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# Molecular dynamics of *de novo* telomere heterochromatin formation in budding yeast

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ABSTRACT

In the budding yeast *Saccharomyces cerevisiae*, heterochromatin structure is found at three chromosome regions, which are homothallic mating-type loci, rDNA regions and telomeres. To address how telomere heterochromatin is assembled under physiological conditions, we employed a *de novo* telomere addition system, and analyzed the dynamic chromatin changes of the *TRP1* reporter gene during telomere elongation. We found that integrating a 255-bp, but not an 81-bp telomeric sequence near the *TRP1* promoter could trigger Sir2 recruitment, active chromatin mark(s)' removal, chromatin compaction and *TRP1* gene silencing, indicating that the length of the telomeric sequence inserted in the internal region of a chromosome is critical for determining the chromatin state at the proximal region. Interestingly, Rif1 but not Rif2 or yKu is indispensable for the formation of intra-chromosomal silent chromatin initiated by telomeric sequence. When an internal short telomeric sequence (e.g., 81 bp) gets exposed to become a *de novo* telomere, the herterochromatin features, such as Sir recruitment, active chromatin mark(s)' removal and chromatin compaction, are detected within a few hours before the *de novo* telomere reaches a stable length. Our results recapitulate the molecular dynamics and reveal a coherent picture of telomere heterochromatin formation.



## 1. Introduction

The genetic information of eukaryotes is packed in chromatin, which is mainly composed of DNA and histones. The basic unit of chromatin is a nucleosome, which contains two molecules of each of the core histones, H2A, H2B, H3 and H4, wrapped by ~147 bp DNA (Luger et al., 1997; Davey et al., 2002). Chromatin has generally been classified as euchromatin or heterochromatin. Euchromatin is usually transcriptionally competent due to its relatively loose structure which facilitates active association of the transcription machinery. Heterochromatin is transcriptionally silent because of its compact structure that prevents the engagement of the transcription machinery (Grewal and Moazed, 2003). In the budding yeast *Saccharomyces cerevisiae*, there are three heterochromatin domains, the rDNA loci, homothallic mating-type (*HM*) loci and telomeres (Rusche et al., 2003).

The telomere heterochromatin domain is localized at the end of a linear chromosome. The formation of telomere heterochromatin relies on the silencer telomeric DNA (Wellinger and Zakian, 2012). The yeast telomeric DNA consists of ~350 bp of $TG_{1\text{-}3}/C_{1\text{-}3}A$ repeats. The G strand extends beyond its complementary strand to form a single-stranded overhang (called G-overhang) (Wellinger and Zakian, 2012). The double strand portion of a telomere is bound by a sequence-specific DNA binding protein Rap1 to initiate the recruitment of silent information regulator (Sir) proteins consisting of Sir2, Sir3 and Sir4 (Moretti et al., 1994; Buck and Shore, 1995; Kueng et al., 2013). Another telomere-associated protein complex, the yKu70/80 heterodimer, has also been shown to recruit Sir proteins to telomeres by interacting with Sir4, providing an additional pathway to recruit silencing factors to telomeres (Tsukamoto et al., 1997; Roy et al., 2004; Kueng et al.,

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2013). Within the Sir complex, Sir2, a histone deacetylase, can remove the acetyl groups on histone H4K16 of nearby nucleosomes (Imai et al., 2000). The deacetylated histone H4K16 enhances the interaction of Sir3 (Johnson et al., 1990; Liou et al., 2005), facilitating Sir complex to spread along the chromosome towards centromere (Hoppe et al., 2002; Luo et al., 2002; Kueng et al., 2013). This amplifying cycle of deacetylation and Sir recruitment eventually enables the formation and spreading of heterochromatin to a distance of several kilobases (Strahl-Bolsinger et al., 1997; Tanny et al., 1999; Imai et al., 2000; Landry et al., 2000a, 2000b; Strahl and Allis, 2000; Hoppe et al., 2002; Luo et al., 2002; Suka et al., 2002; Rusche et al., 2003; Katan-Khaykovich and Struhl, 2005; Liou et al., 2005; Shogren-Knaak et al., 2006; Altaf et al., 2007; Loney et al., 2009; Radman-Livaja et al., 2011; Kitada et al., 2012; Kueng et al., 2013). Besides histone H4K16 acetylation, methyl groups on histone H3K4 and K79 are also removed to promote the maturation of the heterochromatic structure (Grewal and Moazed, 2003; Ng et al., 2003; Katan-Khaykovich and Struhl, 2005; Kitada et al., 2012). Therefore, hypoacetylated H4K16 and hypomethylated H3K4 and K79 are generally believed to be the epigenetic marks of the telomeric heterochromatin. The Sir dependent telomere silencing can be antagonized by two other telomere-associated factors Rif1 and Rif2 (Moretti et al., 1994; Buck and Shore, 1995; Mishra and Shore, 1999; Wotton and Shore, 1997; Levy and Blackburn, 2004). Both Sir proteins and Rif1/2 bind the C-terminal domain of Rap1 in a competitive manner (Moretti et al., 1994; Buck and Shore, 1995; Wotton and Shore, 1997; Feeser and Wolberger, 2008), suggesting a mechanism of how Rif1 and Rif2 repress heterochromatin nucleation at telomeres (Kueng et al., 2013). Rif1 and Rif2 are also involved in the formation of fold-back telomere structures (Poschke et al., 2012), possibly through their multivalent interactions with Rap1 (Shi et al., 2013).

Previous studies by the Zakian laboratory have shown that the insertion of an 81-bp telomeric $TG_{1\text{-}3}/C_{1\text{-}3}A$ repeat sequence into the downstream of a *URA3* reporter gene, which is 20-kb away from a chromosome end, had little influence on *URA3* transcription (Gottschling et al., 1990). Interestingly, insertion of a 276-bp telomeric sequence in the internal region of a chromosome is able to silence nearby genes (Stavenhagen and Zakian, 1994), suggesting that the intra-chromosomal "$C_{1\text{-}3}A$ based silencing" depends on the length of $TG_{1\text{-}3}/C_{1\text{-}3}A$ repeat sequence (Stavenhagen and Zakian, 1994). However, the characteristics of this "$C_{1\text{-}3}A$ based silent" chromatin have not been fully determined probably due to the unavailability of reagents (e.g., anti-histone antibodies) when the work was conducted. A few laboratories have utilized either the inducible over-expression of Sir3 or a temperature sensitive *sir3* allele to investigate the molecular dynamics of heterochromatin formation (Lau et al., 2002; Martins-Taylor et al., 2004; Katan-Khaykovich and Struhl, 2005; Kirchmaier and Rine, 2006; Osborne et al., 2009). They characterized several heterochromatin-associated aspects including protein recruitment, histone modification(s) and cell cycle regulation, which provided invaluable data on how the silencing factors, as well as chromatin marks affect telomere heterochromatin. In order to obtain new information on the dynamic changes of heterochromatin formation, we modified the *de novo* telomere addition system, which was originally developed by the Gottschling lab (Diede and Gottschling, 1999), to induce heterochromatin formation on an elongating short telomere. This system involves neither deletion nor overexpression of silencing factor(s). Thus, our assessment of telomere heterochromatin formation can be more reflective of what happens under physiological conditions. Using this system, we are able to detect the molecular events, such as telomere elongation, histone modifications, chromatin compaction and gene silencing, which are associated with telomere heterochromatin assembly.

## 2. Results

### 2.1. The system for de novo telomere formation

It is generally believed that telomere heterochromatin must be disrupted during DNA replication, and reassembled after telomeric DNA replication is completed. Due to the length heterogeneity of the same telomere in a group of cells, it is technically difficult to study heterochromatin assembly on a specific telomere in wild-type cells. In order to facilitate our investigation on the molecular dynamics during telomere heterochromatin assembly, we exploited the *de novo* telomere addition system that was developed previously by the Gottschling lab (Diede and Gottschling, 1999). In the experiments, we constructed strains in which an 81 bp or a 255 bp of $TG_{1\text{-}3}$ tract was inserted in the *ADH4* locus at the left arm of chromosome VII (Fig. 1A). These two strains were named as TG81 and TG255, respectively. An isogenic strain that does not contain any $TG_{1\text{-}3}$ tracts was also constructed, and served as a non-telomeric control (namely the TG0 strain). Adjacent to the $TG_{1\text{-}3}$ tracts are a *TRP1* reporter gene and an HO (homothallic switching) endonuclease recognition site, with a *LYS2* selection marker at the distal part of the chromosome (Fig. 1A) (Diede and Gottschling, 1999). Instead of placing the telomere sequence downstream of the reporter gene as in previous studies (Stavenhagen and Zakian, 1994), the 81-bp or the 255-bp TG seed was inserted immediately next to the promotor of *TRP1* in our work. This layout probably rendered *TRP1* transcription more sensitive to the molecular changes at the TG sequence, and minimized the influence of the distance between the telomere seed and the *TRP1* reporter gene. When the HO endonuclease is induced by galactose, it will cut the HO recognition sequence in chromosome VII, resulting in a 16-bp non-telomeric tract ending with a TGTT 3′ single strand overhang next to the TG "seed" of either 81 or 255 bp (Fig. 1A). The exposed "seed" could be considered as a telomere (*de novo* telomere) and subjected to elongation by telomerase upon resection of the remaining HO recognition sequence (Fig. 1A). In the Southern blot assay, the genomic DNA was digested with *Eco*R V. The HO-uncut fragments recognized by the *TRP1* probe are ~3.0 and ~3.3 kb in length in the TG81 and the TG255 strains, respectively (Fig. 1B). After HO induction, the distal *LYS2*-containing fragments are lost, and the *TRP1* probing fragments are shifted to ~700 bp and ~900 bp in the TG81 and the TG255 strains, respectively (Fig. 1B). The results showed that *de novo* telomere addition seldom took place at the breaks with no telomere seed in the TG0 strain, while telomere elongation readily occurred at the 81-bp TG seed (Fig. 1B). When the 255-bp telomere seed became the end, its length is similar to that of the wild type telomeres, and appeared to be stably maintained during the increase of culture time (Fig. 1B). These results are consistent with previous reports that short telomeres are preferentially elongated by telomerase (Arneric and Lingner, 2007; Bianchi and Shore, 2007; Hector et al., 2007; Sabourin et al., 2007).

We also performed both telomere PCR and sequencing to examine the *de novo* telomere length of the TG255 strain, as well as the TG255 *rif1*Δ and the TG255 *tlc1*Δ control strains at the point of a 24 h induction (Fig. 1C and D). The PCR products of the wild-type TG255 cells displayed a relatively sharp band, while the TG255 *rif1*Δ and the TG255 *tlc1*Δ cells showed upward and downward smearing patterns, representing elongated and shortened *de novo* telomeres, respectively (Fig. 1C). Though the sequencing results exhibited both lengthening (6 clones) and shortening (8 clones) events in the TG255 cells (Fig. 1D), the average *de novo* telomere length was 244 bp, with only one case showing elongation to about 380 bp and three showing shortening to less than 240 bp (Fig. 1D