Establishment of expression-state boundaries by Rif1 and Taz1 in fission yeast

Toteva, Tea; Mason, Bethany; Kanoh, Yutaka; Brøgger, Peter; Green, Daniel; Hansen, Janne Verhein; Masai, Hisao; Thon, Genevieve

Published in:
Proceedings of the National Academy of Sciences of the United States of America

DOI:
10.1073/pnas.1614837114

Publication date:
2017

Document version
Publisher's PDF, also known as Version of record

Establishment of expression-state boundaries by Rif1 and Taz1 in fission yeast

Tea Toteva[a], Bethany Mason[a], Yutaka Kanoh[b], Peter Brøgger[a], Daniel Green[a], Janne Verhein-Hansen[a], Hisao Masai[b], and Geneviève Thon[a,1]

[a]Department of Biology, BioCenter, University of Copenhagen, 2200 Copenhagen N, Denmark; and [b]Department of Genome Medicine, Tokyo Metropolitan Institute of Medical Science, 2-1-6 Kamkitazawa, Setagaya-ku, Tokyo 156-8506, Japan

Edited by Jasper Rine, University of California, Berkeley, CA, and approved December 16, 2016 (received for review September 4, 2016)

The Shelterin component Rif1 has emerged as a global regulator of the replication-timing program in all eukaryotes examined to date, possibly by modulating the 3D-organization of the genome. In fission yeast a second Shelterin component, Taz1, might share similar functions. Here, we identified unexpected properties for Rif1 and Taz1 by conducting high-throughput genetic screens designed to identify *cis*- and *trans*-acting factors capable of creating heterochromatin–euchromatin boundaries in fission yeast. The preponderance of *cis*-acting elements identified in the screens originated from genomic loci bound by Taz1 and associated with origins of replication whose firing is repressed by Taz1 and Rif1. Boundary formation and gene silencing by these elements required Taz1 and Rif1 and coincided with altered replication timing in the region. Thus, small chromosomal elements sensitive to Taz1 and Rif1 (*STAR*) could simultaneously regulate gene expression and DNA replication over a large domain, at the edge of which they established a heterochromatin–euchromatin boundary. Taz1, Rif1, and Rif1-associated protein phosphatases Sds21 and Dis2 were each sufficient to establish a boundary when tethered to DNA. Moreover, efficient boundary formation required the amino-terminal domain of the Mcm4 replicative helicase onto which the antagonistic activities of the replication-promoting Dbf4-dependent kinase and Rif1-recruited phosphatases are believed to converge to control replication origin firing. Altogether these observations provide an insight into a coordinated control of DNA replication and organization of the genome into expression domains.

heterochromatin | chromatin boundaries | DNA replication program | gene silencing | fission yeast

The functional organization of eukaryotic genomes into distinct transcriptionally competent and inert domains, euchromatin and heterochromatin, provides a powerful mechanism for the coordinated regulation of gene expression at the domain level and orchestrates numerous DNA processes in addition to transcription including replication, recombination, and DNA damage repair. Various DNA elements have the ability to precisely position expression-state boundaries between active and silent chromatin regions. Often multipartite, these elements play essential roles in genome organization by preventing the expansion of heterochromatin or euchromatin, whereas the use of alternate elements or barriers directs differential gene expression during development (reviewed by refs. 1 and 2). For example, some CTCF-binding sites in the vertebrate Hox clusters prevent euchromatin expansion (3). At fission yeast centromeres, the Borderline element prevents heterochromatin expansion (4). The scs and scs' elements in *Drosophila* or the *IR-L* and *IR-R* inverted repeats in fission yeast can prevent the expansion of either active or silent expression states (5, 6).

Methylation of histone 3 lysine 9 (H3K9me) is a hallmark of repressive chromatin in many eukaryotes (7–9). This modification plays an essential role in the assembly of higher-order chromatin by serving as a platform for the recruitment of chromatin proteins. Among its structural roles H3K9me participates in centromere positioning (10) and cohesion, as shown in the fission yeast *Schizosaccharomyces pombe* (11), and it is thus necessary for proper chromosome segregation. Furthermore, H3K9me is responsible for silencing transposable elements in the pericentromeric DNA (12) and retroviruses in embryonic stem cells (13). H3K9me also represses long regions in subtelomeric regions and chromosome arms, such as the olfactory gene clusters in mouse (14) or subtelomeric genes in fission yeast (15), moderating the expression of genes that are essential to cell differentiation and adaptive processes (reviewed by refs. 7 and 9). Mechanistically, H3K9me has attracted attention as a potentially self-templating modification capable of transmitting epigenetic memory (16–18). Through its epigenetic transmission to daughter cells it would ensure the inheritance of structures and expression profiles. A proposed mechanism for epigenetic inheritance is that the association of H3K9 methyltransferases with replicative polymerases would facilitate the modification of newly deposited nucleosomes with marks specific for the replicated region, as might also be the case for other modifications (19–22). Such associations have indicated that replication control could be important for the chromatin domain organization of chromosomes by restricting the spreading of modifying enzymes to specific regions, either in 2D along the chromatin fiber or perhaps in 3D, through the coregulation of replication origins or forks occupying a common subnuclear compartment (23).

We investigated the mechanisms through which chromatin domains with different potential for gene expression are separated from each other in fission yeast using a "boundary trap" for chromosomal elements capable of functionally replacing a natural chromatin boundary, *IR-R*. *IR-R* is one of two repeats that set limits to a ∼20-kb heterochromatic domain in the mating-type region (6, 24) (Fig. 1). Deleting either repeat blurs the naturally sharp border between heterochromatin and euchromatin on the side of the

Significance

In recent years, high-throughput studies have revealed the organization of eukaryotic genomes into chromatin domains, topologically associated domains, and replication domains, laying out important principles for the spatiotemporal organization of the nucleus. Our work shows how customized genetic screens with model organisms can help delineate functional relationships between the different layers of organization and identify the *cis*- and *trans*-acting elements underpinning them. Here, such approaches revealed that DNA elements and proteins that regulate replication-origin firing in yeasts and other eukaryotes can help partition the genome into expressed and repressed domains. We expect this insight acquired with fission yeast will inspire research in other organisms where the proteins we identified, the Shelterin component Rif1 and its effectors, are intensively studied.

Author contributions: T.T., B.M., Y.K., P.B., H.M., and G.T. designed research; T.T., B.M., Y.K., P.B., D.G., J.V.-H., and G.T. performed research; T.T., B.M., Y.K., P.B., D.G., H.M., and G.T. analyzed data; and T.T., B.M., P.B., and G.T. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]To whom correspondence should be addressed. Email: gen@bio.ku.dk.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1614837114/-/DCSupplemental.

deletion (6). In most cells, heterochromatin is weakened, allowing expression of reporter genes placed in the normally silenced heterochromatic region. In a few cells reporter genes placed on the euchromatic side are silenced (6). The latter effect is hard to detect but overexpressing the HP1-like protein Swi6 or deleting its antagonist Epe1 in cells with an impaired boundary element increases heterochromatin expansion, and such experiments have lent credence to the idea that TFIIIC-binding sites within *IR-R* limit heterochromatin expansion (25), similar to a boundary element in *Saccharomyces cerevisiae* (26). Here, we identified chromosomal elements that could functionally replace *IR-R* in an otherwise wild-type background and we went on to identify *trans*-acting factors with which the trapped elements function. The assay was for heterochromatic gene silencing, yet nearly all identified factors have documented roles in DNA replication, suggesting a functional overlap between replication and boundary formation.

Results and Discussion

Within the ~20-kb domain delimited by the inverted repeats *IR-L* and *IR-R* in the chromosome 2 of fission yeast the nucleosomes are methylated at histone H3K9 and hypoacetylated, resulting in gene silencing. The boundary trap relies on the fact that deleting *IR-R* alleviates silencing of an *ade6*$^+$ reporter gene inserted at the edge of the heterochromatic domain (Fig. 1). This provides a visual assay where cells with an intact boundary form red colonies on plates with a low adenine concentration whereas cells lacking a boundary form white colonies due to infringement of euchromatin into the normally silent region. We performed genome-wide screens for genomic elements that, when inserted in the place of *IR-R*, permit heterochromatic repression of *ade6*$^+$ while preserving expression of a *LEU2* gene in the adjacent euchromatin. In addition to the rDNA repeats that we described previously (27), the boundaries of centromere 1 and centromere 3 and the endogenous boundaries of the mating-type region, six unique chromosomal elements originating from the arms of chromosome 1 and 2 were repeatedly isolated, in a total of 35 independent integration events for which representative clones are shown in Fig. 1. In addition, centromeric repeats and the related *cenH* element also exerted a strong, partially bidirectional, repression that extended to *LEU2*, at least for one insert orientation. Eight elements that restored the repression of (*EcoRV*)::*ade6*$^+$ weakly also made it through the screen (Fig. S1), including four Tf2 retrotransposons. The latter elements were isolated only once, possibly due to their weaker phenotype, and they were not characterized further even though some might represent bona fide boundary or silencing elements. TFIIIC-binding sites were not enriched among the recovered elements.

At their endogenous chromosomal locations five of the six elements displayed in Fig. 1 are associated with small patches of H3K9me identified previously in a genome-wide survey as heterochromatic islands (28) (Fig. 1*D*). Most heterochromatic islands contain meiotic genes that are repressed in vegetative cells by the Red1 protein (28). Our screen did not identify Red1-dependent islands, indicating that the ability to form a small H3K9me domain is not sufficient to replace *IR-R*. However, the screen identified nearly all Red1-independent islands, indicating that these elements share a common function or mechanism of action that makes them stand out in our selection procedure.

Each artificial boundary was subjected to a deletion analysis to locate active elements within the large inserts originally captured in the boundary trap. In the case of the five H3K9me-associated elements, subclones as small as 62–128 bp had full boundary activity (Fig. 2 *A* and *B* and Figs. S2 and S3). All active subclones of these five elements contained short repeats of the sequence GGTTA and their boundary activity correlated with the number of repeats (Fig. 2 *A* and *B*). These repeats were previously identified in studies mapping the chromosomal associations of the protein Taz1 (homolog of mammalian TRF1/2), showing Taz1 physically interacts with the elements at their endogenous locations (29, 30). Consistently, in the

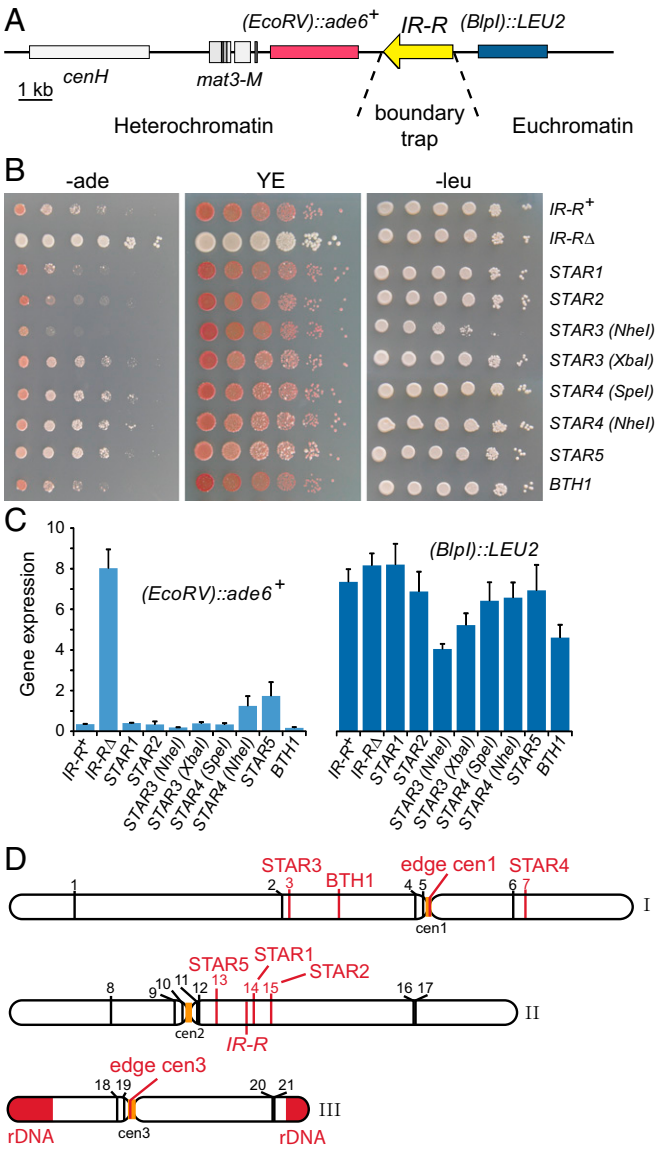

Fig. 1. Boundary trap and trapped elements. (*A*) Edge of heterochromatic domain in the mating-type region showing the two reporters used for the screen: (*EcoRV*)::*ade6*$^+$ and (*BlpI*)::*LEU2*. (*B*) Boundary effects of trapped elements. Ten-fold dilutions of strains with indicated *IR-R* replacements were spotted onto selective media to monitor expression of (*EcoRV*)::*ade6*$^+$ and (*BlpI*)::*LEU2*. Red colony color on YE indicates repression of (*EcoRV*)::*ade6*$^+$. *STAR3* and *STAR4* were each independently isolated from two genomic libraries. *IR-R*$^+$, PG3950; *IR-R*Δ, PG3947. (*C*) Quantification of (*EcoRV*)::*ade6*$^+$ and (*BlpI*)::*LEU2* transcripts by RT–quantitative PCR (RT-QPCR) for strains displayed in *B*, expressed as percent of *act1*$^+$ transcript. (*D*) Endogenous chromosomal locations of trapped elements, shown in red. Corresponding heterochromatic islands are numbered in red; other islands are in black.

mating-type region, Taz1 was required for the boundary effects of GGTTA-containing elements, specifically (Fig. 2 *C*–*E*).

To search for additional effectors of the artificial boundaries, we combined them with an arrayed library of *S. pombe* ORF deletions (Bioneer) in high-throughput genetic crosses. Mutants in which boundaries failed to repress (*EcoRV*)::*ade6*$^+$ were identified. According to their requirements for transacting factors, the five elements that contained GGTTA repeats clustered into one homogeneous class that showed a potent and specific requirement for Rif1 (Fig. 2 *C* and *D*). Rif1 is a chromatin-associated protein with an evolutionarily conserved role in the replication-timing program

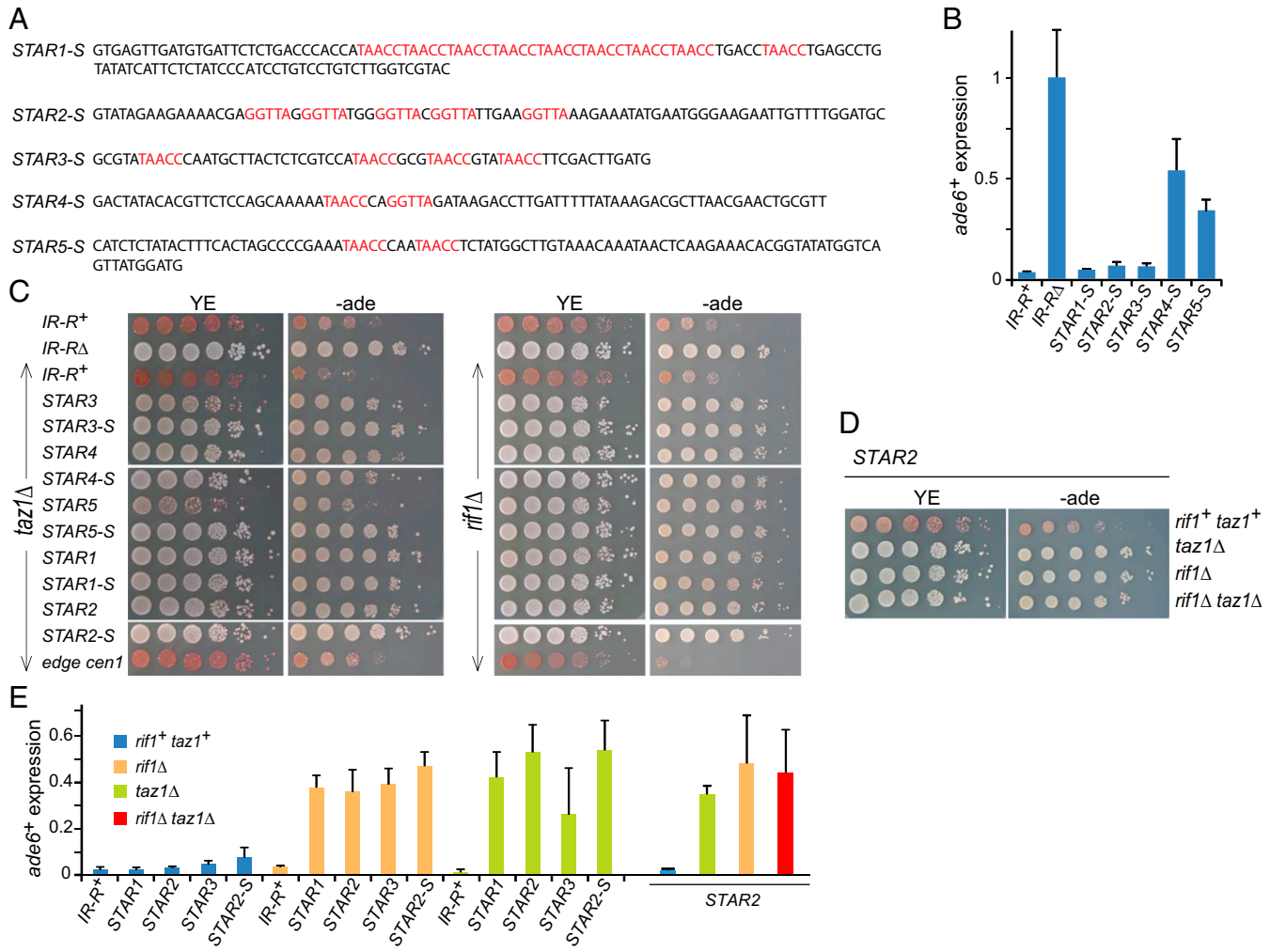

Fig. 2. Boundary formation by *STAR* elements requires Taz1 and Rif1. (*A*) Sequence of smallest subclones of *STAR* elements capable of restoring (*EcoRV*)::*ade6*$^+$ repression, with putative Taz1-binding sites in red. (*B*) Repression of (*EcoRV*)::*ade6*$^+$ by *STAR* subclones, assayed as in Fig. 1*C*. (*C*–*E*) Derepression of (*EcoRV*)::*ade6*$^+$ in *taz1Δ* and *rif1Δ* mutants with indicated boundary elements, and in a double *taz1Δ rif1Δ* mutant with the *STAR2* boundary, assayed as in Fig. 1 *B* and *C*. The (*EcoRV*)::*ade6*$^+$ transcript levels were normalized to *act1*$^+$ measured in the same samples and to the (*EcoRV*)::*ade6*$^+$ transcript in *IR-RΔ* cells propagated in parallel.

(reviewed by refs. 31 and 32). In yeast, due to loss of Rif1 both early-late and late-early switching of origin firing has been documented, and in mammalian cells even more dramatic defects in the replication program have been observed, such as fusing of distinct replication domains (33, 34). Fostering of heterochromatin by the smallest subclones was also strongly dependent on Taz1 and Rif1 (Fig. 2 *C*–*E*). Hence, we refer to them as *STAR* elements (for *S*ensitivity to *T*az1 *A*nd *R*if1) and to the element that is not associated with H3K9me or GGTTA repeats as *BTH1* (*B*oundary *T*o *H*eterochromatin 1). An epistasis analysis performed with the *STAR2* element indicated that Taz1 and Rif1 operate in the same pathway to restore boundary function (Fig. 2 *D* and *E*).

At their endogenous locations the five *STAR* elements are close to origins of replication that fire late in S phase and whose late activation depends on both Taz1 and Rif1 (29, 35). In total, 26 Taz1-dependent late/dormant origins were identified by Tazumi et al. (29) at internal locations in chromosome arms including ori2100 (associated with *STAR2*), ori1097 (associated with STAR3), ori1176 (associated with STAR4), and ori2100 (associated with STAR5). Although STAR1 is not close to a numbered origin, replication at *STAR1* is also inhibited by Taz1 and *STAR1* is bound by Taz1 (35). Taz1 was detected at 16 of the 26 Taz1-dependent late/dormant origins and GGTTA repeats were detected at some of them (29), raising the question of whether these elements might create chromatin boundaries in our assay similar to the isolated *STAR* elements. Testing seven of them, we found that large fragments in the 1.4- to 9-kb range had weak boundary activity, probably too weak for them to stand out in a screen (Fig. S4). Smaller fragments in the 140- to 370-bp range were also inefficient but their effect was enhanced when cloned in two or three copies (Fig. S4). Hence the ability to form chromatin boundaries seems to be shared by the whole class of elements to a various degree, with the isolated *STAR* elements being particularly potent, to an extent correlated with the number of GGTTA repeats and to H3K9me at the endogenous locations. In chromosome arms, whereas all Taz1-dependent origins are also regulated by Rif1, the converse is not true, indicating that Rif1 is necessary for the regulation of the origin timing program and Taz1 attracts Rif1 to some dormant or late-firing origins. This remarkable property of the elements suggested that boundary formation in the mating-type region might be intimately linked to effects on replication.

We expanded on previous studies (36, 37) by monitoring DNA replication over 60 kb encompassing the silent part of the mating-type region, measuring incorporation of BrdU into the DNA of synchronized cells arrested with hydroxyurea (HU) to produce conditions where only early replicating DNA is labeled (Fig. 3). A similar experiment conducted in the absence of HU, where BrdU

incorporation was measured at 25 min and 30 min following synchronous release of cells into M phase, produced essentially the same results (Fig. S5). In wild-type cells, the whole heterochromatic domain replicated early whereas the flanking euchromatin replicated late (Fig. 3*A*). Deletion of *IR-R* eroded the pattern at the edge of the region, correlating with increased *ade6*$^+$ expression. The two artificial boundaries tested, *STAR2-12* (270 bp) and *STAR2-S* (90 bp), induced a major change in replication timing in addition to their effects on gene expression (Fig. 3*A*). Replication was delayed in the whole region; the firing of the adjacent Ori881 as well as Ori879 and Ori880 at the other edge of the silenced domain, ~20 kb away, was reduced (Fig. 3*A*). At a similar distance on the euchromatic side, Ori882 was unaffected. Unlinked early and late origins monitored as controls were unaffected (Fig. S5). In the wild-type mating-type region it has been proposed that early replication is prompted by binding of the DDK activator Dfp1 to the chromodomain protein Swi6 present throughout the domain (37). Our result indicate that *STAR* elements are capable of counteracting this activation throughout the region, together with Taz1, Rif1, and possibly other factors, to create a different replication profile that is still bound by *IR-L* on the left and by the artificial boundary on the right.

Across organisms Rif1 represses origin firing by recruiting protein phosphatase 1 (PP1) through RVxF/SILK docking motifs (32, 38, 39). The recruited phosphatases are believed to antagonize the action of Hsk1 (Dbf4-dependent kinase, or DDK) and Cdc2 (cyclin-dependent kinase, or CDK) at origins, delaying phosphorylation events necessary to origin firing. In *S. pombe*, the PP1 phosphatases Dis2 and Sds21 are recruited by Rif1. Here, we found that a Rif1 mutant whose interaction with Dis2 and Sds21 is impaired (Rif1-PP1, ref. 38) failed to properly support boundary formation by the three elements tested (Fig. 3 *B* and *C* and Fig. S6), consistent with Rif1's establishing a boundary together with Dis2 or Sds21. Moreover, the phosphorylation of Rif1 late in S phase, when the CDK/DDK activity is high, would cause PP1 dissociation, thereby releasing the replication block at late origins (Fig. 3*B*). Consistently, a Rif1 mutant whose predicted phosphorylation sites are mutated to alanine displays enhanced interactions with Dis2 and Sds21 (Rif1-7A, ref. 38) and reinforced boundary activity (Fig. 3*C* and Fig. S6). In contrast, the phosphomimic Rif1-12D whose interactions with Dis2 and Sds21 are reduced (38) alleviated boundary activity and so did the double Rif1-7APP1 mutant, as expected (Fig. 3*C* and Fig. S6). Together these phenotypes strongly point to the boundary activity of Rif1's being executed through its recruitment of PP1 phosphatases. Thus, regulation might occur through the control of DNA replication. Alternatively, Taz1, Rif1, Dis2, and Sds21 might affect chromatin structure independently of DNA replication, perhaps by dephosphorylating histone H3S10 (39), or by recruiting histone-modifying enzymes (30).

To start investigating these possibilities, we turned to examining downstream effectors of Rif1 that would be more specific to DNA replication. In *S. cerevisiae,* deletion of a serine- and threonine-rich amino-terminal domain of Mcm4 abrogates the Rif1-mediated delay of replication origin firing, suggesting that the amino terminus of Mcm4 has an inhibitory effect on helicase activity that is released by DDK-mediated phosphorylation, or maintained by Rif1-associated phosphatases (40–42)). Here, deletion of 148 aa at the structurally similar amino terminus of *S. pombe* Mcm4 alleviated (*EcoRV*)::*ade6*$^+$ repression (Fig. 3 *D* and *E*), favoring models where boundary formation relies on Mcm4-mediated inhibition of origin firing. Not only the Rif1-dependent boundary elements but also the wild-type *IR-R* element failed to function properly in the Mcm4 mutant (Fig. 3*D*), showing that these boundaries ultimately rely on a common mechanism. The loss of silencing was not as pronounced as in *rif1*Δ mutants (compare Fig. 2 *C–E* and Fig. 3*D*), which fits the fact that Mcm4 is not the sole factor mediating the effects of Rif1 on origin

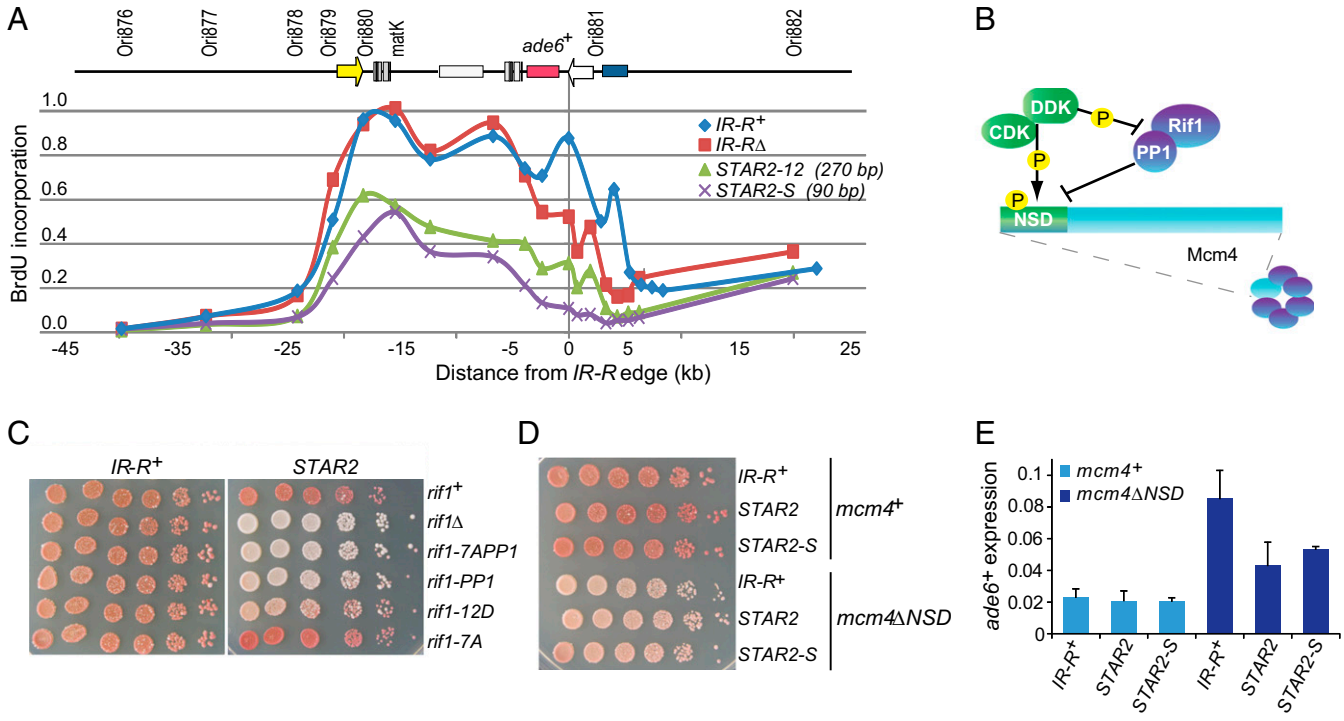

Fig. 3. Regional control of replication by *STAR* elements and requirement for Rif1- and Mcm4 replication regulatory domains for boundary positioning. (*A*) Replication was assayed by BrdU incorporation in the presence of HU in cell-cycle-synchronized cells with the indicated boundaries. (*B*) Proposed regulation of MCM complex by CDK/DDK and Rif1-associated phosphatases (PP1), depicting amino-terminal serine-rich domain (NSD) of Mcm4 that represses origin firing unless phosphorylated. (*C*) Rif1 mutations that reduce its interaction with PP1 (*rif1Δ*; *rif1-7A-PP1*; *rif1-PP1*; and *rif1-12D*) reduce boundary formation by *STAR2* unlike mutations that prevent phosphorylation by CDK/DDK (*rif1-7A*). (*D and E*) Derepression of (*EcoRV*)::*ade6*$^+$ in *mcm4ΔNSD* mutant assayed as in Fig. 2 *C–E*.

firing. Part of the regulation might occur on other MCM subunits or on the initiation factor Sld3 (43, 44). The silencing defects in *mcm4ΔNSD* mutants extend the mutational analyses presented above to reinforce the idea that replication control is necessary to heterochromatic silencing at the edge of the silent domain.

Finally, to test the capacity of Rif1 and associated proteins to form chromatin boundaries locally, as opposed to indirect effects, we tethered these proteins individually to the edge of the mating-type region. Five Gal4 binding sites (5xGBS) were inserted in the place of *IR-R* and proteins fused to the Gal4 DNA-binding domain (GBD) were expressed in the same cells. Fusions of GBD to Dis2, Sds21, Rif1, or Taz1, but not GBD alone, protected the heterochromatic domain (Fig. 4). Tethering Rif1 or the phosphatases in a *taz1*Δ background produced similar results. The fusion proteins had no effect in the absence of Gal4-binding sites (Fig. 4) and they did not induce rearrangements at (*EcoRV*)::*ade6*$^+$ when tethered (Fig. S7). Based on these observations, a plausible model is that Taz1 bound to the GGTTA repeats attracts Rif1 (45) and, in turn, the Dis2 and Sds21 phosphatases, to regulate chromatin structure.

The study of gene silencing in budding yeasts has indicated how proteins with roles in DNA replication might participate in gene silencing independently of their function in DNA replication (46, 47). In the present case, a functional relationship between replication control and heterochromatic silencing is supported beyond the reported experiments by the fact that the Dos2/Clr7 subunit of the *S. pombe* H3K9 methyltransferase complex CLRC associates with the catalytic subunit of DNA polymerase epsilon and that the disruption of this interaction causes defective heterochromatin formation (20). Thus, the control of replication might be required for boundary positioning by regulating the spreading of CLRC. Consistently, our large-scale screens identified factors involved in DNA replication control in addition to Taz1, Rif1, and Mcm4, including the replication fork protection complex and, in the case of the rDNA, the replication blocker Reb1 (27). We speculate that delayed replication at the edge of the domain following boundary deletion (Fig. 3*A*) might lead to the dissociation of CLRC from DNA polymerases at arrested forks, as proposed for other mutants (48), or replication forks incoming from the euchromatic side at a later time might propagate euchromatic marks into the heterochromatic domain in a fraction of the cell population. Taz1 and Rif1 would counteract these effects when attracted to the edge of the domain, by consolidating the heterochromatic domain into a single replication domain. Because other heterochromatic domains are late-replicating in *S. pombe* (36), replication timing per se might not be as relevant to heterochromatin integrity as unimpeded replication. In addition, the Rif1- associated phosphatases or other modifying enzymes bound by Rif1 or Taz1 might directly modify nucleosomes within the defined domain. Heterochromatin can in turn be expected to affect the replication program (49), altogether resulting in reciprocal interplays between replication and heterochromatin formation.

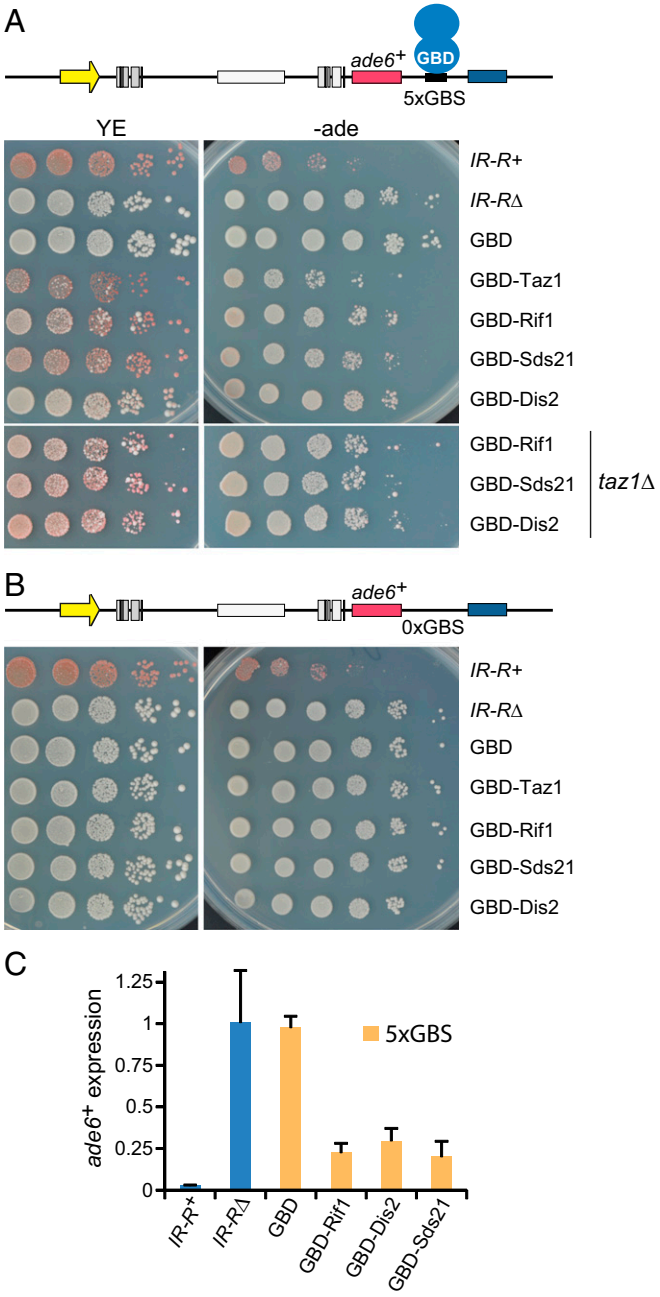

Fig. 4. Restoration of heterochromatic domain integrity by tethered Taz1, Rif1, or protein phosphatases. (*A*) Taz1, Rif1, Sds21, and Dis2 expressed as fusions with the Gal4-binding domain (GBD) mediate (*EcoRV*)::*ade6*$^+$ silencing when targeted to five Gal4-binding sites (5xGBS). Repression by GBD-Rif1, GBD-Sds21, and GBD-Dis2 occurs in the absence of Taz1. (*B*) Repression requires tethering to 5xGBS. (*C*) RT-QPCR analysis of transcripts as in Fig. 2*E*.

Materials and Methods

Strain Constructions and Expression Assays. The previously described *S. pombe* strain PG2897 and plasmid pGT299 (27) were used throughout the study to integrate DNA constructs at the edge of the mating-type region as described in detail in *Supporting Information*. This included the boundary-trap screen, deletion analysis of trapped elements, and insertion of Gal4 binding sites. The genotypes of the produced strains are listed in Table S1 and the sequences of all oligonucleotides used in the study are in Table S2. Table S3 shows the chromosomal coordinates of the trapped boundary elements and Table S4 provides an overview of the primers, plasmids, and strains for the deletion analysis of trapped elements. Expression of the (*EcoRV*)::*ade6*$^+$ and (*BlpI*)::*LEU2* reporter genes was monitored as described previously (27).

Analyses of Replication Efficiency. Strains KYP4001-4004 (under *nda3-KM311* background) expressing thymidine kinase were grown at 20 °C for 5 h and M-phase-arrested cells were released at 30 °C in YES medium containing 25 mM HU and 200 μg/mL BrdU for 60 min. Alternatively, cells were released in the absence of HU and propagated for 25 min or 30 min as indicated. Genomic DNA was isolated with the Qiagen genomic DNA buffer set and Qiagen Genome-tip 100/G and was sheared to an average size of 300 bp by sonication. One microgram of the sheared DNA was denatured at 100 °C for 10 min, and BrdU-substituted DNA was immunoprecipitated by Dynabeads sheep-anti mouse IgG (11031; Thermo Fisher) attached to anti-BrdU antibody (MBL, MI-11-3, 2.5 μg). The beads were washed with lysis buffer and DNA was eluted, as described (50, 51). The immunoprecipitated DNA was treated with proteinase K, purified by a QIAquick PCR purification kit (Qiagen), and used for quantitative PCR performed with SYBR Premix Ex Taq (TaKaRa Bio) on a LightCycler 480 (Roche). The

amount of the immunoprecipitated DNA relative to the input chromatin (percent) is presented as immunoprecipitation efficiency in Fig. 3 and Fig. S5.

ACKNOWLEDGMENTS. We thank the National BioResource Project and Alessandro Bianchi for plasmids and strains and members of our laboratories for discussions. This research was supported by the Novo Nordisk Foundation (G.T.) and by Japan Society for Promotion of Science KAKENHI Grant-in-Aid for Scientific Research (A) Grants 23247031 and 26251004 and Grant-in-Aid for Scientific Research on Priority Areas ("non-coding RNA") Grant 24114520 and the Naito Foundation Continuation Subsidy for Outstanding Projects (H.M.).

1. Barkess G, West AG (2012) Chromatin insulator elements: Establishing barriers to set heterochromatin boundaries. *Epigenomics* 4(1):67–80.
2. Ali T, Renkawitz R, Bartkuhn M (2016) Insulators and domains of gene expression. *Curr Opin Genet Dev* 37:17–26.
3. Narendra V, et al. (2015) CTCF establishes discrete functional chromatin domains at the Hox clusters during differentiation. *Science* 347(6225):1017–1021.
4. Keller C, Kulasegaran-Shylini R, Shimada Y, Hotz HR, Bühler M (2013) Noncoding RNAs prevent spreading of a repressive histone mark. *Nat Struct Mol Biol* 20(8):994–1000.
5. Kellum R, Schedl P (1991) A position-effect assay for boundaries of higher order chromosomal domains. *Cell* 64(5):941–950.
6. Thon G, Bjerling P, Bünner CM, Verhein-Hansen J (2002) Expression-state boundaries in the mating-type region of fission yeast. *Genetics* 161(2):611–622.
7. Becker JS, Nicetto D, Zaret KS (2016) H3K9me3-dependent heterochromatin: Barrier to cell fate changes. *Trends Genet* 32(1):29–41.
8. Mozzetta C, Boyarchuk E, Pontis J, Ait-Si-Ali S (2015) Sound of silence: The properties and functions of repressive Lys methyltransferases. *Nat Rev Mol Cell Biol* 16(8):499–513.
9. Politz JC, Scalzo D, Groudine M (2013) Something silent this way forms: The functional organization of the repressive nuclear compartment. *Annu Rev Cell Dev Biol* 29:241–270.
10. Fukagawa T, Earnshaw WC (2014) The centromere: Chromatin foundation for the kinetochore machinery. *Dev Cell* 30(5):496–508.
11. Bernard P, et al. (2001) Requirement of heterochromatin for cohesion at centromeres. *Science* 294(5551):2539–2542.
12. Martienssen R, Moazed D (2015) RNAi and heterochromatin assembly. *Cold Spring Harb Perspect Biol* 7(8):a019323.
13. Karimi MM, et al. (2011) DNA methylation and SETDB1/H3K9me3 regulate predominantly distinct sets of genes, retroelements, and chimeric transcripts in mESCs. *Cell Stem Cell* 8(6):676–687.
14. Magklara A, et al. (2011) An epigenetic signature for monoallelic olfactory receptor expression. *Cell* 145(4):555–570.
15. Hansen KR, et al. (2005) Global effects on gene expression in fission yeast by silencing and RNA interference machineries. *Mol Cell Biol* 25(2):590–601.
16. Lachner M, O'Carroll D, Rea S, Mechtler K, Jenuwein T (2001) Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. *Nature* 410(6824):116–120.
17. Audergon PN, et al. (2015) Epigenetics. Restricted epigenetic inheritance of H3K9 methylation. *Science* 348(6230):132–135.
18. Ragunathan K, Jih G, Moazed D (2015) Epigenetics. Epigenetic inheritance uncoupled from sequence-specific recruitment. *Science* 348(6230):1258699.
19. Huen MS, Sy SM, van Deursen JM, Chen J (2008) Direct interaction between SET8 and proliferating cell nuclear antigen couples H4-K20 methylation with DNA replication. *J Biol Chem* 283(17):11073–11077.
20. Li F, Martienssen R, Cande WZ (2011) Coordination of DNA replication and histone modification by the Rik1-Dos2 complex. *Nature* 475(7355):244–248.
21. Chuang LS, et al. (1997) Human DNA-(cytosine-5) methyltransferase-PCNA complex as a target for p21WAF1. *Science* 277(5334):1996–2000.
22. Wada M, et al. (2002) The second largest subunit of mouse DNA polymerase epsilon, DPE2, interacts with SAP18 and recruits the Sin3 co-repressor protein to DNA. *J Biochem* 131(3):307–311.
23. Pichugina T, et al. (2016) A diffusion model for the coordination of DNA replication in Schizosaccharomyces pombe. *Sci Rep* 6:18757.
24. Noma K, Allis CD, Grewal SI (2001) Transitions in distinct histone H3 methylation patterns at the heterochromatin domain boundaries. *Science* 293(5532):1150–1155.
25. Noma K, Cam HP, Maraia RJ, Grewal SI (2006) A role for TFIIIC transcription factor complex in genome organization. *Cell* 125(5):859–872.
26. Donze D, Adams CR, Rine J, Kamakaka RT (1999) The boundaries of the silenced HMR domain in Saccharomyces cerevisiae. *Genes Dev* 13(6):698–708.
27. Jakociunas T, et al. (2013) Subnuclear relocalization and silencing of a chromosomal region by an ectopic ribosomal DNA repeat. *Proc Natl Acad Sci USA* 110(47):E4465–E4473.
28. Zofall M, et al. (2012) RNA elimination machinery targeting meiotic mRNAs promotes facultative heterochromatin formation. *Science* 335(6064):96–100.
29. Tazumi A, et al. (2012) Telomere-binding protein Taz1 controls global replication timing through its localization near late replication origins in fission yeast. *Genes Dev* 26(18):2050–2062.
30. Zofall M, Smith DR, Mizuguchi T, Dhakshnamoorthy J, Grewal SI (2016) Taz1-shelterin promotes facultative heterochromatin assembly at chromosome-internal sites containing late replication origins. *Mol Cell* 62(6):862–874.
31. Renard-Guillet C, Kanoh Y, Shirahige K, Masai H (2014) Temporal and spatial regulation of eukaryotic DNA replication: From regulated initiation to genome-scale timing program. *Semin Cell Dev Biol* 30:110–120.
32. Mattarocci S, Hafner L, Lezaja A, Shyian M, Shore D (2016) Rif1: A conserved regulator of DNA replication and repair hijacked by telomeres in yeasts. *Front Genet* 7:45.
33. Cornacchia D, et al. (2012) Mouse Rif1 is a key regulator of the replication-timing programme in mammalian cells. *EMBO J* 31(18):3678–3690.
34. Yamazaki S, et al. (2012) Rif1 regulates the replication timing domains on the human genome. *EMBO J* 31(18):3667–3677.
35. Hayano M, et al. (2012) Rif1 is a global regulator of timing of replication origin firing in fission yeast. *Genes Dev* 26(2):137–150.
36. Kim SM, Dubey DD, Huberman JA (2003) Early-replicating heterochromatin. *Genes Dev* 17(3):330–335.
37. Hayashi MT, Takahashi TS, Nakagawa T, Nakayama J, Masukata H (2009) The heterochromatin protein Swi6/HP1 activates replication origins at the pericentromeric region and silent mating-type locus. *Nat Cell Biol* 11(3):357–362.
38. Davé A, Cooley C, Garg M, Bianchi A (2014) Protein phosphatase 1 recruitment by Rif1 regulates DNA replication origin firing by counteracting DDK activity. *Cell Reports* 7(1):53–61.
39. Sreesankar E, Bharathi V, Mishra RK, Mishra K (2015) Drosophila Rif1 is an essential gene and controls late developmental events by direct interaction with PP1-87B. *Sci Rep* 5:10679.
40. Hiraga S, et al. (2014) Rif1 controls DNA replication by directing Protein Phosphatase 1 to reverse Cdc7-mediated phosphorylation of the MCM complex. *Genes Dev* 28(4):372–383.
41. Sheu YJ, Stillman B (2006) Cdc7-Dbf4 phosphorylates MCM proteins via a docking site-mediated mechanism to promote S phase progression. *Mol Cell* 24(1):101–113.
42. Sheu YJ, Stillman B (2010) The Dbf4-Cdc7 kinase promotes S phase by alleviating an inhibitory activity in Mcm4. *Nature* 463(7277):113–117.
43. Masai H, et al. (2006) Phosphorylation of MCM4 by Cdc7 kinase facilitates its interaction with Cdc45 on the chromatin. *J Biol Chem* 281(51):39249–39261.
44. Bruck I, Kaplan DL (2015) Conserved mechanism for coordinating replication fork helicase assembly with phosphorylation of the helicase. *Proc Natl Acad Sci USA* 112(36):11223–11228.
45. Kanoh J, Ishikawa F (2001) spRap1 and spRif1, recruited to telomeres by Taz1, are essential for telomere function in fission yeast. *Curr Biol* 11(20):1624–1630.
46. Liachko I, Tye BK (2009) Mcm10 mediates the interaction between DNA replication and silencing machineries. *Genetics* 181(2):379–391.
47. Hickman MA, Froyd CA, Rusche LN (2011) Reinventing heterochromatin in budding yeasts: Sir2 and the origin recognition complex take center stage. *Eukaryot Cell* 10(9):1183–1192.
48. Zaratiegui M, et al. (2011) RNAi promotes heterochromatic silencing through replication-coupled release of RNA Pol II. *Nature* 479(7371):135–138.
49. Kaykov A, Taillefumier T, Bensimon A, Nurse P (2016) Molecular combing of single DNA molecules on the 10 megabase scale. *Sci Rep* 6:19636.
50. Katou Y, et al. (2003) S-phase checkpoint proteins Tof1 and Mrc1 form a stable replication-pausing complex. *Nature* 424(6952):1078–1083.
51. Kanoh Y, et al. (2015) Rif1 binds to G quadruplexes and suppresses replication over long distances. *Nat Struct Mol Biol* 22(11):889–897.
52. Ekwall K, Thon G (2016) Genetic analysis of Schizosaccharomyces pombe. *Fission Yeast: A Laboratory Manual*, eds Hagan I, Carr AM, Grallert A, Nurse P (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY), Chap 3.
53. Kim DU, et al. (2010) Analysis of a genome-wide set of gene deletions in the fission yeast Schizosaccharomyces pombe. *Nat Biotechnol* 28(6):617–623.
54. Baryshnikova A, et al. (2010) Synthetic genetic array (SGA) analysis in Saccharomyces cerevisiae and Schizosaccharomyces pombe. *Methods Enzymol* 470:145–179.
55. Matsuyama A, et al. (2004) pDUAL, a multipurpose, multicopy vector capable of chromosomal integration in fission yeast. *Yeast* 21(15):1289–1305.
56. Matsuyama A, et al. (2006) ORFeome cloning and global analysis of protein localization in the fission yeast Schizosaccharomyces pombe. *Nat Biotechnol* 24(7):841–847.
57. Lyne R, et al. (2003) Whole-genome microarrays of fission yeast: Characteristics, accuracy, reproducibility, and processing of array data. *BMC Genomics* 4(1):27.