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Brief Communication

Rif1 phosphorylation site analysis in telomere length regulation and the response to damaged telomeres

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ABSTRACT

Telomeres, the ends of eukaryotic chromosomes, consist of repetitive DNA sequences and their bound proteins that protect the end from the DNA damage response. Short telomeres with fewer repeats are preferentially elongated by telomerase. Tel1, the yeast homolog of human ATM kinase, is preferentially recruited to short telomeres and Tel1 kinase activity is required for telomere elongation. Rif1, a telomere-binding protein, negatively regulates telomere length by forming a complex with two other telomere binding proteins, Rap1 and Rif2, to block telomerase recruitment. Rif1 has 14 SQ/TQ consensus phosphorylation sites for ATM kinases, including 6 in a SQ/TQ Cluster Domain (SCD) similar to other DNA damage response proteins. These 14 sites were analyzed as N-terminal, SCD and C-terminal domains. Mutating some sites to non-phosphorylatable residues increased telomere length in cells lacking Tel1 while a different set of phosphomimetic mutants increased telomere length in cells lacking Rif2, suggesting that Rif1 phosphorylation has both positive and negative effects on length regulation. While these mutations did not alter the sensitivity to DNA damaging agents, inducing telomere-specific damage by growing cells lacking *YKU70* at high temperature revealed a role for the SCD. Mass spectrometry of Rif1 from wild type cells or those induced for telomere-specific DNA damage revealed increased phosphorylation in cells with telomere damage at an ATM consensus site in the SCD, S1351, and non-ATM sites S181 and S1637. A phosphomimetic *rif1-S1351E* mutation caused an increase in telomere length at synthetic telomeres but not natural telomeres. These results indicate that the Rif1 SCD can modulate Rif1 function. As all Rif1 orthologs have one or more SCD domains, these results for yeast Rif1 have implications for the regulation of Rif1 function in humans and other organisms.

1. Introduction

Telomeres, the natural ends of linear chromosomes in eukaryotes, are important to maintain genome integrity and protect the ends from the DNA damage response (DDR)[1]. Short telomeres (i.e. telomeres with fewer tracts of telomere repeats) have been found associated with human diseases, such as carcinogenesis, aplastic anemia, pulmonary fibrosis and dyskeratosis congenital [2]. Telomere structure and the basic cellular mechanisms for telomere length regulation are conserved from yeasts to humans. In *Saccharomyces cerevisiae*, the Rap1 binds to duplex telomeric DNA, and both Rif1 and Rif2 bind to telomeres through Rap1 C-terminus, preventing telomerase access to the ends [3,4]. Moreover, telomerase was found preferentially recruited to short telomeres [5,6]; however, many of the steps by which short telomeres are targeted for elongation are unknown.

Tel1^{ATM} and Mec1^{ATR} are ATM family kinase members that are recruited to DNA damage sites and short telomeres [7–9]. Deletion of *TEL1* and *MEC1* prevents telomerase access to chromosome ends [10], and Tel1 is preferentially recruited to short telomeres where its kinase activity is required to recruit telomerase [6,8]. ATM family kinases usually phosphorylate Serine or Threonine followed by Glutamine (SQ/TQ)[11]. Rif1 has 14 SQ/TQ motifs, with six of them clustered in a 108 amino acid SQ/TQ Cluster Domain (SCD), a common feature for substrates phosphorylated by ATM in response to DNA damage [12] and a

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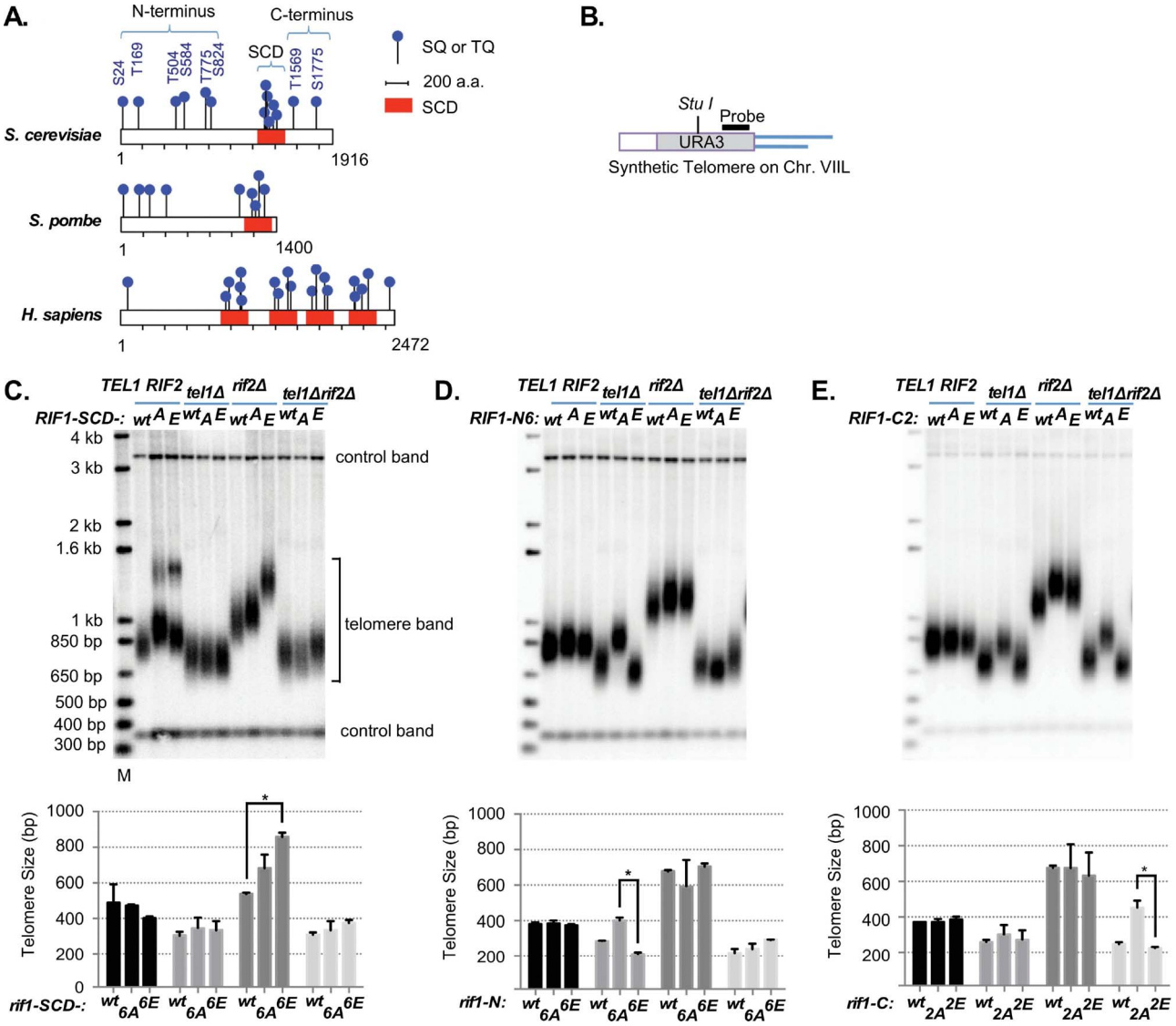

Fig. 1. Mutation of Rif1 ATM consensus sites alters telomere length.

A. SQ/TQ motifs and clustered domains are conserved from yeasts to humans. *S. cerevisiae* Rif1 has 14 SQ/TQ motifs, with 6 of them in a 108 a. a. SCD (SQ/TQ motif clustered domain, defined as 3 or more sites with 100 amino acids [12]). The presence of SCDs in Rif1 is evolutionarily conserved in *Schizosaccharomyces pombe* and humans. These *S. cerevisiae* motifs were divided into three groups for mutant analysis, N-terminus, SCD and C-terminus. The locations of the S and T residues in the SQ/TQ motifs are shown. The SQ/TQ motifs in the Rif1 SCD are S1308, T1316, S1330, S1351, T1386, and T1417. **B. The telomere on Chromosome VIIL was replaced by a synthetic telomere with a marker gene *URA3*.** After digestion with *Stu I*, the terminal restriction fragments were measured on Southern blot by a probe against *URA3* gene to calculate telomere length. **C. Telomere length is increased for *rif1-SCD-6E* in *rif2*Δ cells.** Telomere lengths of the Rif1 SCD mutations with or without the *TEL1* or *RIF2* genes were measured by Southern blotting using *URA3* probe. The cells were grown for 100 generations after isolation of the mutant strains by serial dilution. The bar graph shows the averaged telomere size from 2 independent isolates, but only one representative Southern blot from one isolate was shown. The control bands are from internal loci and used as internal size standards for accurate measurement of the telomere band. M: DNA marker lane. * $p < 0.05$ by two-way ANOVA analysis. **D. Telomere length is increased for *rif1-N6A* in *tel1*Δ cells.** Similar as in panel C but for Rif1 mutations at N-terminus. **E. Telomere length is increased for *rif1-C2A* in *tel1*Δ *rif2*Δ cells.** Similar as in panel C but for Rif1 mutations at C-terminus.

conserved feature of Rif1 orthologs (Fig. 1A). One proteomic study showed that Rif1 Serine 1351 (S1351) in the SCD is phosphorylated in response to the DNA damaging agent MMS in a Tel1/Mec1 dependent manner [13]. Tel1 and Mec1 action on Rif1 is also implied by yeast genetics because these two kinases are required for telomere elongation [10], while loss of Rif1 and Rif2 results in unregulated telomere elongation by telomerase [4], indicating that Rif1 and Rif2 block telomerase access to the chromosome end. Cells lacking Rif1 or Rif2 show telomerase interaction with the telomere throughout the cell cycle, suggesting that telomere end sequestration by Rif1 and Rif2 can be reversibly regulated [14]. As Rif2 has no SQ/TQ motifs, the simplest hypothesis is that Tel1 or Mec1 phosphorylates Rif1, which then allows telomerase recruitment to add new telomeric repeats to the ends. Subsequent dephosphorylation of Rif1 would block telomerase access to the telomere.

Rif1 is conserved from yeasts to mammals, and recent studies showed that in mammalian cells, Rif1 is recruited to DNA damage sites through an interaction with 53BP1 phosphorylated by ATM kinase, which prevents DNA resection and thus promotes non-homologous end-joining [15–18]. However, in *S. cerevisiae*, Rif1's role in the DDR is unclear. Rif1 was found to prevent checkpoint activation at damaged telomeres and DSBs [19–21]. Since *S. cerevisiae* Rif1 was phosphorylated after DNA damage [13], Rif1 phosphorylation may be involved in inhibiting checkpoint activation at damaged telomeres and DSBs.

To test if Tel1 or Mec1 regulates Rif1 functions via phosphorylation, we mutated the residues in the SCD and the domains N-terminal and C-terminal to the SCD to non-phosphorylatable or phosphomimetic residues to ascertain the effect on telomere length regulation. None of