Telomere-binding protein Taz1 controls global replication timing through its localization near late replication origins in fission yeast

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In eukaryotes, the replication of chromosome DNA is coordinated by a replication timing program that temporally regulates the firing of individual replication origins. However, the molecular mechanism underlying the program remains elusive. Here, we report that the telomere-binding protein Taz1 plays a crucial role in the control of replication timing in fission yeast. A DNA element located proximal to a late origin in the chromosome arm represses initiation from the origin in early S phase. Systematic deletion and substitution experiments demonstrated that two tandem telomeric repeats are essential for this repression. The telomeric repeats recruit Taz1, a counterpart of human TRF1 and TRF2, to the locus. Genome-wide analysis revealed that Taz1 regulates about half of chromosomal late origins, including those in subtelomeres. The Taz1-mediated mechanism prevents Dbf4-dependent kinase (DDK)-dependent Sld3 loading onto the origins. Our results demonstrate that the replication timing program in fission yeast uses the internal telomeric repeats and binding of Taz1.

Keywords: Taz1; chromosome; initiation of replication; replication timing; telomere

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DNA replication is fundamental and essential for the inheritance of genetic information through generations. For complete replication of large eukaryotic chromosomes to occur within the limited period of S phase, DNA replication initiates at a large number of chromosome loci known as replication origins. Replication origins do not fire simultaneously at the onset of S phase but do so at distinct time points during S phase. Control of replication timing, in which replication initiates from each origin at a prefixed time during S phase, is common in eukaryotic chromosomes from yeast to metazoan [MacAlpine and Bell 2005; Gilbert et al. 2010]. In mammalian cells, it is suggested that the replication timing program is established during a discrete time window in G1 phase, the timing decision point (TDP) [Dimitrova and Gilbert 1999]. The fact that replication timings are changed coupled with alterations in histone modifications and gene expression during metazoan development suggests the importance of replication timing control RTC [Hiratani et al. 2004, 2008; Lande-Diner et al. 2009]. The initiation of replication in eukaryotes consists of several distinct steps that are regulated by cell cycle. In G1 phase, the origin recognition complex (ORC), together with Cdc6 and Cdt1, promotes loading of a minichromosome maintenance (MCM) complex, composed of heterohexamer Mcm2–7, onto replication origins, resulting in a prereplicative complex [pre-RC] [Bell and Dutta 2002]. Activation of two essential kinases, cyclin-dependent kinase (CDK) and Dbf4-dependent kinase (DDK), at the onset of S phase promotes the assembly of several other replication factors, including Sld3, Cdc45, and GINS, leading to the unwinding of origin DNA and the initiation of DNA synthesis [Remus and Diffley 2009; Labib 2010; Tanaka and Araki 2010]. In budding yeast and fission yeast, pre-RCs are formed at all of the origins, whereas specific origins are activated in early S phase [Raghuraman et al. 2001; Wyrick et al. 2001; Hayashi et al. 2007]. Recent studies have suggested that DDK-dependent origin loading of Sld3 and Cdc45 is a key step in determining the timing
of initiation [Tanaka et al. 2011]. Therefore, the replication timing program seems to regulate the activation of pre-RCs in these yeasts.

It is generally agreed that the status of chromatin has an impact on the timing of origin activation. Replication origins in euchromatin domains, where genes are actively transcribed, initiate early, while those in heterochromatin, where genes are repressed, replicate in late S phase (MacAlpine et al. 2004; Hiratani and Gilbert 2009). However, genome-wide identification of replication origins in fission yeast indicate that a subset of euchromatic origins do not fire in early S phase [Hayashi et al. 2007]. Moreover, centromeric heterochromatin and the silent mating type \( [\text{mat}] \) locus replicate in early S phase [Kim et al. 2003; Hayashi et al. 2009], and interestingly, Swi6, a conserved heterochromatin protein 1 (HP1), stimulates their early replication [Hayashi et al. 2009]. On the other hand, subtelomeric heterochromatin replicates very late in S phase in the presence or absence of Swi6 [Hayashi et al. 2009], suggesting that a certain mechanism independent from heterochromatin regulates replication timing. Although the checkpoint pathway represses late origins under the depletion of nucleotides by hydroxyurea (HU) in budding yeast and fission yeast, it does not affect the replication timing of the origins [Santocanale and Diffley 1998; Santocanale et al. 1999; Hayashi et al. 2007].

In budding yeast, proximity to the telomere confers late replication of some origins [Ferguson and Fangman 1992]. Telomeric DNA, consisting of \( \text{TTAGGG} \) with species-specific variations, becomes shorter as a result of lagging strand synthesis, and shortened telomeres are elongated by telomerase, the telomere-specific reverse transcriptase. Interestingly, long telomeres cause a delay in the replication timing of telomere-proximal origins [Bianchi and Shore 2007], suggesting the involvement of telomere DNA and probably the telomere-binding proteins in the control of replication timing in the regions proximal to telomeres. In the internal arm regions, although the importance of some unique sequence elements associating with late origins has been reported [Friedman et al. 1996; Sharma et al. 2001; Yompakdee and Huberman 2004], the molecular mechanisms underlying the control of replication timing have not been elucidated.

In the present study, we demonstrate that the telomere-binding protein Taz1 plays an essential role in the control of replication timing in fission yeast. Taz1, an ortholog of mammalian TRF1 and TRF2, binds to telomeres at the ends of fission yeast chromosomes and is required for the protection and maintenance of the telomeres [Cooper et al. 1997; Kanoh and Ishikawa 2001]. Although Taz1 has been shown to be required for the passage of replication forks through telomeric DNA [Miller et al. 2006], the involvement of Taz1 in the regulation of replication initiation in the chromosome arm regions has not been considered. Here, we show that a DNA fragment proximal to the late origin, \( \text{AT2088} \), when inserted at ectopic sites, repressed firing from neighboring early origins in early S phase. The essential sequence for repression determined by deletions and substitutions resembles two tandem copies of a telomeric repeat. Interestingly, similar telomeric repeats were found near a subset of late origins. The telomeric repeats proximal to \( \text{AT2088} \) and another late origin, \( \text{AT2035} \), recruit Taz1 to the loci, and the recruitment of Taz1 was essential for the timing control of these late origins. A 50-base-pair (bp) fragment containing two copies of the essential sequence is sufficient for the recruitment of Taz1 and the timing control of a nearby origin. Remarkably, in the \( \text{taz1A} \) strain, about half of the previously identified late origins, including those in the subtelomeres, fire in early S phase. These results indicate that Taz1, a well-defined telomere-binding protein, plays an essential role in RTC through binding to the telomeric repeats in the vicinity of internal late origins in fission yeast.

Results

Identification of the DNA element that controls replication timing

To elucidate the mechanism that regulates timing of firing at individual replication origins, we compared two replication origins, \( \text{ars2004} \) and \( \text{AT2088/ARS745} \), which share properties of strong replication origins but differ in replication timing. \( \text{ars2004} \), located in the left arm of chromosome II (Fig. 1A), is a well-characterized early origin [Okuno et al. 1999; Takahashi et al. 2003] where initiation factors assemble efficiently at the onset of S phase [Yamada et al. 2004; Yabuuchi et al. 2006]. \( \text{AT2088/ARS745} \) [Maundrell et al. 1988; Segurado et al. 2003] is a late/dormant origin located in the right arm of chromosome II (Fig. 1A). Despite the efficient formation of pre-RC at \( \text{AT2088} \), 5-bromo-2′-deoxyuridine (BrdU), a heavy-density analog of thymidine, was not incorporated in HU-treated early S-phase cells [Hayashi et al. 2007]. Both origins are located in large intergenic regions flanked by divergent promoters and contain long AT-rich regions that are essential for the replication origin activity. Moreover, they exhibit efficient ARS activity when the respective fragment is cloned on a plasmid (Supplemental Fig. S1A).

Since the previous study of early and late origins was carried out using HU-treated cells where the replication checkpoint was activated [Hayashi et al. 2007], we examined the replication timing of \( \text{ars2004} \) and \( \text{AT2088} \) in unperturbed S phase. Cells synchronously released from G2/M block by a temperature-sensitive \( \text{cdc25-22} \) mutation were labeled for indicated periods with BrdU. Genes containing the heavy–light (HL) and light–light (LL) DNA, separated by cesium chloride (CsCl) density gradient centrifugation, were determined by real-time PCR (qPCR) using primers amplifying \( \text{ars2004} \) and \( \text{AT2088} \), together with \( \text{AT2024} \) (internal early origin control), \( \text{AT2035} \) (late origin control), and \( \text{TAS59} \), which is \( 20 \text{ kb} \) distant from the right end of chromosome II (Fig. 1A). Replication kinetics showed that \( \text{ars2004} \) and \( \text{AT2024} \) replicated \( 10 \text{ min} \) earlier than \( \text{AT2035} \) and \( \text{AT2088} \), while \( \text{TAS59} \) replicated much later than the others (Fig. 1B). These results demonstrated that the replication timings of \( \text{ars2004} \) and \( \text{AT2088} \) differ in unperturbed S phase.
Figure 1. Replication timings of ars2004 and AT2088 origin fragments are maintained at ectopic loci. (A) The locations of early and late replication origins on Schizosaccharomyces pombe chromosome II are presented schematically. The positions of early origins AT2024 (gray) and ars2004 (red), cen2, late origins AT2035 (green) and AT2088 (blue), and a subtelomeric origin, TAS59 (purple) are shown. For the ars2004 and AT2088 loci, the locations of the genes, along with the direction of transcription (arrow) and fragments [3.2-kb ars2004 (red), 3.6-kb AT2088 (blue)] used for translocation, are presented. Relevant restriction fragments [EcoT22I (E), FbaI (F), and NcoI (N)] analyzed by two-dimensional (2D) gel electrophoresis are shown below the maps. (B) Replication kinetics of early and late origins. Fission yeast cdc25-22 nmt1-TK+ cells arrested at the G2/M boundary for 3 h at 36°C were released at 25°C in the presence of BrdU (200 μM). At the indicated time points, the replicated heavy–light (HL) DNA was separated from light–light (LL) DNA using cesium chloride (CsCl) density gradient centrifugation, and the amount of DNA of AT2024 (black), AT2035 (green), ars2004 (red), AT2088 (blue), and TAS59 (purple) in the LL and HL densities was determined by qPCR. The replication kinetics of each origin are presented. The results of biologically independent experiments are shown in Supplemental Figure S1B. (C,D) The replication kinetics of ars2004 (red), inserted at the AT2088 locus (C), and AT2088, inserted at the ars2004 locus (D), were obtained as described in B. The results of biologically independent experiments are shown in Supplemental Figure S1, C and D. The right panels show the results of 2D gel analysis of ars2004 (FbaI–NcoI fragment) at the AT2088 locus (C) and of AT2088 (EcoT22I fragment) at the ars2004 locus (D) prepared at 90 min after G2/M release in the presence of HU (10 mM). The membrane was rehybridized with the early origin AT2046 probe. An arrowhead indicates the bubble arc. (E) Comparison of the time required for replication in half of the cell population (T1/2). T1/2 (in minutes) for each origin was obtained from the results presented in B–D and Supplemental Figure S1B–D. The difference in T1/2 between an origin and AT2024 (early origin control) is presented. AT2035 and AT2088 replicate 6–12 min later than ars2004 and AT2024, regardless of their locations on the chromosome.
To investigate whether the replication timings of *ars2004* and *AT2088* are intrinsic to the origins, the intergenic fragment containing each origin was translocated into an ectopic chromosomal context. The 3.2-kb fragment containing *ars2004* inserted at the *AT2088* locus (*ars2004/AT2088*) replicated as early as the control early origin *AT2024* [Fig. 1C]. In contrast, the *AT2088* fragment inserted at the *ars2004* locus (*AT2088/ars2004*) replicated as late as the control late origin *AT2035* [Fig. 1D]. Although the absolute times of replication vary between experiments, probably due to the difference in the period required for re-entry into the cell cycle from the G2/M block, we confirmed that the difference in T1/2, the time required for replication in half of a cell population for an origin relative to that of the early origin control *AT2024*, was maintained [Fig. 1E]. The results of two-dimensional (2D) gel electrophoresis analysis indicated that replication initiated efficiently from the *ars2004* placed at the *AT2088* locus, as shown by the bubble arc [Fig. 1C], and that replication was rarely initiated from the *AT2088* fragment at the *ars2004* locus in early S phase [Fig. 1D]. Moreover, the plasmids pARS2004 and pAT2088, carrying the corresponding fragments, replicated early and late in S phase, respectively (Supplemental Fig. S1E). These results demonstrate that the replication timings are intrinsic to the *ars2004* and *AT2088* fragments.

If an element located in the *AT2088* fragment forces the origin not to fire in early S phase, it might also repress another origin placed closely to the fragment. To test this possibility, the *AT2088* fragment was inserted in the vicinity of *ars2004* on the chromosome [Fig. 2A], and the amounts of BrdU-incorporated DNA in HU-treated early S-phase cells were analyzed for *ars2004* locus and other origins. In HU-treated wild-type cells without insertion, both *ars2004* and *AT2024* showed robust replication, while *AT2035* did not replicate efficiently [Fig. 2B]. Although the insertion of the *ura4* fragment alone did not cause a significant change [Fig. 2B], the insertion of *AT2088* markedly decreased replication of *ars2004*, indicating that the *AT2088* fragment repressed the neighboring *ars2004* [Fig. 2B]. This was due to a decrease in initiation from *ars2004*, as shown by the reduced amount of bubble arc in 2D gel analysis [Fig. 2C]. Furthermore, the *AT2088* fragment, when inserted at a site close to another early origin *ORI12* on chromosome I (Segurado et al. 2003), also repressed the early replication of the origin (Supplemental Fig. S2B). These results showed that the *AT2088* fragment has the ability to control the replication timing of proximal origins. Accordingly, we named the activity “replication timing control” (RTC) activity.

**Figure 2.** Repression of early initiation from the *ars2004* origin by the adjoining *AT2088* fragment. (A) Schematic presentation of the *ars2004* locus carrying the *ura4* -*AT2088* or *ura4* insertion. (B) *cdc25-22 nmt1-TK* derivatives with or without the insertion of *ura4*-*AT2088* or *ura4* alone next to the *ars2004* locus were released from the G2/M block and labeled with BrdU for 120 min in the presence of HU (10 mM). Replication (percentage) in the presence of HU was determined for *ars2004* (red) as well as the control early and late origins *AT2024* (gray) and *AT2035* (green), respectively. The mean ± SD obtained from multiple measurements in qPCR is presented. The results of a biologically independent experiment are presented in Supplemental Figure S2A. (C) The replication intermediates of *ars2004* (Styl fragment) and a control early origin, *AT2047* (Styl fragment), prepared at 90 min after G2/M release of the HU-treated *AT2088-ars2004* cells were analyzed by 2D gel electrophoresis. An arrowhead shows the bubble arc.
ARS activity [Supplemental Fig. S3B], confirming that RTC is distinct from the origin activity.

We here examined whether the RTC controls replication timing at the original AT2088 locus. A 210-bp fragment containing the RTC essential sequence was deleted from the AT2088 locus (AT2088Δ210), and replication initiation was analyzed by 2D gel electrophoresis. In contrast to a faint Y arc from the AT2088 locus in HU-treated wild-type cells, a bubble arc, indicative of the initiation, was generated along with a strong transition Y arc resulting from initiation from an asymmetrical position within the restriction fragment in AT2088Δ210 [Fig. 3E]. These results indicate that the RTC is required for the control of replication timing at the native AT2088 locus.

**Telomeric repeats are essential for repression of internal late replication origins**

In fission yeast, telomeric repeats consist of GGTTAC followed by A(G)_{0–1}C(G)_{0–1}G variations [Sugawara 1989; Cooper et al. 1997; Hiraoka et al. 1998]. We found that the RTC essential sequence contains two tandem copies of a telomeric repeat, with a substitution of T for the last C [Fig. 4B]. We examined whether the telomeric repeats are essential for the control of replication timing at the native AT2088 locus. Base substitution of the essential sequence [AT2088-S2632] promoted early replication specifically at AT2088 [Fig. 4B]. Because the telomeric repeats in the RTC essential sequence contain an incomplete match to the consensus, we confirmed that two copies of the telomere consensus sequence GGGTTACAGGGTTAC AGGG replaced functionally the incomplete sequence [Fig. 4B, AT2088-teIF]. Insertion of the consensus in the opposite direction also restored the RTC [Fig. 4B, AT2088-teIR], suggesting that the telomeric repeats act as a discrete element, such as the protein-binding site.

To explore the general role of telomeric repeats in the control of replication timing in fission yeast, we searched the fission yeast genome for two tandem telomeric sequences—GGTTAY-N_{1–3}-GGTTAY, where Y represents pyrimidine—using the “fuzznuc” program [see the Materials and Methods]. Such telomeric repeats are located at 26 loci, exclusively in the intergenic regions [Supplemental Tables S1, S2]. Interestingly, 13 of them exist within the same intergenic spaces with late origins, which were identified in a previous genome-wide study.
with standard error (SE) obtained from three biologically independent experiments. (B) The mean ± SD obtained in multiple measurements in qPCR is presented. The results of biologically independent experiments are shown in Supplemental Figure S4A. (C) Replication of the AT2035 was examined as described in B. The nucleotide sequences of the region (1954–1993, complementary strand) containing telomere-like repeats (blue underlining) and base substitutions (red) in AT2035-S1959 and AT2035-S1985 are presented. The results of biologically independent experiments are shown in Supplemental Figure S4B. (D) Flag-Taz1 was immunoprecipitated from asynchronously cultured wild-type, AT2035-S1959, and AT2088-S2632 cells. Immunoprecipitation recovery of the chromosome locus against the total cellular DNA was measured by qPCR for ade6 (gray), AT2035 (green), AT2088 (blue), and Tel-0.3 (purple). Bars indicate the mean of immunoprecipitation recovery (percentage) ± SE. [Right] The results, except for those of Tel-0.3, are presented for a better comparison of Taz1 localization at the internal sites.

Hayashi et al. (2007). To investigate whether the telomeric repeats have a general role in RTC, we disrupted the one located near AT2035 on chromosome II [Fig. 4C]. Base substitutions in the telomeric repeats caused robust early replication specifically at AT2035 [Fig. 4C, AT2035-S1959]. These results strongly suggest that the telomeric repeats present near internal late origins play a general role in RTC.

Taz1 binds to the telomeric repeats near the internal late origins

At the ends of chromosomes, Taz1, a fission yeast ortholog of human TRF1 and TRF2, binds to two tandem copies of the telomeric repeat (Hayashi et al. 2007). To elucidate the role of telomeric repeats located near the internal late origins in the control of replication timing, we analyzed localization of 6Flag-tagged Taz1 at the internal chromosome regions using a chromatin immunoprecipitation (ChIP) assay. ChIP recovery of DNA for ade6, AT2035, AT2088, and the telomere-proximal fragment (Tel-0.3) was determined by qPCR [Fig. 4D]. In wild-type cells, the recovery of Tel-0.3 was ~50-fold higher than that of ade6 [Fig. 4D, left], indicating the accumulation of Taz1 at the telomeres. Taz1 was also localized at telomeric repeats near AT2035 and AT2088, as shown by recovery threefold and fourfold higher than that of ade6, respectively [Fig. 4D, right]. The localization of Taz1 at AT2035 was impaired by AT2035-S1959 base substitution, while the localization at AT2088 was not affected [Fig. 4D, right]. Moreover, AT2088-S2632 substitution abolished the Taz1 localization specifically at the AT2088 locus [Fig. 4D, right]. These results demonstrate that the telomeric repeats near AT2035 and AT2088 recruit Taz1 to the corresponding loci.

Two copies of RTC essential sequence are sufficient for control of the replication timing of the nearby replication origin

Because telomeric repeats are essential for the timing control of AT2088 and AT2035 [Fig. 4B,C], we tested whether telomeric repeats are sufficient for control of late replication timing. To this end, two copies of the 25 bp of the DNA fragment (2RTC25) containing the RTC essential sequence of AT2088 were integrated next to the ars2004 locus, and the replication of the ars2004 in HU-treated cells was analyzed [Fig. 5A]. A BrdU-incorporation
assay showed that the insertion of 2RTC25 decreased the replication of ars2004, indicating that the telomeric repeats are sufficient for the repression of early replication of the neighboring origin [Fig. 5B]. Then, we analyzed whether the 2RTC25 recruited Taz1 to the locus using a ChIP assay (Fig. 5C). In wild-type cells without insertion, Taz1 was not localized at the locus near ars2004 [Fig. 5C, WT]. In contrast, Taz1 was localized to the locus in the 2RTC25-ars2004 cells [Fig. 5C, 2RTC25-ars2004]. These results indicate that the telomeric repeats are sufficient for the recruitment of Taz1 and for the timing control of a nearby replication origin.

**Taz1 plays an important role in the global control of replication timing**

We then asked whether Taz1 is required for RTC at late replication origins. BrdU incorporation was determined in the wild-type and taz1Δ strains for AT2035 and AT2088, which are associated with telomeric repeats, as well as late origins AT2080 and ARS727, which are not associated with telomeric repeats, and the subtelomeric TASS9. In HU-treated wild-type cells, the early origin ars2004, but none of the late origins, replicated significantly [Fig. 6A]. In contrast, in HU-treated taz1Δ cells, AT2035 and AT2088 showed robust replication to a level comparable with ars2004, whereas AT2080 or ARS727 did not replicate significantly [Fig. 6A]. TASS9, which is located ~20-kb distant from the telomere, replicated more efficiently than ars2004 [Fig. 6A]. These results indicate that Taz1 is required for RTC of a subset of late origins that is associated with telomeric repeats.

To investigate the role of Taz1 in the global control of replication timing, we analyzed the replication profile of all chromosomes. The BrdU-labeled DNA prepared from HU-treated wild-type and taz1Δ cells was subjected to sequencing by oligonucleotide ligation and detection (SOLiD) [Fig. 6B; Supplemental Fig. S5]. Comparison of the replication profiles in wild-type and taz1Δ cells revealed that among 156 late/dormant origins, 26 in chromosome arm regions and 46 in subtelomerics fired in the HU-treated taz1Δ cells [Fig. 6D, Supplemental Fig. S5, Supplemental Table S1]. Genome-wide analysis of Taz1 localization showed that Taz1 was localized at 16 of 26 internally located late origins that fired in taz1Δ cells, although Taz1 was localized only in the vicinity of the telomere [Fig. 6C; Supplemental Fig. S5]. These results suggest that Taz1 plays an important role in the control of global replication timing in fission yeast.

**Taz1-mediated RTC prevents loading of Sld3 to late origins**

Because pre-RCs are formed at both early and late replication origins, we assumed the process of pre-RC activation to be regulated by the Taz1-mediated mechanism. To elucidate the step that is regulated, we analyzed the localizations of a pre-RC component (Mcm6) and Sld3, which is recruited to pre-RC in the most upstream step of the origin activation process in S phase [Yabuuchi et al. 2006]. Because Sld3 localization occurs only transiently under wild-type conditions, we employed the cold-sensitive nda4-108 mcm5 mutation that allows Sld3 loading but prevents the initiation of replication [Yamada et al. 2004]. In cdc25-22 nda4-108 sld3-Flag cells carrying the native AT2088 [wild type] released synchronously...
into S phase from G2/M, Mcm6 was efficiently localized at all the origins but not in the nonorigin (nonARS) region (Fig. 7A, left). Sld3 was preferentially enriched at the ars2004 but not at late origins or non-ARS (Fig. 7B, left). In AT2088-S2632 cells lacking the telomeric repeat near AT2088, localization of Sld3 increased specifically...
at AT2088 [Fig. 7B, middle], whereas Mcm6 localizations were not significantly different from those in the wild type [Fig. 7A, middle]. These results show that the telomeric repeat-dependent RTC activity represses Sld3 loading. We then examined whether Taz1 impacted the localization of Mcm6 or Sld3. In the taz1Δ strain, localization of Sld3 increased specifically at AT2088 and TAS59 but not at AT2035 or ARS727 [Fig. 7B, right], while Mcm6 was localized at the origins, similarly to those in wild type [Fig. 7A, right]. These results show that Taz1-mediated control of replication timing does not affect Mcm6 localization but prevents Sld3 loading at specific late origins.

Discussion

A replication timing program that regulates individual replication origins to fire at a prefixed time during S phase exists in all eukaryotes. In this study, we demonstrate that replication timing at a late origin is intrinsically determined by a proximal segment in fission yeast. The segment has the activity to repress initiation from nearby origins, even at ectopic sites, in early S phase, and two copies of the telomeric repeat are essential for this repression. A conserved telomere-binding protein, Taz1, binds to the internal telomeric repeats and plays an essential role in RTC. Genome-wide analysis revealed that about half of late origins are regulated by the Taz1-mediated mechanism. Remarkably, almost all origins in subtelomeric regions ~100 kb long were found to be regulated by Taz1. Our findings provide novel insights into the mechanism of RTC, especially how chromosome regions are distinctly regulated.

Roles of Taz1 in the control of replication timing

Our results demonstrate that two tandem copies of a telomeric repeat located in the vicinity of two late origins, AT2088 and AT2035, are essential for the control of replication timing at these origins. The results of ChIP analysis demonstrated that the telomeric repeats, which are the binding sites for a homodimeric Taz1 (Cooper et al. 1997; Spink et al. 2000), indeed recruit Taz1 to the internal chromosome loci [Fig. 4]. Moreover, Taz1 is required for the timing control of about half of the late origins (72 among 156), including all of the subtelomeric origins (46 origins). Genome-wide analysis of Taz1 localization showed that Taz1 binds to the vicinity of 16 among 26 internal late origins that are regulated in a Taz1-dependent manner [Fig. 6; Supplemental Fig. S5; Supplemental Table S1]. Because 13 of the 16 origins are associated with telomeric repeats (including three that were manually found), Taz1 plays a role in the control of replication timing through binding to telomeric repeats near these origins. On the other hand, Taz1 localization was not detected at 10 internal late origins that are activated in taz1Δ cells, leaving the possibility that Taz1 can participate in the control from a distance through a certain mechanism. It is also remarkable that control of all of the subtelomeric late origins are dependent on Taz1, despite the absence of Taz1 localization at the individual origins except for in the vicinity of the telomeres [Supplemental Fig. S5; Supplemental Table S1; Kanoh et al. 2005]. It is likely that Taz1 that binds to the telomere may play a role in the control of subtelomeric origins. Because the amount of telomere-bound Taz1 varies depending on the length of the individual telomere, Taz1 monitors the telomere length and impacts the timing of subtelomeric replication. This is consistent with the observations that shortened telomeres induce early firing of subtelomeric replication origins in budding yeast, in which Rap1 plays the role of Taz1 [Bianchi and Shore 2007; Shore and Bianchi 2009; Lian et al. 2011]. The timing of replication fork passage through the telomere is important for the stable association of the telomerase and the elongation of the telomere [Dionne and Wellerling 1998; Marcand et al. 2000; Gallardo et al. 2011]. Thus, Taz1 is likely to play crucial roles in the feedback loop of replication-mediated maintenance of telomere length in addition to its role in the passage of the replication forks through the telomeres [Miller et al. 2006].

Two copies of the 25-bp RTC essential sequence containing four telomeric repeats are sufficient for late timing control of a nearby early origin [Fig. 5], suggesting the critical role of the telomeric sequence and binding of Taz1. In addition to the telomeric sequence, a flanking AAGAGAGA sequence has some importance for the
RTC activity [Fig. 3D, data not shown]. A similar purine cluster is found flanking the telomeric sequence near AT2035 [Fig. 4C]. A purine-rich sequence may either stimulate Taz1 binding or recruit a factor involved in the repression of replication initiation. In addition, a region of ~60 bp (2557–2617) adjacent to the essential sequence contributes to origin repression to some extent (Fig. 3). Since the region is included in the scaffold/matrix-associating regions (SAR/MARs) [Amati and Gasser 1990], association with the nuclear scaffold may restrict the access of replication factors or increase the local concentration of inhibitory factors.

What is the significance of Taz1-dependent RTC in the internal chromosome regions? About half of the telomeric repeat-associated late origins [nine among 13], including ori2100, ori2114, and AT2088/ori2114, are flanked by the genes that are silenced in normal growth conditions and induced under various stresses or during meiosis [Supplemental Table S2]. Since gene expression at these loci might be regulated by the mechanisms involving specific transcription factors and histone modifications, control of replication timing may contribute to the maintenance or alteration of histone modifications. Although none of the Taz1-dependent internal late origins, except several subtelomeric origins in the vicinity to the telomeres, are activated in early premeiotic S phase [Heichinger et al. 2006], further investigations of relations between replication timing and gene expression will be needed. In metazoan, histone acetylation status is distinctly regulated during replication in the early and late S phases, suggesting that a change in replication timing can play a role in the alteration of gene expression during development [Lande-Diner et al. 2009].

Recently, the observation that Rif1, another telomere-binding protein in fission yeast, is involved in the global control of replication timing has been reported [Hayano et al. 2012]. Genome-wide analysis of replication in taz1Δ as well as rif1Δ revealed that all of the 72 late origins that fire in taz1Δ are also activated in rif1Δ [Supplemental Figs. S5, S7, Supplemental Table S1]. Moreover, 47 late origins that do not fire in taz1Δ, including AT2080 and ARS727/AT2013, are activated in rif1Δ [Supplemental Figs. S5, S7, Supplemental Table S1]. These observations suggest that there are at least Taz1-dependent and Taz1-independent pathways for timing control and both require Rif1. It is likely that Rif1 acts downstream from Taz1. It has been reported that the ARS727 fragment cloned on a plasmid forces late replication of a concurrently cloned early origin and that several copies of the late consensus sequence (LCS) A/G-G/T-G/T-GGGGA-A/T are required for the repression [Yompakdee and Huberman 2004]. The LCS might be involved in a Taz1-independent Rif1-dependent pathway [Hayano et al. 2012]. At present, it is not known whether Taz1 recruits Rif1 to Taz1-dependent late origins such as AT2088. Further examination will be needed to understanding the mechanisms in which telomere-binding proteins Taz1 and Rif1 function to repress initiation of replication.

The Taz1-dependent pathway regulates DDK-dependent Sld3 loading in the initiation of replication

In the Taz1-dependent control of replication timing described here, Taz1 that binds in the vicinity of late origin impacts the process of initiation. The effects diminish by distance because an early origin 20 kb from the Taz1-binding site is not inhibited [Supplemental Fig. S5]. These characteristics suggest that a catalytic reaction or a semidiffusible factor may be involved in the interference with the process of replication initiation. The heterochromatin formation does not play a significant role because late replication of AT2088 is maintained in the heterochromatin-deficient swi6Δ or clr4Δ strain [A Tazumi, unpubl.]. Checkpoint pathways, which are proficient in taz1Δ [Miller and Cooper 2003], may not play a major role in the Taz1-dependent timing control. Therefore, the Taz1-dependent regulation seems to involve a novel mechanism that affects the initiation process.

In this study, we showed that loading of Sld3 onto pre-RC is prevented at late origins by a Taz1-mediated control of replication timing. In fission yeast, loading of Sld3 onto replication origins is dependent on pre-RC and DDK and is a prerequisite for CDK-dependent assembly of other replication factors [Yabuuchi et al. 2006; Fukuura et al. 2011]. A recent in vitro study in budding yeast has shown that the origin association of Sld3 is dependent on DDK and is distinct from those that require CDK [Heller et al. 2011]. Moreover, an in vivo study in budding yeast suggests that the origin association of low-abundance Sld3, Sld7, and Cdc45, which is dependent on DDK, is crucial for the temporal order of origin firing [Tanaka et al. 2011]. The following evidence in fission yeast further emphasizes the importance of DDK recruitment in RTC. Swi6/HF1-dependent recruitment of Dfp1, a regulatory subunit of DDK, stimulates early activation of origins in pericentromeric heterochromatin and the silent mat locus [Hayashi et al. 2009]. Furthermore, tethering of Dfp1 to the late origin ARS727, which is Taz1-independent and Rif1-dependent, causes early firing of the origin [Patel et al. 2008]. Because DDK preferentially phosphorlates subunits of the chromatin-bound Mcm2–7 complex [Francis et al. 2009], both Taz1-dependent and -independent regulation may interfere with the access of DDK or the phosphorylation of the chromatin-bound Mcm2–7 complex by DDK [Francis et al. 2009].

Although recruitment of DDK and loading of Sld3 occur in S phase, a process that occurs in G1 phase may impact the reactions. Correlation of the origin association timing of the ORC and MCM in G1 phase with initiation timing has been reported in fission yeast [Wu and Nurse 2009]. In mammalian cells, it has been proposed that a replication timing program is established during a discrete window of time in G1 phase, the TDP [Dimitrova and Gilbert 1999]. It is possible that an early event such as chromatin modifications around individual replication origins could affect the recruitment of DDK and subsequent loading of replication factors at the origins in S phase.
Our results show that fission yeast uses a set of telomeric sequences and the binding protein Taz1 for regulation of replication timing. Recent studies revealed novel genome-wide functions of telomere-binding proteins in mammals. Mouse Rap1 binds to two tandem copies of the telomere repeat outside telomeric regions and regulates the expression of the genes located in the vicinity of the repeats [Martinez et al. 2010]. In addition, mammalian Rap1 has also been identified as a regulator of the NF-κB signaling pathway that mediates the transcriptional responses to various stresses [Teo et al. 2010]. Since telomeric sequences and telomere-binding proteins are highly conserved among eukaryotes, it would be interesting to investigate whether they could be involved in the regulation of DNA replication in other organisms.

**Materials and methods**

*Strains and media*

Strains and media used in this study are described in the Supplemental Material and are listed in Supplemental Table S3.

*BrdU incorporation and replication timing analysis*

Fission yeast strains carrying Pntmt1-TK cdc25-22 were cultured in EMM medium lacking thiamine to induce transcription of the TK gene for 18 h at 25°C to 5 × 10⁶ cells per milliliter. Cells arrested at the G2/M boundary by cdc25-22 mutation for 3 h at 36°C were released at 25°C and cultured for up to 20 h, after which DNA synthesis was measured by the incorporation of BrdU into DNA. Cell extracts were used for ChIP using anti-Flag and Mcm6 antibodies.

*Taz1 ChIP, cdc25Δ, and mcm5Δ strains and separated by CsCl density gradient centrifugation and those obtained by Flag-Taz1 immunoprecipitation were used for SOLiD deep-sequencing analysis, which was performed according to the manufacturer’s standard protocol [Applied Biosystems].

**Accession numbers**

Sequence data have been deposited to the Sequence Read Archive [http://www.ncbi.nlm.nih.gov/Traces/sra] with the accession number SRP011412.

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Telomere-binding protein Taz1 controls global replication timing through its localization near late replication origins in fission yeast

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