM. 2012. Forkhead transcription factors establish origin timing and long-range clustering in *S. cerevisiae*. Cell 148:99–111. https://doi.org/10.1016/j.cell.2011.12.012.
13. Tazumi A, Fukuura M, Nakato R, Kishimoto A, Takenaka T, Ogawa S, Song JH, Takahashi TS, Nakagawa T, Shirahige K, Masukata H. 2012. Telomere-binding protein Taz1 controls global replication timing through its localization near late replication origins in fission yeast. Genes Dev 26:2050–2062. https://doi.org/10.1101/gad.194282.112.
14. Yamazaki S, Ishii A, Kanoh Y, Oda M, Nishito Y, Masai H. 2012. Rif1 regulates the replication timing domains on the human genome. EMBO J 31:3667–3677. https://doi.org/10.1038/emboj.2012.180.
15. Yoshida K, Poveda A, Pasero P. 2013. Time to be versatile: regulation of

the replication timing program in budding yeast. J Mol Biol 425:4696–4705. https://doi.org/10.1016/j.jmb.2013.09.020.
16. Dave A, Cooley C, Garg M, Bianchi A. 2014. Protein phosphatase 1 recruitment by Rif1 regulates DNA replication origin firing by counteracting DDK activity. Cell Rep 7:53–61. https://doi.org/10.1016/j.celrep.2014.02.019.
17. Mattarocci S, Shyian M, Lemmens L, Damay P, Altintas DM, Shi T, Bartholomew CR, Thoma NH, Hardy CF, Shore D. 2014. Rif1 controls DNA replication timing in yeast through the PP1 phosphatase Glc7. Cell Rep 7:62–69. https://doi.org/10.1016/j.celrep.2014.03.010.
18. Matsumoto S, Shimmoto M, Kakusho N, Yokoyama M, Kanoh Y, Hayano M, Russell P, Masai H. 2010. Hsk1 kinase and Cdc45 regulate replication stress-induced checkpoint responses in fission yeast. Cell Cycle 9:4627–4637. https://doi.org/10.4161/cc.9.23.13937.
19. Koren A, Soifer I, Barkai N. 2010. MRC1-dependent scaling of the budding yeast DNA replication timing program. Genome Res 20:781–790. https://doi.org/10.1101/gr.102764.109.
20. Gispan A, Carmi M, Barkai N. 2014. Checkpoint-independent scaling of the *Saccharomyces cerevisiae* DNA replication program. BMC Biol 12:79. https://doi.org/10.1186/s12915-014-0079-z.
21. Masai H, Miyake T, Arai K. 1995. *hsk1*$^{+}$, a *Schizosaccharomyces pombe* gene related to *Saccharomyces cerevisiae CDC7*, is required for chromosomal replication. EMBO J 14:3094–3104.
22. Hayano M, Kanoh Y, Matsumoto S, Masai H. 2011. Mrc1 marks early-firing origins and coordinates timing and efficiency of initiation in fission yeast. Mol Cell Biol 31:2380–2391. https://doi.org/10.1128/MCB.01239-10.
23. Zhao H, Tanaka K, Nogochi E, Nogochi C, Russell P. 2003. Replication checkpoint protein Mrc1 is regulated by Rad3 and Tel1 in fission yeast. Mol Cell Biol 23:8395–8403. https://doi.org/10.1128/MCB.23.22.8395-8403.2003.
24. Xu YJ, Davenport M, Kelly TJ. 2006. Two-stage mechanism for activation of the DNA replication checkpoint kinase Cds1 in fission yeast. Genes Dev 20:990–1003. https://doi.org/10.1101/gad.1406706.
25. Shimmoto M, Matsumoto S, Odagiri Y, Noguchi E, Russell P, Masai H. 2009. Interactions between Swi1-Swi3, Mrc1 and S phase kinase, Hsk1 may regulate cellular responses to stalled replication forks in fission yeast. Genes Cells 14:669–682. https://doi.org/10.1111/j.1365-2443.2009.01300.x.
26. Masai H, Taniyama C, Ogino K, Matsui E, Kakusho N, Matsumoto S, Kim J, Ishii A, Tanaka T, Kobayashi T, Tamai K, Ohtani K, Arai K. 2006. Phosphorylation of MCM4 by Cdc7 kinase facilitates its interaction with Cdc45 on the chromatin. J Biol Chem 281:39249–39261. https://doi.org/10.1074/jbc.M608935200.
27. Cho WH, Lee YJ, Kong SI, Hurwitz J, Lee JK. 2006. CDC7 kinase phosphorylates serine residues adjacent to acidic amino acids in the minichromosome maintenance 2 protein. Proc Natl Acad Sci U S A 103:11521–11526. https://doi.org/10.1073/pnas.0604990103.
28. Szyjka SJ, Viggiani CJ, Aparicio OM. 2005. Mrc1 is required for normal progression of replication forks throughout chromatin in *S. cerevisiae*. Mol Cell 19:691–697. https://doi.org/10.1016/j.molcel.2005.06.037.
29. Petermann E, Helleday T, Caldecott KW. 2008. Claspin promotes normal replication fork rates in human cells. Mol Biol Cell 19:2373–2378. https://doi.org/10.1091/mbc.E07-10-1035.
30. Scorah J, McGowan CH. 2009. Claspin and Chk1 regulate replication fork stability by different mechanisms. Cell Cycle 8:1036–1043. https://doi.org/10.4161/cc.8.7.8040.
31. Heller RC, Kang S, Lam WM, Chen S, Chan CS, Bell SP. 2011. Eukaryotic origin-dependent DNA replication in vitro reveals sequential action of DDK and S-CDK kinases. Cell 146:80–91. https://doi.org/10.1016/j.cell.2011.06.012.
32. Yeeles JT, Deegan TD, Janska A, Early A, Diffley JF. 2015. Regulated eukaryotic DNA replication origin firing with purified proteins. Nature 519:431–435. https://doi.org/10.1038/nature14285.
33. Duzdevich D, Warner MD, Ticau S, Ivica NA, Bell SP, Greene EC. 2015. The dynamics of eukaryotic replication initiation: origin specificity, licensing, and firing at the single-molecule level. Mol Cell 58:483–494. https://doi.org/10.1016/j.molcel.2015.03.017.
34. Yang CC, Suzuki M, Yamakawa S, Uno S, Ishii A, Yamazaki S, Fukatsu R, Fujisawa R, Sakimura K, Tsurimoto T, Masai H. 2016. Claspin recruits Cdc7 kinase for initiation of DNA replication in human cells. Nat Commun 7:12135. https://doi.org/10.1038/ncomms12135.
35. Alfa C, Fantes P, Hyams J, McLeod M, Warbrick E. 1993. Experiments with fission yeast: a laboratory course manual. Cold Spring Harbor Laboratory Press, Plainview, NY.
36. Moreno S, Klar A, Nurse P. 1991. Molecular genetic analysis of fission yeast Schizosaccharomyces pombe. Methods Enzymol 194:795–823. https://doi.org/10.1016/0076-6879(91)94059-L.
37. Katou Y, Kanoh Y, Bando M, Noguchi H, Tanaka H, Ashikari T, Sugimoto K, Shirahige K. 2003. S-phase checkpoint proteins Tof1 and Mrc1 form a stable replication-pausing complex. Nature 424:1078–1083. https://doi.org/10.1038/nature01900.
38. Langmead B, Trapnell C, Pop M, Salzberg SL. 2009. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol 10:R25. https://doi.org/10.1186/gb-2009-10-3-r25.
39. Zhang Y, Liu T, Meyer CA, Eeckhoute J, Johnson DS, Bernstein BE, Nusbaum C, Myers RM, Brown M, Li W, Liu XS. 2008. Model-based analysis of ChIP-Seq (MACS). Genome Biol 9:R137. https://doi.org/10.1186/gb-2008-9-9-r137.
40. Nicol JW, Helt GA, Blanchard SGJ, Raja A, Loraine AE. 2009. The Integrated Genome Browser: free software for distribution and exploration of genome-scale datasets. Bioinformatics 25:2730–2731. https://doi.org/10.1093/bioinformatics/btp472.
41. Uno S, You Z, Masai H. 2012. Purification of replication factors using insect and mammalian cell expression systems. Methods 57:214–221. https://doi.org/10.1016/j.ymeth.2012.06.016.
42. Tanaka T, Yokoyama M, Matsumoto S, Fukatsu R, You Z, Masai H. 2010. Fission yeast Swi1-Swi3 complex facilitates DNA binding of Mrc1. J Biol Chem 285:39609–39622. https://doi.org/10.1074/jbc.M110.173344.