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JB Review

G-quadruplex binding protein Rif1, a key regulator of replication timing

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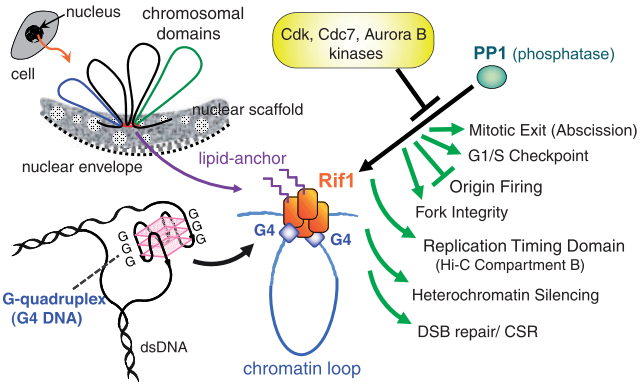
DNA replication is spatially and temporally regulated during S phase to execute efficient and coordinated duplication of entire genome. Various epigenomic mechanisms operate to regulate the timing and locations of replication. Among them, Rif1 plays a major role to shape the 'replication domains' that dictate which segments of the genome are replicated when and where in the nuclei. Rif1 achieves this task by generating higher-order chromatin architecture near nuclear membrane and by recruiting a protein phosphatase. Rif1 is a G4 binding protein, and G4 binding activity of Rif1 is essential for replication timing regulation in fission yeast. In this article, we first summarize strategies by which cells regulate their replication timing and then describe how Rif1 and its interaction with G4 contribute to regulation of chromatin architecture and replication timing.

Keywords: chromatin structure; DNA replication; G-quadruplexes; DNA–protein interaction; Rap1 interacting factor 1 (Rif1); replication timing.

Abbreviations: ars, *a*utonomously *r*eplicating *s*equences; 4C analyses, Circularized Chromosome Conformation Capture; CDK, cyclin-dependent kinase; ChIP-seq, chromatin immunoprecipitation-sequencing; CMG helicase, Cdc45–MCM–GINS helicase; CTCF,CCCTC-binding factor; CTD, C-terminal domain; CTR, constant timing region; *D. melanogaster*, *Drosophila melanogaster*; DSB, double-stranded DNA break; ERCEs, early replication control elements; ERVs, endogenous retrovirus; ESCs, embryonic stem cells; G4, G-quadruplex; GINS complex, from the Japanese Go-Ichi-Ni-San meaning 5-1-2-3 for the four subunits of the complex, Sld5, Psf1, Psf2 and Psf3; H3K9me3, covalent modification of histone protein, trimethylation of lysine 9 on histone H3; HEAT, huntingtin, elongation factor 3, a subunit of protein phosphatase 2A, and TOR/Armadillo-like helical repeats; Hp1,heterochromatin protein 1; hTERT, telomerase reverse transcriptase; IDP, intrinsically disordered polypeptide; lncRNA, long non-coding RNAs; MCM, mini-chromosome maintenance protein complexes; NHEJ, non-homologous end-joining; NTD, N-terminal domain; ORC, origin recognition complex; *oriC*, *origin* of chromosome replication; PP1, protein phosphatase 1; pre-IC,pre-initiation complex; Pre-RCs, pre-replicative complexes; RAD, Rif1-associated domains; Rif1, Rap1 interacting factor 1; Rif1BS, Rif1 binding site; Rif1CSs, Rif1 consensus sequences; *S. cerevisiae*, *Saccharomyces cerevisiae*; *S. pombe*, *Schizosaccharomyces pombe*; SMN, survival motor neuron; SUUR, SUppressor of UnderReplication; TADs, topologically associated domains; TDP, timing decision point; TRF1, telomeric repeat-binding factor 1; UFBs, ultrafine anaphase bridge.

Graphical Abstract



Rap1 interacting factor 1 (Rif1), originally discovered as a telomere binding protein in yeast, was rediscovered as a crucial protein in replication timing regulation in both yeasts and mammalian cells. Rif1 is also known to play crucial roles in selection of DSB (double-stranded DNA break) repair pathways. It also affects transcription of gene clusters. Rif1 binds to DNA with notable specificity to G-quadruplex (G4)

and the distinct functions of Rif1 may be related to its ability to bind G4. This review focuses on Rif1 protein which regulates DNA replication timing by forming specific chromatin architecture and by recruiting a phosphatase, and discusses its potential mechanisms.

Regulation of DNA Replication

Genetic information needs to be accurately duplicated to maintain the genomic integrity and to ensure the precise inheritance of phenotypic traits of cells and organisms (*1*). Although the mechanism of replication is evolutionarily conserved, its regulation is significantly different in eukaryotes in comparison to prokaryotes or even between higher and lower eukaryotes (*2*). Complex arrays of proteins are sequentially implemented for coordinated execution of DNA replication steps including initiation, elongation and termination (*2*). The whole process of replication needs to be tightly controlled (*3*).

For most of the prokaryotic organisms, replication starts from a single loci called the origin of chromosome replication (*oriC*) and is usually terminated opposite to its initiation spot (*4*). For larger genomes of most eukaryotes, genetic information are distributed across multiple chromosomes and there are many initiation sites (origins of replications) on each chromosome (*2*, *3*).

Two subsequent steps are required for initiation of eukaryotic DNA replication. The replication *origin licensing* occurs at late-M to -G1 phase in which double hexameric mini-chromosome maintenance protein complexes (MCM) are loaded onto replication origins which stay inactive until the beginning of S phase. Several MCM-loading factors, including Cdc6, Cdt1 and the origin recognition complex (ORC) are required in this multi-step process for the assembly of pre-replicative complexes (pre-RCs) [reviewed in Refs. *5–12* and a comprehensive book on DNA replication (*13*)].

Second step, *origin firing*, requires the implementation of a cascade of proteins to interact and deliver Cdc45 and the four-subunit GINS complex to MCM, resulting in formation of an active CMG helicase (Cdc45–MCM–GINS) and activation of DNA synthesis. Two kinases are involved in this process. First, Cdc7-Dbf4 kinase (also called Dbf4-dependent kinase; DDK) phosphorylates MCM subunits. Sld3 in cooperation with Sld7 and Cdc45 recognizes Mcm4 as well as other Mcm subunits that are phosphorylated by Cdc7 (*14–16*). Second kinase, cyclin-dependent kinase (CDK), functions subsequently in phosphorylating Sld2 and Sld3, which facilitates their interactions with Dpb11 in partnership with Pol ε and GINS (*13*, *17–19*). At the G1–S-phase transition, pre-RCs are converted to pre-initiation complexes (pre-ICs), in which the replicative helicase is activated, leading to unwinding of duplex DNA and synthesis of both strands (*11*).

Generation of replication forks from an origin more than once during one cell cycle leads to re-replication, an event that would create multiple copies of a specific genomic region (*3*). This leads to gene amplification and eventually to genome instability (*20*), a phenomenon observed in many human cancers (*21*).

In the S phase, more origins are licensed than needed. The dormant origins act as backups and they would be activated to replicate the unreplicated segment, if the replication fork stalls (*22*, *23*). It should be noted that the firing event at each origin may be stochastically regulated, but epigenomic regulations also apply to regulate timing and activity of origin firing (*24*).

Spatio-Temporal Regulation of DNA Replication: Timing of Origin Activation, Early- and Late-Replicating Domains

Replication of eukaryotic chromosomes follows a temporal order which is called 'replication timing program'. This process is mainly mediated by generation of several hundred kilobase to megabase sized genome segments that contain origins fired during the specific period of S phase (*2*, *25*) (for a review, see Ref. *26*). There are segments in the genome that replicate early during S phase. Early replication occurs in the transcriptionally active gene-rich domains. On the other hand, late replication is generally associated with gene-poor regions with repressive epigenetic marks.

It is important to note that, during any cell cycle, only a subset of the licensed origins is fired. In mammalian and yeast cells, origin usage is flexible in which the choice of activated origins is different from cell to cell or even in the same cell from one cycle to another (*11*). Factors that influence the timing of replication may include origin organization, several other associated factors that regulate folding and loop structures of chromosomes in the nucleus, epigenomic states, regulation of the transcriptional program and concentrations of limiting replication factors (*11*). Several mechanisms have been proposed by which cells achieve timing regulation of replication origin firing.

First, the origins of DNA replication are marked by the pre-RC components through specific covalent modification or a factor(s) that selectively interacts with a specific pre-RC prior to initiation. Fkh1 protein recruits Cdc7 kinase specifically to early-firing origins and facilitates the association of Cdc45 with these origins (*27*, *28*). In fission yeast (*Schizosaccharomyes pombe*; *S. pombe*), Mrc1 appears to bind selectively to early-firing origins prior to firing by Cdc7 kinase, potentially marking them for early firing. This role of Mrc1 is independent of its function in checkpoint control (*29*).

Second, origin firing is closely related to the chromatin regulation. Early firing is promoted by open chromatin structure or by euchromatic marks.

Third, availability of limiting initiation factors affects timing of origin firing by defining the rate of the transition from early origin initiation to late origin initiation (*30*). In early-S phase, only permissive origins are activated, whereas activation of other origins is delayed until initiation factors are recycled.

Finally, firing of mid-/late-S origins is prevented by Rif1 (*31*). It does so by two non-exclusive mechanisms. (i) Rif1 recruits protein phosphatase 1 (PP1)

which inhibits firing of surrounding origins by counteracting the phosphorylation events catalyzed by Cdc7, essential for replication initiation (*32*). (ii) Rif1 generates chromatin domains possibly through chromatin looping. This will make the chromatin compact and less mobile, enabling the nearby origin clusters to fire with similar timing (*33*).

Proteins and Chromatin Features Affecting Replication Timing

Cis-acting elements

In budding yeast (*Saccharomyces cerevisiae*; *S. cerevisiae*), early replication origins are neighboured by forkhead consensus sequences (*34*). Fkh1/Fkh2 transcription factors bind to them, and activate early-firing origins. Moreover, Fkh1/Fkh2 promote relocalization and clustering of early origins (*27*, *28*). This spatial reorganization stimulates Cdc7-dependent recruitment of the key initiation factor, Cdc45, in G1 phase, leading to pre-IC formation for early-S phase origin activation (*11*).

A subset of late-replication origins in fission yeast are associated with consensus sequences for telomere length regulator, Taz1 (*35*). It is the ortholog of human telomeric repeat-binding factor 1 (TRF1) and TRF2, and is responsible for telomere maintenance (*36*). It suppresses loading of DNA replication regulator Sld3, thus preventing the formation of pre-IC (*35*, *37*). Taz1 facilitates the association of late-firing origins with telomere, Rif1 and nuclear periphery, thereby establishes heterochromatin–euchromatin boundaries that suppress initiation through recruited PP1 (*11*, *38*, *39*).

Yompakdee *et al*. reported LCS (late consensus sequence; RKKGGGGGAW [R: A or G; W: A or T; K: G or T]) on the basis of its ability to enforce late-replication to *ars* (*a*utonomously *r*eplicating *s*equences) on a plasmid (*40*). A G-rich consensus sequence, Rif1CS (Rif1 consensus sequence), was identified as a target of Rif1 protein, an evolutionally conserved factor that regulates late replication on a genome-wide basis (*33*, *41*). This sequence will be described in details in later sections.

Human chromosome 6 and 15 encode monoallelically expressed long non-coding RNAs (lncRNA), termed ASAR6 and ASAR15 that, when disrupted, result in delayed replication of entire chromosomes. Notably, antisense strand of an L1 retrotransposon within ASAR6 is essential for chromosome-wide replication timing control (*42*). Moreover, another lncRNA, ASAR6-141, is transcribed monoallelically from the opposite chromosome 6 homolog, and is responsible for proper replication timing of the chromosome expressing ASAR6-141 (*43*). It would be interesting to examine whether Rif1 is involved in this regulation through its ability to interact with RNA (*44*).

A recent study identified ERCEs (early replication control elements) bearing super enhancer-like property in mouse ESCs (embryonic stem cells). Deletion of ERCEs resulted in a domain-wide early-to-late shift of replication timing, and their clustering seemed essential for the timing control (*45*).

Trans-acting elements

Histone acetylation has a positive role in early replication timing (*46*), and ectopic hyperacetylation of heterochromatin (which is known as chromocenters in mouse cells) leads to early replication of these regions. Also, constitutive heterochromatin regions are enriched in H3K9me3 (covalent modification of histone protein, trimethylation of lysine 9 on histone H3) and poor in histone acetylation, and thus replicate late (*46*, *47*).

Heterochromatin protein 1 (Hp1) in *Drosophila melanogaster* (*D.melanogaster*) (*48*) and its ortholog Swi6 in fission yeast (*49*) regulate replication timing in heterochromatin. Hp1 is responsible for very late replication of centromeric DNA through promoting heterochromatin formation, and thus shielding these chromatin regions from replication initiation and/or activation factors (*48*). Interestingly, Hp1 is also responsible for replication of early-replicating regions (*50*) such as pericentromeric DNA in *D.melanogaster* (*48*) or the pericentromeric region and silent mating-type locus in fission yeast (*49*). It is believed that the direct association of Hp1 with ORC (*51*) or Cdc7 might stimulate licensing or activation of replication origins, respectively (*49*).

Another key *trans*-acting factor that regulates replication timing in yeast (*31*, *52*), human (*53*) and other vertebrates (*54*) is Rif1 (*55*). Origin activation by Cdc7 kinase is inhibited by Rif1 (*31*). Global alterations of replication timing are caused by Rif1 depletion concomitant with loss of the mid-S-phase replication pattern of replication foci. Rif1 prevents MCM phosphorylation by recruiting PP1 to counteract Cdc7 kinase activity (*32*, *56–59*). Therefore, depletion of Rif1 induces premature activation of MCM, deregulating timing of origin firing (*53*, *54*).

The timing of replication is also influenced by the availability of limiting factors that are involved in activation of DNA replication. Several activating factors might become more available in the late-S phase, as they are steadily reused after early replication, resulting in increased probability of late-replicating regions activation that are less permissive (*60*). These activating factors include Cdc45 (*61*, *62*), Cdc7-Dbf4 (*62*) and the CDK targets, Sld2, Sld3 and Dbp11 which are generally present in limited amounts (*30*, *63*).

Rif1: Structure and Function

Rif1 structure

The Rif1 protein consists of three regions; namely N-terminal, C-terminal and the intrinsically disordered polypeptide (IDP) domains as depicted in Fig. 1A.

N-terminal domain (*NTD*)*:* It is predicted that in the NTD of human and mouse Rif1, there are 14–21 HEAT[1] (huntingtin, elongation factor 3, a subunit of protein phosphatase 2A and TOR)/Armadillo-like helical repeats (length varies with species) (*64*) and

1 The HEAT unit consists of 35–45 amino acids, bearing loosely conserved Asp and Arg at 19th and 25th residues, respectively, and forms a pair of antiparallel α-helices and a turn surrounding a virtual axis.

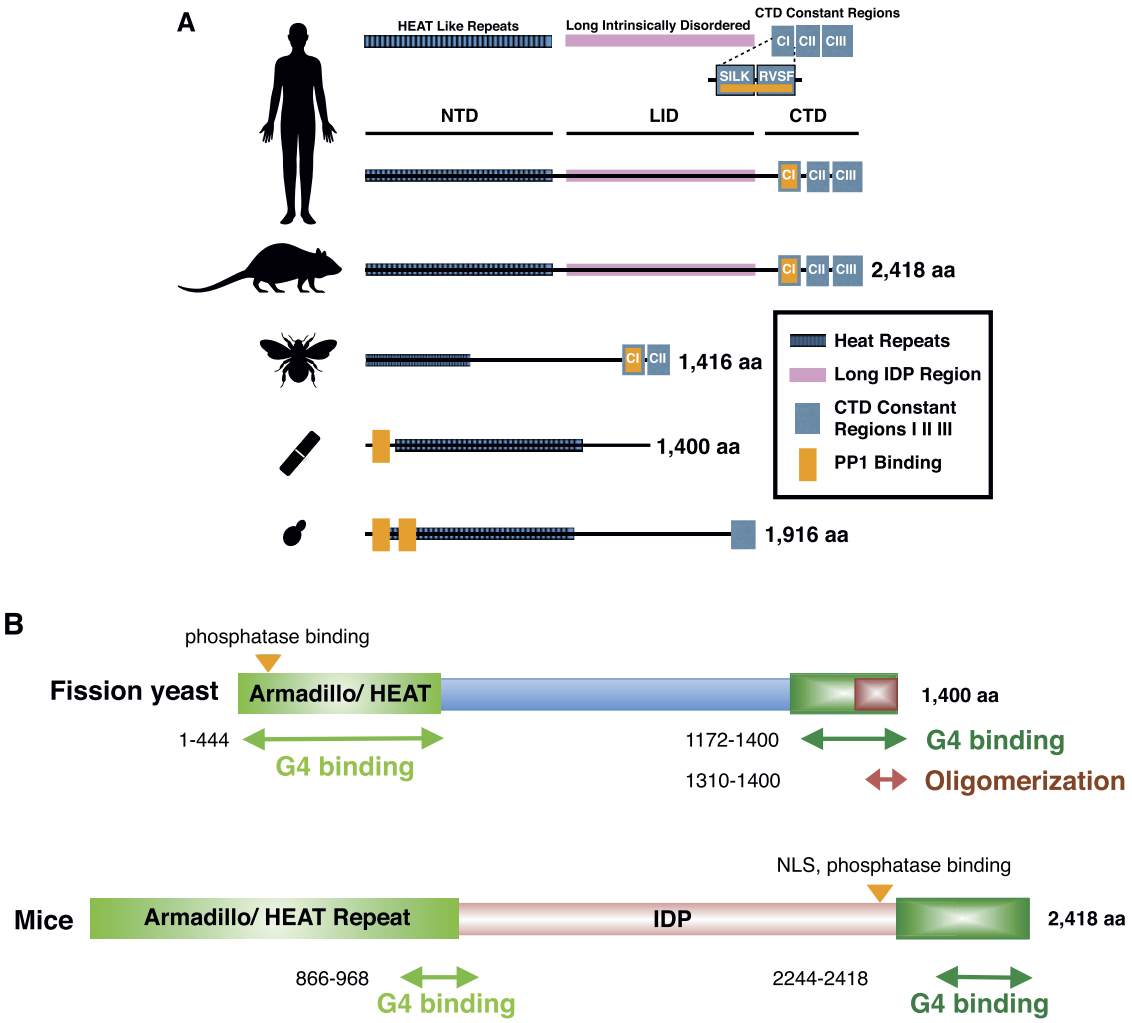


Fig. 1. Comparison of Rif1 structure in different eukaryotic species. (**A**) Schematic drawings of the structures of Rif1 orthologs from *Homo Sapiens* (Human), *Mus musculus* (Mouse), *D.melanogaster* (Fruit Fly), *S.pombe* (Fission Yeast) and *S.cerevisiae* (Budding Yeast). They consist of NTD, CTD and a long intrinsically disordered (LID) structure found in higher eukaryotes between NTD and CTD. NTD consists of HEAT like repeats (patterned blue blocks), and C-terminal subdomains consisting of constant regions I, II and III (blue blocks). CII was reported to carry DNA-binding activity. The PP1 binding motifs are present at different locations on the proteins. (**B**) Fission yeast and mouse Rif1 form oligomers and contain two G4 binding domains. Further analysis indicated the presence of two G4 binding domains. The C-terminal G4 binding domain of fission yeast or mice Rif1 was narrowed down to 229 or 175 amino acids, respectively, and overlaps with the oligomerization domains. In fission yeast Rif1, the oligomerization domain was mapped to the 91 amino acids.

among Rif1 homologs of diverse eukaryotic species, the 133 residues (from 172 to 304) are particularly well conserved and constitutes the core conserved region of the Rif1 HEAT repeat (*65*). Striped blue segments in Fig. 1A represent Rif1-NTD in different species. The crystal structure and biochemical analyses of the conserved 125-kDa NTD of Rif1 from *S.cerevisiae* revealed the dsDNA-binding activity associated with this domain (*66*). The corresponding NTDs from mouse and fission yeast Rif1 proteins exhibit G4 DNA-binding activity (*67*, *68*). The budding yeast Rif1 is anchored to the membrane through lipid modification (palmitoylation) in its N-terminal segment. However, it is not known if this feature is also conserved in Rif1 from higher eukaryotes (*69*, *70*).

IDP: The IDP region is a long stretch of amino acids between the NTD and C-terminal domain (CTD) of Rif1 in higher eukaryotes that are unusually acidic and predicted to constitute IDP structure under physiological conditions (Fig. 1A) (*65*). The functional significance of IDP is unknown, despite the fact that a number of phosphorylation sites have been mapped in this domain (*71*).

Although the isolated IDP does not show any DNA-binding activity, removal of this segment from Rif1 decreased its G4 DNA-binding affinity (*67*, *68*). Resolving the functional and structural roles of the IDP in Rif1 will be an important topic to be studied in the future.

CTD: The CTD of Rif1 can interact with dsDNA with higher affinity than with ssDNA *in vitro* (*64*). This direct interaction of C-terminal segment of Rif1 with DNA was shown to play a key role in DNA damage repair (Iguchi, Itoh et al. unpublished data).

Sukackaite *et al.* characterized the mouse Rif1-CTD and showed that it contains three segments (CI, CII and CIII). Among them, CI and CII are conserved in all organisms but CIII is conserved only in

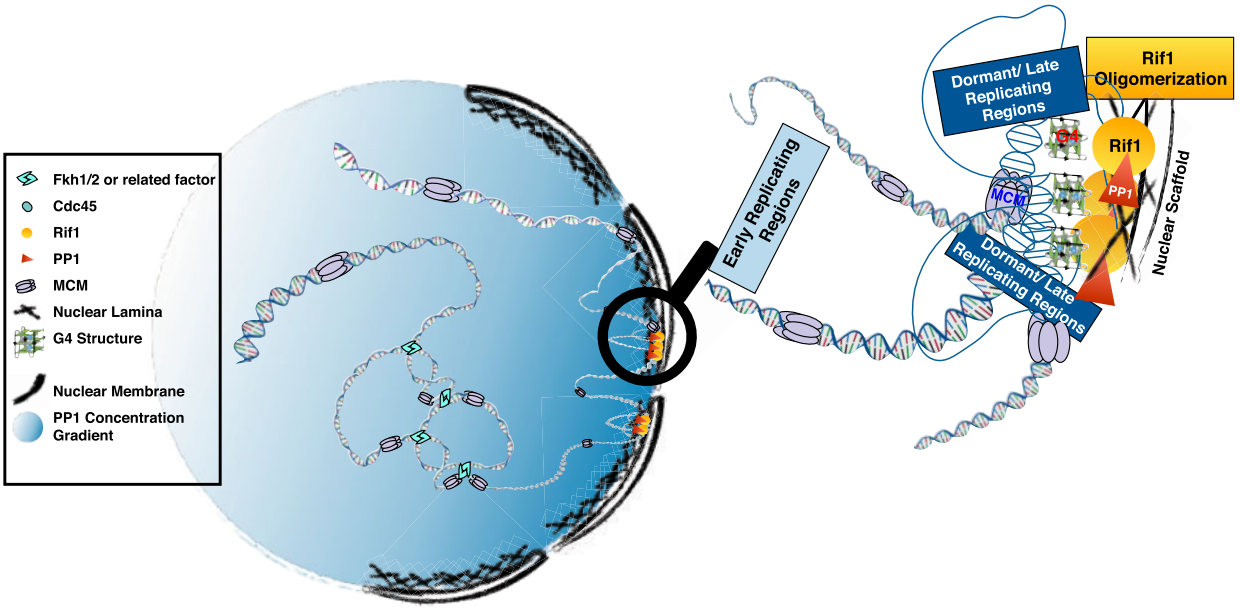


Fig. 2. Rif1 protein in regulation of chromatin architecture and replication timing: a speculative model. Timing decision takes place in G1. In budding yeast, Fkh1/2 may tether the early-firing origins in the nuclear interior in which high concentrations of initiation factor is present (left side representing the interior area of nuclei; dark blue). On the other hand, Rif1 can associate with late-replicating regions at the onset of G1 and may hold together multiple chromatin fibers through its oligomerization capability at the nuclear periphery, generating multiple chromatin loops (*68*, *146*,*147*). Rif1 specifically interacts with sequences in the DNA that can form specific G4 structures (*at least in fission yeast*). These structures are generated at specific inter-origin and intergenic regions (magnified picture on the right). PP1 is recruited by Rif1 to counteract the phosphorylation reactions required for replication initiation. Thus, it is suggested that PP1 concentration coincides with Rif1 concentration in the nucleus [the blue colour gradient for the nucleus background is intended to show the PP1 concentration from high (*dark blue*) to low (*light blue*)]. Please note that the role of G4 in chromatin binding of Rif1 was clearly shown in fission yeast, but not in other species. This speculative model was developed by combining the findings in fission yeast and mammalian cells. It should be noted that Rif1 may associate with chromatin through recognition of other motifs or other proteins in mammalian cells, although the purified mammalian Rif1 protein binds specifically to G4 *in vitro* (*67*).

vertebrates (Fig. 1A) (*72*). They showed that CII is partially folded and can bind DNA (*64*, *72*). In budding yeast, CII is required for interaction with Rap1 (*73*), Cdc7 (*58*) and tetramerization function (Fig. 1A) (*73*). The residues involved in the latter function are overlapping and show weak homology to DNA-binding domains of human and mouse Rif1 (*64*).

The crystal structure of the budding yeast Rif1 C-terminal segment (from 1,857 to 1,916 aa), which is conserved among eukaryotes, revealed a tetramerization function (*73*). We reported that both mouse and fission yeast Rif1 are highly oligomerized and it forms an elongated shape (*67*, *68*). Both N-terminal and C-terminal segments of mouse and human Rif1 form oligomers and both segments bind to G4. The C-terminal G4 binding domains of fission yeast and mouse Rif1 were mapped to the 229 and 175 amino acid segments, respectively (*67*, *68* and our unpublished data). The oligomerization domain of fission yeast Rif1 was localized to the C-terminal 91 amino acid segment (Fig. 1B).

Another conserved feature of Rif1 is the presence of PP1 interacting motifs. CI containing an intrinsically disordered structure is closely associated with a PP1 binding site in Metazoa (*64*, *71*). Nearly all Rif1 homologs have both SILK and RVxF/W PP1-docking motifs with a short stretch of amino acids in between (shown as green boxes in Fig. 1A) (*65*). Metazoan Rif1 has this motif near the IDP-CTD boundary, whereas yeast Rif1 bears this motif near the N-terminus of the HEAT repeat domain (*65*). PP1-interacting motifs are missing in the plant Rif1. Functional significance of the PP1-binding sequences has been proven in budding/fission yeasts and human (*32*, *56–59*). It was recently reported that mouse Rif1 is a high-affinity PP1 adaptor that can outcompete PP1 inhibitors *in vitro* (*74*).

Rif1 functions

Mammalian Rif1 orthologs have been shown to play roles in cellular response to DSB (*75–79*) and also in defining mouse ESCs' identity (*80–82*). Budding yeast Rif1was also shown to have a role in DSB repair (*83*). Recently, Fontana *et al.* have suggested that Rif1 S-palmitoylation is important for efficient non-homologous end-joining (NHEJ) in yeast, and have implicated palmitoyl acyltransferase, Pfa4 (protein fatty acyltransferase 4), in this regulation, showing the importance of inner membrane association in DSB repair (*84*). In addition to the regulation of telomere maintenance, Rif1 has also been shown to be involved in transcriptional silencing at yeast telomeres and ribosomal DNA (*85*).

Rif1 in mammalian cells seems neither to bind normal telomeres nor to have a direct role in telomere homeostasis (*86–88*). On the other hand, Rif1 protects

nascent DNA at blocked replication forks both in yeast and human cells (*89–91*). Human Rif1 protects nascent DNA from DNA2-WRN[2]-mediated inappropriate degradation when replication fork stalls, contributing to the maintenance of the genome stability (*90*, *91*). *Drosophila* Rif1 does not seem to participate in DSB responses, but is essential for normal fly development (*92*). Cell lineage primarily affects replication timing program, and Rif1 functions in a tissue-specific manner to control replication timing in cooperation with PP1 (*93*). In *Drosophila* polyploid cells, SUppressor of UnderReplication (SUUR) protein inhibits replication fork progression within specific genomic regions to promote DNA underreplication. Rif1 localizes to active replication forks in a partially SUUR-dependent manner and directly inhibits replication fork progression (*94*).

Rif1 plays diverse roles in transcriptional regulation in ESCs and in cancer progression. Rif1 is required for heterochromatin silencing at subtelomeric and pericentromeric regions in mouse ESCs (*95*). Rif1 directly occupies ERV (endogenous retrovirus) regions in mouse and human ESCs, and assembles repressive histone mark (H3K9me3 and H3K27me3) and DNA methylation, thereby repressing ERV activation (*96*). The HEAT domain of Rif1 is essential for this repression. Rif1 promotes epithelial ovarian cancer progression by activating hTERT (telomerase reverse transcriptase) via direct binding to its promoter (*97*). hTERT promoter bears multiple Rif1CS-like sequences that are required for Rif1-induced activation. In non-small-cell lung carcinoma, Rif1 promotes tumour growth by PP1-mediated activation of Wnt/β-catenin signalling (*98*).

Phosphorylation of Rif1 is involved in dissociation of PP1 from its docking motif. A low level of ATR and Chk1 kinase signalling suppresses dormant origin firing throughout the unperturbed S phase. ATR and Chk1 kinase inhibitors induce Rif1 phosphorylation at the PP1-docking motif that depends on Cdk1 but not on Cdk2. This phosphorylation disrupts an interaction between Rif1 and PP1, leading to dormant origin firing (*99*). A Wee1 kinase inhibitor induces Cdk1-dependent Rif1 phosphorylation and Cdk2- and Cdc7-dependent origin firing in G1 and S phases (*100*). Thus, Rif1–PP1 interaction is a shared target of both G1/S checkpoint enforced by Wee1 and S/G2 checkpoint enforced by ATR. Notably, another cell cycle checkpoint, known as abscission checkpoint at M/G1 transition, also involves Rif1–PP1 interaction in human cells (*101*). Rif1 aligned along a subset of the midzone microtubules between the separating chromosomes in anaphase (*87*). Rif1 is recruited to the ultrafine anaphase bridges (UFBs) in a PICH[3]-dependent fashion, and promotes the resolution of UFBs (*102*). UFBs activate Aurora B[4]-driven abscission checkpoint until chromosome separation completes. Rif1 promotes cytokinesis through recruitment of PP1 to the midbody, which then counteracts Aurora B kinase activity, thereby dephosphorylates and activates CHMP4C, an abscission timing regulator (*101*). In fission yeast, Rif1 also promotes resolution of UFBs (*103*). Such an anaphase role for Rif1 is separated from its role in G1/S or S/G2. In all cases, however, a recruited PP1 is crucial, indicating the presence of spatially and temporally distinct Rif1-regulated phosphatase substrates. In the absence of Rif1, chromatin loops are more relaxed and this supports the concept of a connection between replication timing and nuclear structure (*33*, *53*).

Rif1 Regulates Replication Timing Through Organizing the 3D Structure of Chromatin

There are many shreds of evidence on the correlation between replication timing and nuclear structure (*104*, *105*). Rif1 may play a key role in the regulation of molecular mechanisms linking 3D (three-dimensional) organization of chromatin and replication timing, even though the mechanism itself is still not clear (*106*). There are structurally distinct chromatin domains in which chromatin fibers interact with those in the same domain but not with those in other domains (*107*). 3D-folded ~megabase-long chromatin domains in which internal interaction of DNA fibers occurs have been characterized and are called topologically associated domains (TADs) (*108*). Interestingly, it was observed that the boundaries of replication timing domains remarkably overlap with TAD borders. Thus, the idea that a number of origins within the same structural domains are coordinately activated in a temporally regulated manner was put forward (*107*). However, the latest studies suggest that replication timing domains are organized differentially from TADs. Depletion of a cohesin subunit or its loading factor disrupts global TAD organization, but causes little effect on either transcriptional profiles or replication timing domains (*109*, *110*). CCCTC-binding factor (CTCF) ablation also disorganizes TAD borders and insulation capability, with negligible influences on replication timing regulation (*45*, *105*, *111*). Instead of TADs, Hi-C compartments A/B seem to correspond to early/late-replication domains (*105*). Rif1 supports subsets of inter-chromatin contacts (*112*) that serve to organize the proper Hi-C compartments.

Evidence strongly suggests that the nuclear organization and subnuclear localization of chromatin play crucial roles in replication timing determination (*106*). Fission yeast and mammalian Rif1 protein fractionates into Triton- and nuclease-insoluble fractions (*41*). At late-M to early-G1, Rif1 firmly binds to

2 DNA2, acting with the Werner's helicase protein WRN, is found to be the major nuclease–helicase complex that drives uncontrolled resection of nascent DNA in the absence of RIF1 (*83*).

3 Plk1-interacting checkpoint helicase, a DNA translocase specially adapted for processing anaphase bridge DNA.

4 A member of the Aurora family of serine/threonine protein kinases, a key player in chromosome segregation.

nuclear-insoluble structures. These nuclease-insoluble bindings are mainly localized at nuclear and nucleolar periphery in the close proximity to the mid-S replication foci. It was proposed that Rif1 creates the mid-S replication domains where replication is restrained during early-S phase (*33*, *53*). Thus, late origins are concentrated near the nuclear membrane already in G1 phase, and on the other hand, the early-firing origins are scattered inside the nucleus (Fig. 2) (*113*). Therefore, early-replicating domains reside in the nuclear interior, which is more permissive to transcription, and late activation happens in the nuclear periphery or other transcriptionally repressed compartments (*114*). These and other results strongly indicate that Rif1 plays a critical role in regulation of DNA replication timing through controlling higher-order chromatin architecture (*33*).

Sequences were identified in fission yeast that can render nearby origins to fire late. One of them is the binding site of Taz1, a telomere binding protein (see above). The other is the Rif1 binding site (Rif1BS), which suppresses the origin firing over segment encompassing >50 kb. These sites could induce chromatin alteration that would facilitate long-range origin suppression. Although the chromatin alterations occur near nuclear periphery, artificial tethering of early origins to the nuclear membranes was not sufficient to turn them into late-firing origins in *S. cerevisiae* (*115*). Thus, a current model for replication timing regulation involves specific chromatin organizations generated by the nuclear periphery-tethered late-replicating segments and local chromatin structures (histone modification, binding of transcription factors, etc.) which determine the firing efficiency and, ultimately, the global timing of DNA replication (Fig. 2) (*106*).

In mammalian cells as well, replication timing appears to be regulated by the subnuclear organization and 3D structure of DNA. In fact, genomic profiles of the constant timing regions (CTR) are well matched with the DNA 3D structures (*25*, *105*, *107*, *114*). A strong connection between the lamina nuclear domain and replicated regions was reported (*116*). In human, Rif1 is localized at nuclease-insoluble structures at the nuclear membranes and the periphery of the nucleoli, reminiscent to the mid-S replicating foci. When Rif1 is depleted, these foci are particularly lost. Therefore, it was proposed that Rif1 may tether mid-/late-S replicating origins to special compartments in the nucleus and generate special chromatin compartment refractory to the actions of initiation factors, so they are suppressed until mid-S phase (*33*, *106*). Rif1 co-localizes with survival motor neuron (SMN), a Cajal body protein, and recruits Ddx1, an RNA-helicase (*117*). It will be interesting to examine potential roles of Rif1 in a phase-separated nuclear body (*118*), where Rif1 may interact with nucleic acids.

It was shown [through Circularized Chromosome Conformation Capture (4C) analyses] that early origins have a propensity to contact with each other in *S.cerevisiae* (*119*). Factors like Fkh1/2 can form dimers, and promote 3D clustering of early origins through their self-association, causing clustered early firing (Fig. 2) (*120*). Similarly, early-replicating domains in mammalian cells interact frequently according to 4C experiments, even when they are far away from each other (*121*). It was shown that genomic rearrangement very often happens between early-replicating domains (*122*). This suggests that early-replicating domains cluster inside the nucleus and that different replication timing domains may spatially cluster in separate compartments in nucleus (*123*). Limiting initiation factors like Sld3, Sld7 and Cdc45 can create a local concentration gradient (*30*) and by this, at the beginning of S phase, they are readily accessible to early CTR but not mid or late CTR. With the progression of the cell cycle, the latter domains will become more accessible possibly through conformational changes (*106*).

Phosphorylation plays a key role in regulation of initiation. Dephosphorylation of MCM complex and Sld3 by PP1 (which is recruited by Rif1) represses the origins from firing and repression is reversed by Rif1 phosphorylation by CDK/DDK (*32*, *56*, *57*). In budding yeast, Rif1 recruits PP1 to also control telomere length (*124*). The Cdc7-mediated phosphorylation of MCM components in pre-RC is essential for initiation, and PP1 recruited by Rif1 counteracts this phosphorylation (*57*). Mutations at Rif1BS that disrupt Rif1 binding causes deregulation of DNA replication over 50–100 kb segment spanning the mutated Rif1BS (*41*). Foti *et al.* showed that Rif1 is responsible for chromatin interactions within the segment where origin firing is coregulated, because Rif1 deletion leads to loss of spatial constraints of inter-chromatin interactions, followed by replication timing program disruption (*112*). When Rif1 is depleted, mid-S replication foci disappear and late origins are activated early concomitant with loss of chromatin loops and increased mobility and decondensation of chromatin near nuclear periphery (*53*, our unpublished data). In fission yeast, firing of some early origins are delayed, presumably by the depletion of initiation factors due to precocious firing of origins normally fired late (*41*). Thus, Rif1 links nuclear architecture and replication timing establishment (*112*).

In addition to its crucial role in replication timing, Rif1's diverse roles in DSB repair, fork protection, chromosome separation at anaphase, cytokinesis and others raise an issue on how Rif1 regulates these multiple events. Does Rif1 regulate each process in totally unrelated mechanism? Or does Rif1 act on each process in a common manner possibly by serving as an 'effector'? Through interaction with many key proteins and DNA structures, Rif1 may recruit PP1 and generate specific chromatin architecture at the sites of actions to regulate these processes. Recently, DNA replication and homologous replication-dependent repair at high IR doses were reported to be inhibited by Rif1 through a common mechanism involving the recruitment of PP1 (*125*). To further elucidate the mechanisms of Rif1 actions in various chromosome events, detailed dissection of functional domains of Rif1 protein will be needed.

G-Quadruplex in Biological Reactions

> ***'If G-quadruplexes form so readily in vitro, nature will have found a way of using them in vivo'***

At >30 years after this statement was made by Aaron Klug, four-stranded helical structures called G-quadruplex (G4) have come into view from being a structural curiosity *in vitro* to being identified as a possible nucleic acid-based mechanism for regulating multiple biological processes *in vivo*. Many researches are ongoing to clarify the roles of G-quadruplexes in cells (*126–128*) [see Valton and Prioleau for a recent review (*129*)]. Even though mapping their occurrence *in vivo* has been difficult, accumulating evidence points to their formation on both DNA and RNA (*130*), supporting the notion that higher-order structures of DNA and RNA rather than their primary sequences provide a nucleic acid-based regulatory mechanism for transcription (*131*), replication (*132*), translation (*133*) and telomere structure (*134*).

Recent efforts to map G4 DNA in the whole human genome utilizing a G4-specific, BG4 antibody uncovered the presence of ~10,000 G4 structures in the chromatin of transformed human cells, predominantly in regulatory, nucleosome-depleted regions (*135*, *136*). Mao *et al*. showed that G4 formation sequesters DNMT1, and protects certain CGIs (CpG islands) from methylation, inhibiting local methylation in human K562 chronic myelogenous leukaemia cells (*137*). Hou *et al*. noticed that ~2,500 G4 peaks are located in TAD boundaries (among total of 7,250 boundaries) (*138*). Moreover, the peaks of CTCF and cohesin tended to locate around G4 peaks, and adjacent boundaries containing G4s frequently contacted each other, suggesting an architectural role for G4s in chromatin looping.

Not only the formation but also the resolution of G4 needs to be regulated in a strict spatio-temporal manner (*139*, *140*). Specific interacting proteins, chaperones and helicases bind to G4 to regulate their functions, formation and resolution (*141*, *142*). However, it remains to be determined whether all or a subset of potential G4 motifs function in cells and in what biological functions they are involved (*128*). It would be of prime importance to elucidate where, when and how G4s are formed in the cells and how dynamic they appear and disappear. It would also be important how the bindings of G4 stabilizing proteins or G4 resolving helicases are related to cellular G4 profiles (*142*). Development of new methods to detect and map the chromosomal G4 in cells would also help dissolve these issues (*143*).

In avian DT40 cells, association of G4 and replication origins have been investigated and G4 was found to be important for origin firing analysed by a physical method (*144*). It is yet to be analysed that how a distinct G4 structure is generated on a chromosome and is recognized by its relevant binding factors to potentiate its functions (*41*). Below, we would like to discuss mode of interaction between G4 and its binding protein by taking Rif1 of fission yeast, a recently identified G4 binding protein, as an example.

Interactions of Rif1 with G-Quadruplexes

Using chromatin immunoprecipitation-sequencing method (ChIP-seq), 35 high-affinity Rif1BSs were identified (*41*). Rif1BSs have the following special characteristics.

Locations: Rif1BSs are located in the intergenic regions near late-firing or dormant origins that are converted to early in *rif1*Δ cells and do not exactly overlap with the locations of pre-RC (MCM binding sites) or promoter sequences.

Sequence features: Rif1BSs tend to contain at least two copies of a conserved motif, CNWWGTGGGGG[5] (Rif1CS) and change of the guanine residues within these motifs resulted in the complete loss of Rif1 binding as well as deregulation of origin firing near the mutated site. Mutations of other G tracts near Rif1CSs also led to reduced Rif1 binding, showing that both Rif1CSs and additional G tracts in their vicinity are important for Rif1 binding.

Structure: Rif1BSs adopt G4-like structures *in vitro* in a manner dependent not only on the Rif1CS but also on other G tracts. There is a strong correlation between G4 formation *in vitro* and interaction with Rif1 both *in vitro* and *in vivo*. The precise structure formed by Rif1BSs remains to be determined, but *in vitro* analyses of various truncated sequences of Rif1BS show that many of truncated sequences can form parallel-type G4, which are bound by Rif1 (*41*, *145*, *146*). Rif1BSs appear to be capable of forming alternative forms of G4 depending on which sequences are utilized for G-quartet formation. The sequences of Rif1BS G4 are vastly diversified from canonical G4 motifs. For example, predicted loop sequences are quite longer (up to 29 nucleotides) than those of the canonical G4 forming sequences. The presence of long loop structures was experimentally verified on representative Rif1BS sequences (*145*).

Replacement of the loop sequence with other sequences did not affect the G4 forming efficiency and both *in vitro* and *in vivo* binding of Rif1 (*147*), unpublished data. Only two of the 35 Rif1BSs overlap with the 450 G4 forming sequences predicted from nucleotide sequences (*148*). This suggests diversity of G4-forming sequences *in vivo* which is currently difficult to predict.

Rif1 binds to G4 structures through two G4 binding domains, N-terminal HEAT repeats and C-terminal unknown domains, both of which can bind to G4 independently (*67*, *68*). The maximum binding of Rif1 to chromatin is observed throughout G1 and decreases during S phase (*31*, *41*). Various structured DNAs (including duplex, forked, Y-fork, G4 or cruciform DNA) were used to examine interaction with Rif1. It showed that Rif1 binds to oligomerized forms of parallel-type G4 DNA with the highest affinity (*41*, *67*, *68*, *145*, *146*). Rif1, through its highly oligomerized structure, simultaneously binds to multiple G4 (*146*).

5 N, any nucleotide; W, A or T.

On the basis of these results, a model has been proposed that Rif1 binds to multiple G4 structures generated in the intergenic regions and condense chromatin fibers to form chromatin compartments that are suppressive to origin firing (*67*, *68*).

Biochemical properties of fission yeast Rif1 and mammalian Rif1 appear to be very similar including their G4 binding and oligomerization activities. The chromatin binding profiles of Rif1, as revealed by ChIP-seq analyses, indicate the overlap of the Rif1's distribution with late-replicating regions. Rif1 exhibits broad binding over the late-replicating regions, but is generally absent in early-replicating regions. A small fraction of Rif1 exhibits sharp binding peaks and those bindings are correlated with the GC content, enriched at CpG-rich transcription start sites, and to a lesser extent, with the presence of G4-forming sequences (*112*). These data suggest that although a fraction of Rif1 may bind chromatin through direct recognition of G4-related structures, other modes of recognition could contribute to chromatin binding of Rif1 in mammalian cells.

Concluding Remarks and Speculations

Rif1 is a conserved protein with multifaceted functions. It is a critical regulator of replication timing and an important element in organizing replication domains (by regulating chromatin architecture), and it also plays crucial roles in DSB repair, transcription as well as other chromosome dynamics.

During the past decade, the 'replication domain model' has been presented which states that temporal regulation of replication is related to its spatial regulation. A key player in this phenomenon is now considered to be Rif1 protein. The ability of G4 to bind G4 and emerging roles of G4s in genome functions raise many interesting questions.

Hi-C compartments A/B largely overlaps with early/late-replication timing domains. Rif1-associated domains (RAD) are localized at the nucleolar periphery and nuclear lamina which coincide with late-replicating domains. Strikingly, RADs coincides with high concentrations of PP1 (Fig. 2), since Rif1 recruits PP1 and suppresses initiation of DNA replication. Rif1 also exerts constraints on replication timing domains through regulating chromatin interactions. Thus, the deletion of Rif1 leads to deregulation of replication timing through disruption of nuclear architecture and failure of PP1 recruitment. The ability of Rif1 to generate chromatin domains may facilitate the effect of PP1 on a long range. One hypothesis is that Rif1 may generate specific nucleoprotein structures at nuclear and nucleolar periphery that involves extended chromatin segments (*33*). A possibility of liquid-phase separation mediated by Rif1, which contains a long IDP, and interacting G4 sequences (see below) is of interest.

It was previously proposed that replication timing is established at very early G1 [timing decision point (TDP)]. On the basis of the early G1 binding of Rif1 at nuclear and nucleolar periphery, we proposed a model that Rif1 may determine the replication timing at the time of TDP at least on some of the chromosomes, if not on all the chromosome segments (*33*). However, at present, we cannot rule out the possibility that Rif1 executes the pre-existing timing program at a later time point.

Even though G4 has previously been indicated to be a hazardous element, recent studies have indicated its association with various important biological functions (*150*). Different sets of G4 may function for specific chromosome transactions in conjunction with proteins that recognize specific sets of G4.

The G4 binding affinity of Rif1, as measured by dissociation constant (K*d*), was reported to be less than nM by gel shift analyses (*41*, *67*, *68*), but more accurate methods like surface plasmon resonance can be used to quantify this interaction. These methods can be implemented as a platform on which many other future studies including drug discovery can be investigated.

Future works will be needed to uncover the labyrinth of Rif1 functional networks. These will include the domains and detailed structural information of Rif1, profiling of interacting G4s, interacting proteins, dynamic aspects of structural transition, functional modifications of the protein and cellular dynamics of G4 and Rif1. Understanding regulation of Rif1 during cell cycle and in response to the environmental changes as well as its structural basis would be of prime importance to fully understand the Rif1-mediated chromatin regulation and its biological implication.

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Conflict of Interest

None declared.

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