

The proper connection between shelterin components is required for telomeric heterochromatin assembly

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Telomeric regions contain prominent sites of heterochromatin, which is associated with unique histone modification profiles such as the methylation of histone H3 at Lys9 (H3K9me). In fission yeast, the conserved telomeric shelterin complex recruits the histone H3K9 methyltransferase complex CLRC to establish subtelomeric heterochromatin. Although many shelterin mutations affect subtelomeric heterochromatin assembly, the mechanism remains elusive due to the diverse functions of shelterin. Through affinity purification, we found that shelterin directly associates with CLRC through the Ccq1 subunit. Surprisingly, mutations that disrupt interactions between shelterin subunits compromise subtelomeric heterochromatin without affecting CLRC interaction with shelterin component Pot1, located at chromosome ends. We further discovered that telomeric repeats are refractory to heterochromatin spreading and that artificial restoration of shelterin connections or increased heterochromatin spreading rescued heterochromatin defects in these shelterin mutants. Thus, subtelomeric heterochromatin assembly requires both the recruitment of CLRC by shelterin to chromosome ends and the proper connection of shelterin components, which allows CLRC to skip telomeric repeats to internal regions.

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Eukaryotic genomes contain large amounts of repetitive DNA elements at centromeres and telomeres that assemble constitutive heterochromatin, which is critical for preventing homologous recombination between repeats to maintain genome integrity (Blasco 2007; Grewal and Jia 2007). These heterochromatic regions are associated with distinct histone modification profiles and chromatin proteins, including low levels of acetylation, high levels of methylation at histone H3 Lys9 (H3K9me), and heterochromatin protein 1 (HP1) family proteins (Grewal and Jia 2007). Heterochromatin formation requires concerted actions of diverse histone-modifying activities, which are classified into three distinct steps: initiation, spreading, and maintenance (Wang et al. 2014a). Initiation of heterochromatin assembly takes place at nucleation centers via targeting of histone H3K9 methyltransferases by sequence-specific DNA-binding proteins or noncoding RNAs, leading to H3K9me (Castel and Martienssen 2013; Cohen and Jia 2014). Heterochromatin then spreads from these nucleation centers through a positive feedback

loop involving H3K9me-mediated recruitment of additional histone H3K9 methyltransferases that methylate adjacent nucleosomes, resulting in an “inch worm”-like spreading (Talbert and Henikoff 2006; Wang et al. 2014a). Similarly, the recruitment of H3K9 methyltransferases by pre-existing H3K9me on old histones allows the newly incorporated histones during DNA replication to be methylated as well, resulting in stable maintenance of heterochromatin domains through cell divisions even without the initiation signal (Audergon et al. 2015; Ragunathan et al. 2015). Although the mechanisms of heterochromatin assembly at centromeres have been extensively studied, those that regulate initiation and spreading of heterochromatin at telomeres are not well understood.

The fission yeast *Schizosaccharomyces pombe* serves as a perfect model organism for the study of heterochromatin assembly and telomere biology. The

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heterochromatin assembly machinery in fission yeast is highly conserved compared with those of higher eukaryotes (Grewal and Jia 2007). The SUV39 family SET domain protein Clr4, the sole H3K9 methyltransferase in this organism, is critical for heterochromatin assembly (Rea et al. 2000; Nakayama et al. 2001). Clr4 associates with an E3 ubiquitin ligase (composed of Cul4, Rik1, Raf1, and Raf2) to form the CLRC complex, which is required for H3K9me in vivo (Rea et al. 2000; Nakayama et al. 2001; Hong et al. 2005; Horn et al. 2005; Jia et al. 2005). H3K9me recruits HP1 family proteins Swi6 and Chp2 to further compact chromatin (Nakayama et al. 2001; Sadaie et al. 2004). These HP1 proteins in turn recruit additional chromatin-modifying activities, such as the SHREC complex (composed of the histone deacetylase Clr3, chromatin remodeling protein Mit1, and additional proteins Clr1, Clr2, and Ccq1) to regulate histone acetylation and nucleosome positioning at heterochromatin (Sugiyama et al. 2007; Motamedi et al. 2008). Heterochromatin in fission yeast is mainly present at pericentric regions, subtelomeres, and the silent mating-type locus, all of which contain a similar repeat element that initiates heterochromatin through an RNAi-mediated pathway (Grewal and Jia 2007). In addition, DNA-binding proteins such as stress-activated ATF/CREB proteins Atf1/Pcr1 initiate heterochromatin at the silent mating type region, and the telomere-binding protein complex shelterin initiates heterochromatin at telomeres (Jia et al. 2004; Kim et al. 2004; Kanoh et al. 2005).

Telomeres and the telomere-binding shelterin complex in fission yeast are also highly conserved with respect to those of mammals (Jain and Cooper 2010). Telomeric DNA is composed of dsDNA repeats followed by a single-stranded overhang. Shelterin contains six subunits: Taz1, Rap1, Poz1, Tpz1, Pot1, and Ccq1 (Miyoshi et al. 2008). Taz1 (homolog of mammalian TRF1/TRF2) binds to the double-stranded portion of telomeric DNA (Cooper et al. 1997). The Tpz1–Pot1 subcomplex (homologous to the mammalian TTP1–POT1 complex) binds to the ssDNA overhang (Baumann and Cech 2001; Miyoshi et al. 2008). Rap1 and Poz1 (similar to mammalian RAP1 and TIN2, respectively) bridge Taz1 with Tpz1–Pot1 (Miyoshi et al. 2008). Tpz1 also associates with Ccq1, which is involved in telomerase recruitment and activation (Miyoshi et al. 2008; Tomita and Cooper 2008; Moser et al. 2011; Yamazaki et al. 2012; Jun et al. 2013; Armstrong et al. 2014; Harland et al. 2014).

Shelterin is required for heterochromatin assembly near native telomeres. For example, mutations of Taz1, Rap1, Poz1, Tpz1, and Ccq1 all resulted in silencing defects of reporter genes inserted near telomeric repeats (Cooper et al. 1997; Nimmo et al. 1998; Kanoh and Ishikawa 2001; Kanoh et al. 2005; Sugiyama et al. 2007; Fujita et al. 2012; Tadeo et al. 2013; Kallgren et al. 2014). In addition, insertion of telomeric repeats at an internal chromosome location or artificial recruitment of shelterin component Rap1 to DNA induces ectopic heterochromatin in a shelterin- and CLRC-dependent manner, suggesting that shelterin recruits CLRC to telomeric DNA (Kanoh et al. 2005; Tadeo et al. 2013). However, mechanis-

tic dissection of subtelomeric heterochromatin assembly has been difficult due to the multiple functions that shelterin performs. For instance, the Tpz1–Pot1 subcomplex is required for chromosome end protection, and their null mutations result in the loss of telomeric DNA and chromosome circularization (Baumann and Cech 2001; Miyoshi et al. 2008). In addition, shelterin is required for telomere length control. Mutations such as *taz1Δ*, *rap1Δ*, or *poz1Δ* result in massive elongation of telomeres (Cooper et al. 1997; Chikashige and Hiraoka 2001; Kanoh and Ishikawa 2001; Fujita et al. 2012; Jun et al. 2013; Harland et al. 2014). In contrast, *ccq1Δ* results in defective telomerase recruitment and activation, leading to telomere shortening (Miyoshi et al. 2008; Tomita and Cooper 2008; Moser et al. 2011; Yamazaki et al. 2012; Jun et al. 2013; Armstrong et al. 2014; Harland et al. 2014). In addition, shelterin components are recruited to telomeres through both dsDNA and ssDNA, and the deletion of a single component of shelterin generally does not abolish the recruitment of other shelterin subunits to telomeres (Miyoshi et al. 2008). Therefore, it is unlikely that all of the known shelterin mutations block CLRC recruitment, and shelterin may play additional roles in subtelomeric heterochromatin assembly in addition to recruiting CLRC.

Here we show that shelterin physically associates with CLRC, mediated by the Ccq1 subunit. Interestingly, aside from *ccq1Δ*, mutations in shelterin components that affect subtelomeric heterochromatin still maintain CLRC interaction with shelterin subunit Pot1, which binds to the single-stranded end of telomeric DNA. A common feature of these mutations is that they affect the connections between the CLRC and double-stranded telomeric DNA, and forced restoration of their proper connections rescues subtelomeric heterochromatin defects. We further showed that insertion of telomeric repeats into an ectopic heterochromatin domain blocks heterochromatin spreading when these repeats do not recruit Clr4. Moreover, enhancing heterochromatin spreading alleviates subtelomeric heterochromatin defects in shelterin connection mutants. These results suggest that the recruitment of CLRC to the ssDNA portion of telomeres is not sufficient for heterochromatin assembly at subtelomeric regions due to the effects of telomeric repeats in blocking CLRC spreading and that the proper connections between shelterin components play a vital role in promoting CLRC spreading over telomeric repeats into subtelomeric regions.

Results

Shelterin associates with CLRC

To understand the mechanism by which shelterin recruits heterochromatin assembly factors to telomeres, we generated a Flag-tagged version of shelterin component Poz1 at its endogenous chromosome location (Tadeo et al. 2013). Affinity purification of Poz1-Flag followed by mass spectrometry identified high levels of peptides corresponding to shelterin components such as Rap1, Tpz1, Pot1,

Ccq1, and, to a lesser extent, Taz1 (Fig. 1A,B). Interestingly, members of CLRC, such as Clr4, Cul4, Rik1, Raf1, and Raf2, were also identified. Similarly, affinity purification of another shelterin component, Rap1-Flag, also identified both shelterin and CLRC components (Fig. 1A,B). Likewise, affinity purification of Flag-Clr4 identified CLRC and most of the shelterin subunits as well (Fig. 1A,B).

To confirm the shelterin-CLRC interaction, we performed coimmunoprecipitation (co-IP) analyses using extracts from a strain expressing both Flag-Clr4 and Poz1-myc at their endogenous chromosomal loci. When immunoprecipitation was performed with a Flag antibody, the specific association of Poz1-myc was also detected. Moreover, the interaction was resistant to ethidium bromide treatment, indicating that their interaction is independent of DNA (Fig. 1C). These results demonstrate that shelterin and CLRC physically associate with each other *in vivo*.

Ccq1 mediates interaction between shelterin and CLRC

We then used co-IP analyses to determine which subunit of shelterin mediates interaction with CLRC. We found that Clr4-Poz1 interaction remained intact in *taz1Δ*, *rap1Δ*, and *pot1Δ* cells but was lost in *tpz1Δ* and *ccq1Δ* cells (Fig. 2A). In addition, the Clr4-Pot1 interaction was not affected by *taz1Δ*, *rap1Δ*, or *pot1Δ* but was abolished by *tpz1Δ* and *ccq1Δ* (Fig. 2B). Furthermore, the Clr4-Tpz1 interaction was disrupted in *ccq1Δ* cells, but the Clr4-Ccq1 interaction was unaffected in *tpz1Δ* cells (Fig. 2C, D), indicating that Ccq1 mediates the interaction between CLRC and shelterin. To confirm this idea, we performed affinity purifications of Flag-Clr4 and Poz1-Flag in *ccq1Δ* followed by mass spectrometry to identify associated proteins. We found that, in the absence of Ccq1, none of the shelterin subunits was identified in Flag-Clr4 purification, and none of the CLRC subunits was present in Poz1-Flag purification (Fig. 2E).

We also attempted to identify the subunit of CLRC that mediates interaction with shelterin through co-IP analyses. However, Clr4-Ccq1 interaction was significantly reduced in *raf1Δ*, *raf2Δ*, or *rik1Δ* cells (data not shown),

suggesting that each of these CLRC subunits is likely required for the assembly of CLRC or that multiple CLRC subunits make independent contributions to shelterin-CLRC interaction. We then performed a yeast two-hybrid assay by fusing Ccq1 with the Gal4-activating domain (GAD) and each CLRC subunit with the Gal4-binding domain (GBD). We found that Rik1 and Raf2, but not any other CLRC subunit, interact with Ccq1 in this assay (Fig. 2F).

In addition to being a component of shelterin, Ccq1 copurifies with the SHREC histone deacetylase complex (Sugiyama et al. 2007; Motamedi et al. 2008). Although other members of SHREC are required for heterochromatin assembly at various chromosomal locations, Ccq1 is only required for telomeric heterochromatin assembly and is proposed to recruit SHREC to telomeric regions (Sugiyama et al. 2007). We consistently detected the SHREC subunit Clr3 in our mass spectrometry analyses of Poz1-Flag, Rap1-Flag, and Flag-Clr4 purifications but not when Poz1-Flag was purified from *ccq1Δ* cells (Supplemental Fig. S1A). Interestingly, other SHREC components were not detected or were present only at very low levels. Chromatin immunoprecipitation (ChIP) analyses showed that both Clr4 and Clr3 were delocalized from sequences immediately next to native telomeres (telomere-associated sequences [TAS1]) in *ccq1Δ* cells (Fig. 2G; Supplemental Fig. S1C). Furthermore, the localization of Clr4 to telomeres was unaffected by *clr3Δ*. However, Clr3 was reduced at, although not abolished from, TAS1 in *clr4Δ* cells (Fig. 2G; Supplemental Fig. S1D), consistent with previous studies showing that the localization of Clr3 to heterochromatin requires HP1 proteins (Yamada et al. 2005; Motamedi et al. 2008; Sadaie et al. 2008). Therefore, the effect of *ccq1Δ* on Clr4 localization to telomeres directly results from the loss of interaction between shelterin and Clr4 rather than an indirect effect on Clr3 localization.

We next examined silencing of *TEL::ura4⁺*, in which the *ura4⁺* reporter is inserted immediately adjacent to telomeric repeats on a minichromosome (Nimmo et al. 1994). There is no subtelomeric DNA near this reporter, thus making it highly sensitive to shelterin mutations

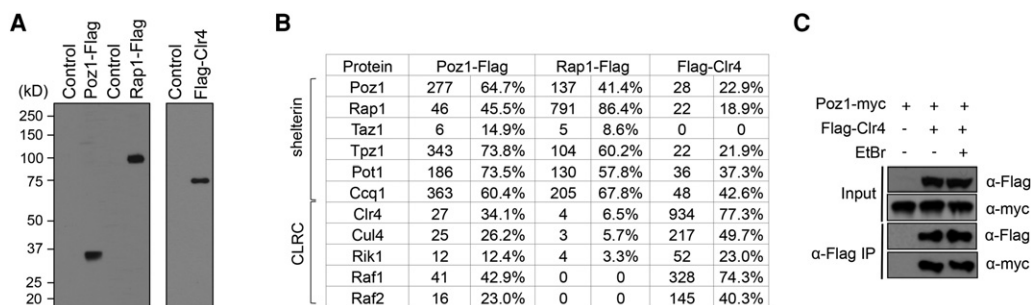


Figure 1. Shelterin associates with CLRC *in vivo*. (A) Western blot analyses of affinity-purified protein complexes with a Flag antibody. (B) Mass spectrometry analyses of purified protein complexes. The spectral count numbers (*left*), which are normalized to the amino acid number of each protein, and the sequence coverage of each protein (*right*) are indicated. (C) Coimmunoprecipitation analyses of Clr4 and Poz1. Immunoprecipitation was performed with Flag-agarose beads, and Western blot analyses were performed with Flag and myc antibodies.

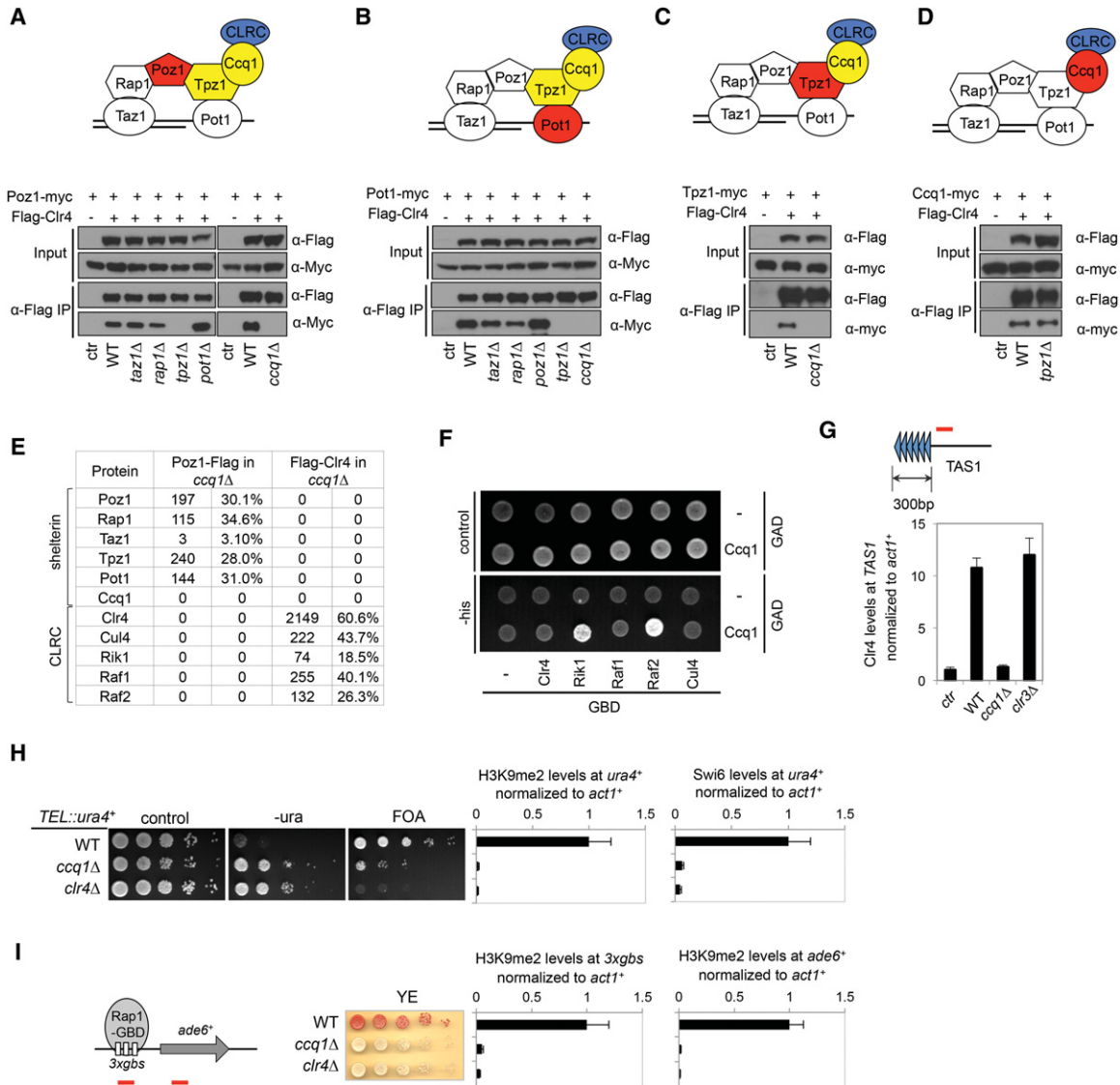


Figure 2. Ccq1 mediates shelterin–CLRC interaction. (A–D) Co-IP analyses of shelterin–CLRC interaction. Flag-Clr4 (blue) was used for immunoprecipitation (IP) with Flag-agarose beads, and myc-tagged shelterin subunits (red) were used for co-IP detection. The shelterin subunits required for interaction are labeled in yellow. (E) Mass spectrometry analyses of purified protein complexes. The spectral counts (left), which were normalized to the amino acid number of each protein, and the sequence coverage of each protein (right) are indicated. (F) Yeast two-hybrid assay to measure the interaction between the Ccq1 and CLRC subunits. Each individual CLRC subunit was fused with the Gal4 DNA-binding domain, and Ccq1 was fused with the Gal4 DNA activation domain. Interaction between CLRC and Ccq1 resulted in the activation of a *HIS3* reporter gene, allowing cells to grow in the absence of histidine. (G) Chromatin immunoprecipitation (ChIP) analyses of Flag-Clr4 levels at *TAS1* (indicated by a red bar), which is immediately adjacent to telomeric repeats, normalized to *act1*⁺. (H, left) Tenfold serial dilution analyses of the indicated yeast strains grown on the indicated media to measure the expression of *TEL::ura4*⁺. (Right) ChIP analyses of H3K9me2 and Swi6 levels at *TEL::ura4*⁺, normalized to *act1*⁺. (I, left) Tenfold serial dilution analyses of indicated yeast strains grown on low-adenine medium (YE) to measure the expression of *3gbs-ade6*⁺. (Right) ChIP analyses of H3K9me2 levels at *3gbs* and *ade6*⁺ (indicated by red bars at the left), normalized to *act1*⁺.

and avoiding complications of redundant heterochromatin initiation pathways within subtelomeric DNA. Consistent with the requirement of Ccq1 for the localization of Clr4 to telomeres, we found that silencing of *TEL::ura4*⁺ was defective in *ccq1Δ* cells, accompanied by the complete loss of H3K9me2 and Swi6 at the reporter and *TAS1*, similar to *clr4Δ* cells (Fig. 2H; Supplemental Fig. S1B). The residual growth on FOA medium is mainly due to increased telo-

mere recombination in *ccq1Δ* cells, which resulted in the loss of the *ura4*⁺ reporter gene (confirmed by PCR analysis of individual FOA-resistant colonies) (data not shown).

Ectopic recruitment of the Rap1-GBD fusion protein to Gal4-binding sites silences an adjacent *ade6*⁺ reporter gene (*3xgbs-ade6*⁺) in a CLRC-dependent manner, resulting in the formation of red colonies when cells are grown on low-adenine (YE) medium (Tadeo et al. 2013).

We found that Rap1-GBD failed to silence *3xgbs-ade6⁺* in *ccq1Δ* cells, as indicated by the formation of white colonies on YE medium, with the complete loss of H3K9me2 at the body of the *ade6⁺* gene as well as the Gal4-binding sites (Fig. 2I). Moreover, H3K9me2 depends on Poz1 and Tpz1, which are required to link Ccq1 with Rap1, but independent of Taz1 or Pot1, which are not expected to affect Ccq1–Rap1 interaction (Supplemental Fig. S2; Tadeo et al. 2013). These results further support the role of Ccq1 in mediating CLRC–shelterin interaction.

The interaction between shelterin components is required for subtelomeric heterochromatin assembly

We previously isolated a *poz1-W209A* mutant that affects subtelomeric heterochromatin assembly without obvious impact on telomere length control (Tadeo et al. 2013).

Co-IP analyses showed that Poz1-W209A still interacts with CLRC to a degree comparable with wild-type Poz1 (Fig. 3A). Moreover, *Poz1-W209A* had no effect on Poz1–Tpz1 interaction (Fig. 3B,C). Therefore, the *Poz1-W209A* mutation specifically affects the interaction between Rap1 and Poz1 (Fig. 3D). While a strong interaction of Rap1–Poz1 is required for telomere silencing, the residual Rap1–Poz1 interaction seems strong enough for telomere length maintenance. These results raise the possibility that the proper connection of shelterin components is essential for heterochromatin assembly at subtelomeres. To test this idea, we investigated other mutations that selectively disrupt interactions between shelterin subunits. A previous study showed that *Tpz1-I501R* specifically ablates Poz1–Tpz1 interaction and that *Tpz1-I200R* specifically disrupts Tpz1–Pot1 interaction (Fig. 3D; Jun et al.

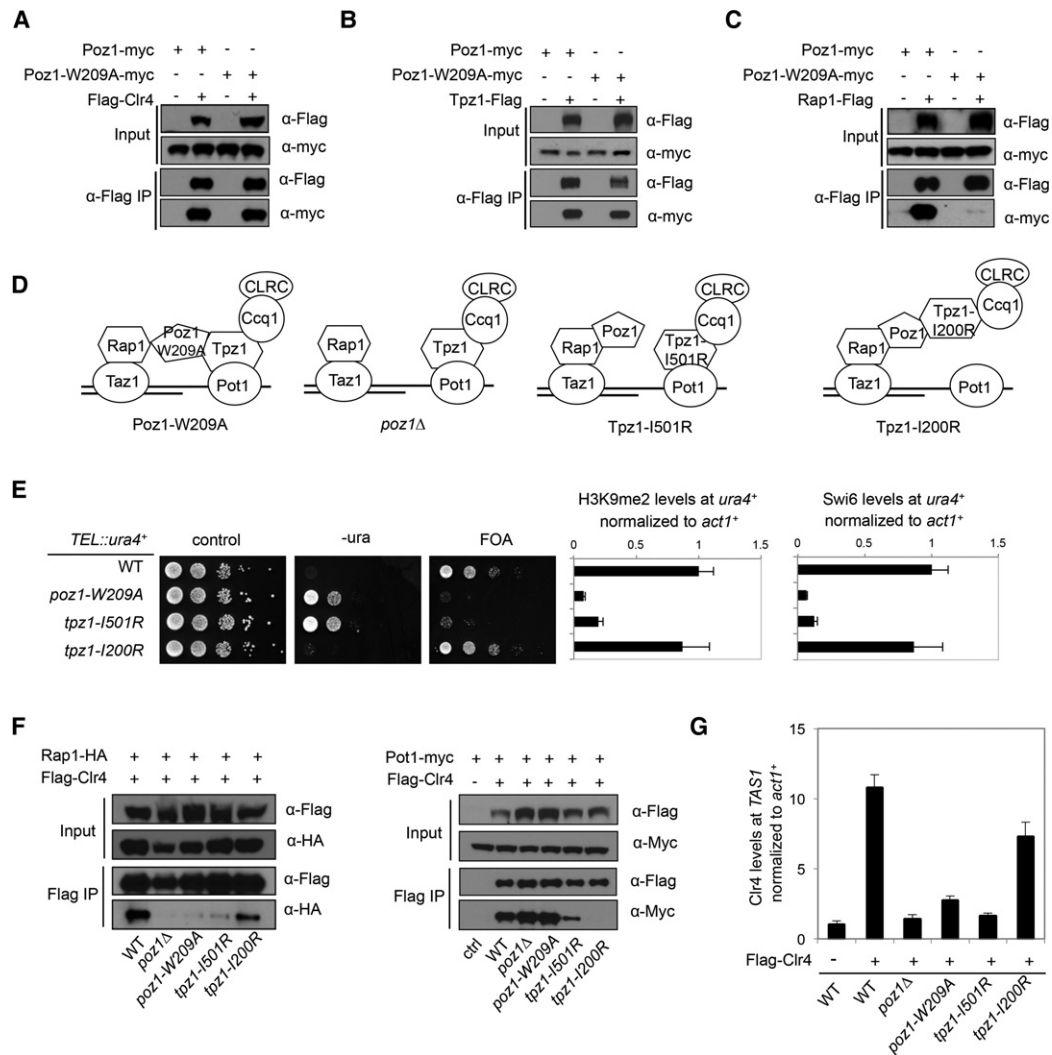


Figure 3. Proper connection between shelterin subunits is critical for telomeric heterochromatin assembly. (A,B,C,F) Co-IP analyses to examine the effect of *poz1-W209A* on shelterin–CLRC or shelterin subunit interactions. (D) Schematic diagram of the shelterin–CLRC interaction in each mutant background. (E, left) Tenfold serial dilution analyses of the indicated yeast strains grown on indicated the media to measure the expression of *TEL::ura4⁺*. (Right) ChIP analysis of H3K9me2 and Swi6 levels at *TEL::ura4⁺*, normalized to *act1⁺*. (G) ChIP analyses of Flag-Clr4 levels at *TAS1*, normalized to *act1⁺*.

2013]. Like *poz1-W209A*, *tpz1-I501R* resulted in loss of silencing of *TEL::ura4⁺* and loss of H3K9me2 and Swi6 at the reporter (Fig. 3E). However, *tpz1-I200R* has no silencing defects at *TEL::ura4⁺*, and ChIP analyses showed normal H3K9me2 and Swi6 levels at the *ura4⁺* reporter gene (Fig. 3E). These results suggest that the proper connection from Rap1 to Tpz1, but not the Tpz1–Pot1 connection, is required for subtelomeric heterochromatin formation. To further confirm this idea, we examined the requirement of shelterin connections in Rap1-GBD-mediated ectopic heterochromatin assembly. Supporting our hypothesis, H3K9me2 levels were abolished in all mutants that are defective in subtelomeric heterochromatin formation (*poz1Δ*, *poz1-W209A*, *tpz1Δ*, and *tpz1-I501R*) but remained unchanged in *tpz1-I200R* cells (Supplemental Fig. S2B).

Recruitment of CLRC to double-stranded telomeric DNA is required for subtelomeric heterochromatin

The differences in the ability of *poz1-W209A*, *tpz1-I501R*, and *tpz1-I200R* mutants to assemble heterochromatin raise the possibility that interaction between CLRC and dsDNA-binding proteins, but not ssDNA-binding protein Pot1, is required for subtelomeric heterochromatin. To test this idea, we performed co-IP analyses and found that *poz1-W209A*, *tpz1-I501R*, and *poz1Δ*, which showed loss of silencing at *TEL::ura4⁺*, all disrupted the interaction between Clr4 and Rap1 but still maintained the interaction between Clr4 and Pot1 (Fig. 3F). On the other hand, the Clr4–Pot1 interaction, but not the Clr4–Rap1 interaction, was disrupted in *tpz1-I200R* cells, which had no silencing defects at *TEL::ura4⁺* (Fig. 3F). Furthermore, ChIP analysis showed that Flag-Clr4 enrichment at *TAS1* and *TEL::ura4⁺* was abolished in *poz1-W209A*, *tpz1-I501R*, and *poz1Δ* cells but remained intact in *tpz1-I200R* cells (Fig. 3G; Supplemental Fig. S3A). Clr4 levels at more telomere-distal regions were not affected due to the presence of an RNAi-dependent pathway to recruit CLRC (Supplemental Fig. S3C; Kanoh et al. 2005). These results suggest that the association of CLRC with the dsDNA-binding part of shelterin is essential for subtelomeric heterochromatin assembly. In addition, Clr3 enrichment at subtelomeres was also reduced in all shelterin mutants that affected Clr4 recruitment but remained at wild-type levels in the *tpz1-I200R* mutant, which has no effects on Clr4 recruitment to telomeres (Supplemental Fig. S1C,D).

Restoring the proper association between shelterin components rescues subtelomeric heterochromatin assembly defects of shelterin mutants

To examine whether a compromised Poz1–Rap1 interaction is responsible for heterochromatin assembly defects in *poz1-W209A* cells, we generated a strain expressing Poz1-W209A with a GBP (GFP-binding protein) tag (Rothbauer et al. 2008) and crossed it into a strain expressing Rap1-GFP. The interaction between GBP and GFP is expected to restore the interaction between Rap1 and

Poz1-W209A. Indeed, the *TEL::ura4⁺* reporter was efficiently silenced in *poz1-W209A-GBP rap1-GFP* cells, accompanied by the restoration of heterochromatin hallmarks such as H3K9me2 and Swi6 (Fig. 4A). Similarly, *tpz1-I501R-GBP poz1-GFP* cells, which are expected to restore interaction between Tpz1 and Poz1, rescued silencing at *TEL::ura4⁺* as well as H3K9me2 and Swi6 levels (Fig. 4B). In addition to the restoration of subtelomeric heterochromatin, *tpz1-I501R-GBP poz1-GFP* cells also alleviated telomere length regulation defects associated with *tpz1-I501R*, further indicative of the restoration of interaction between Tpz1 and Poz1 (Supplemental Fig. S4). Interestingly, forced Tpz1–Rap1 interaction through Tpz1-GBP and Rap1-GFP in *poz1Δ* cells was able to silence *TEL::ura4⁺* and restored H3K9me2 and Swi6 levels at the reporter gene (Fig. 4C). Moreover, the telomere length was gradually reduced to wild-type level in *tpz1-GBP rap1-GFP poz1Δ* cells compared with *poz1Δ* cells (Supplemental Fig. S4). The fact that restoration of the linkage between the dsDNA and ssDNA telomere-binding complexes successfully rescues silencing defects caused by point mutants that specifically disrupt these interactions suggests that the overall architecture of the shelterin complex rather than the identity or specialized structure of the interaction is crucial for subtelomeric heterochromatin assembly. A recent study showing mini-shelterin, in which Taz1 is artificially fused to Tpz1, can maintain telomere length homeostasis and subtelomeric silencing even in the absence of Rap1 and Poz1 further supports this view (Pan et al. 2015).

Telomeric repeats block heterochromatin spreading

The fact that CLRC maintains its interaction with Pot1 in *poz1Δ*, *tpz1-I501R*, and *poz1-W209A* cells suggests that the subtelomeric silencing defect is not a result of impaired CLRC recruitment but is instead due to a failure of CLRC to spread from the chromosome ends over telomeric repeats. Telomere elongation increases the distance between Pot1-bound Clr4 and *TEL::ura4⁺* in *poz1Δ* and *tpz1-I501R* cells, which might contribute to the inability of Clr4 spreading to silence the reporter. However, *poz1-W209A* has very little effect on telomere length (Tadeo et al. 2013). The fact that *poz1-W209A* lost telomere silencing similar to *poz1Δ* and *tpz1-I501R* indicates that even the normal length of telomeric repeats is sufficient to block the spreading of Clr4 from the chromosome ends to internal regions when the connection between CLRC and the dsDNA region of the telomeric repeat is compromised.

We then directly examined whether telomeric repeats block heterochromatin spreading at an ectopic heterochromatin domain. Artificially targeting a GBD-Clr4-ΔCD fusion protein to three copies of Gal4-binding sites (*3xgbs*) silences an adjacent *ade6⁺* reporter gene (*3xgbs-ade6⁺*), as indicated by red colony color when cells were grown on YE medium (Supplemental Fig. S5A; Kagansky et al. 2009). We inserted between *3xgbs* and *ade6⁺* either 257 base pairs (bp) of telomeric repeat DNA (*3xgbs-tel-ade6⁺*) or 300 bp of control DNA from the *leu1* locus

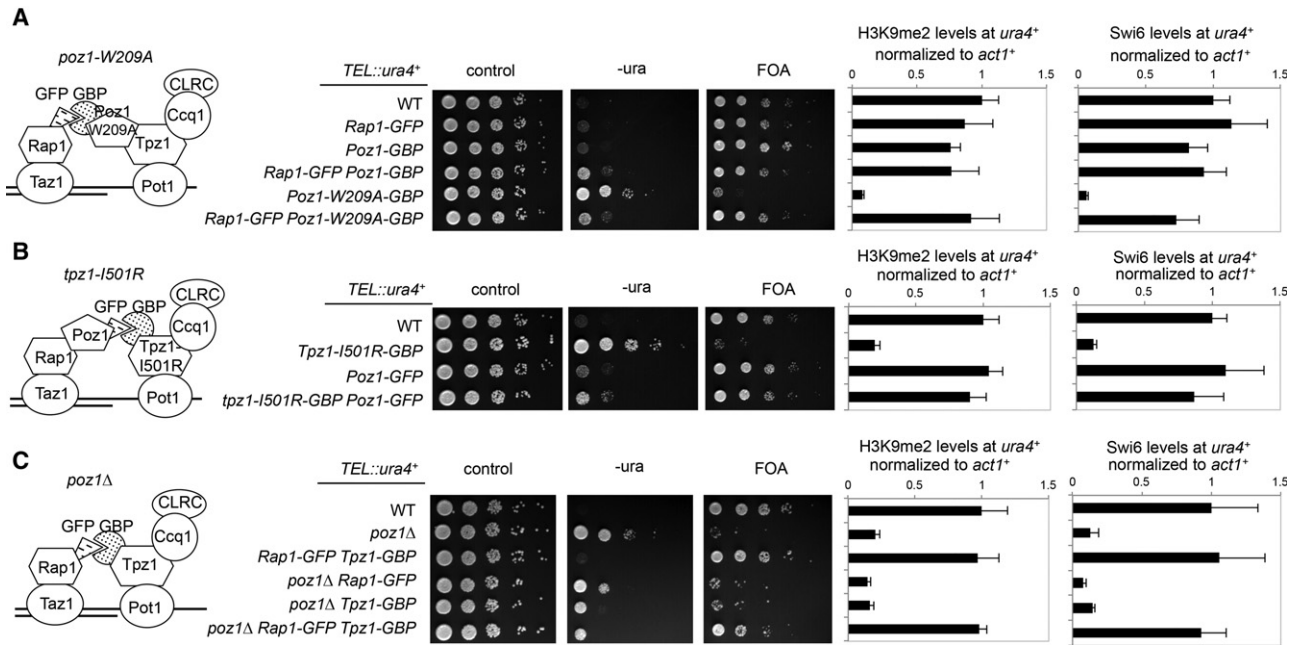


Figure 4. Proper connection between shelterin subunits is critical for subtelomeric heterochromatin assembly. (Left) Schematic diagram of the constructs. (Middle) Tenfold serial dilution analyses of indicated yeast strains grown on the indicated media to measure the expression of *TEL::ura4+*. (Right) ChIP analyses of H3K9me2 and Swi6 levels at *TEL::ura4+*, normalized to *act1+*.

(*3xgbs-control-ade6+*) (Fig. 5A). The control sequence had no effects on silencing of the *ade6+* reporter by GBD-Clr4- Δ CD, indicating efficient CLRC spreading (Supplemental Fig. S5A). In the absence of GBD-Clr4- Δ CD, the telomeric repeats silenced the *ade6+* reporter, and silencing was lost in *taz1Δ*, *rap1Δ*, *poz1Δ*, and *ccq1Δ* cells (Supplemental Fig. S5B). Such a result is consistent with shelterin-mediated recruitment of CLRC to telomeric repeats. Interestingly, in *ccq1Δ* cells, which abolished shelterin-CLRC interaction, GBD-Clr4- Δ CD failed to silence *3xgbs-tel-ade6+* (Fig. 5B). Moreover, ChIP analyses showed that H3K9me2 and Swi6 were enriched at *3xgbs*, but not *ade6+*, in *ccq1Δ* cells (Fig. 5B), suggesting that CLRC failed to spread over the telomeric repeats. In contrast, GBD-Clr4- Δ CD efficiently silences *3xgbs-control-ade6+* in *ccq1Δ* cells, and H3K9me2 and Swi6 spread to the *ade6+* reporter (Fig. 5B). These results suggest that shelterin-bound telomeric repeats are refractory to heterochromatin spreading.

In both budding and fission yeasts, telomeric DNA has been suggested to form nonnucleosomal structures (Chikashige et al. 1989; Wright et al. 1992). We performed MNase digestion of chromatin followed by Southern blot analyses with probes derived from pericentric and telomeric repeats. Pericentric chromatin showed a characteristic nucleosomal ladder pattern, indicative of well-positioned nucleosomes (Supplemental Fig. S6). In contrast, telomeric chromatin showed a diffuse pattern, indicative of low nucleosome density or alternative nucleosome structures (Supplemental Fig. S6). Interestingly, GBD-Clr4- Δ CD silenced *3xgbs-tel-ade6+* in *taz1Δ* cells (Supplemental Fig. S5C). Given that Taz1 is responsible

for recruiting other shelterin components in this setting, the results suggest that telomeric repeats and its associated chromatin structure, at least when they are only 257 bp long, are insufficient to block CLRC spreading in the absence of bound shelterin. Telomeres associate with the nuclear periphery through the interaction between Rap1 and inner nuclear membrane proteins Bqt3-Bqt4 (Chikashige et al. 2009). GBD-Clr4- Δ CD failed to silence *3xgbs-tel-ade6+* in *ccq1Δ bqt3Δ* cells (Supplemental Fig. S5C), suggesting that the ability of shelterin-bound telomeric repeats to block CLRC spreading is not due to the tethering of these repeats to the nuclear periphery but rather that the bound shelterin creates a physical barrier.

We also found that *poz1Δ* had stronger silencing defects at *TEL::ura4+* compared with *taz1Δ* (Supplemental Fig. S5D). Given that *taz1Δ* and *poz1Δ* have similar effects on telomere length, such a difference might be attributed to the binding of Taz1 to telomeric repeats in *poz1Δ* cells, which more efficiently blocks CLRC spreading. Consistent with this idea, *poz1Δ taz1Δ* partially rescued silencing defects of *TEL::ura4+* compared with *poz1Δ* (Supplemental Fig. S5D).

Increased heterochromatin spreading restores silencing at subtelomeres in shelterin mutants

If the heterochromatin defects of shelterin connection mutations are due to the failure of CLRC spreading over telomeric repeats, we expect that a genetic background that is more permissive to heterochromatin spreading might overcome this barrier and rescue silencing defects in shelterin mutants that disrupt shelterin interactions. Our

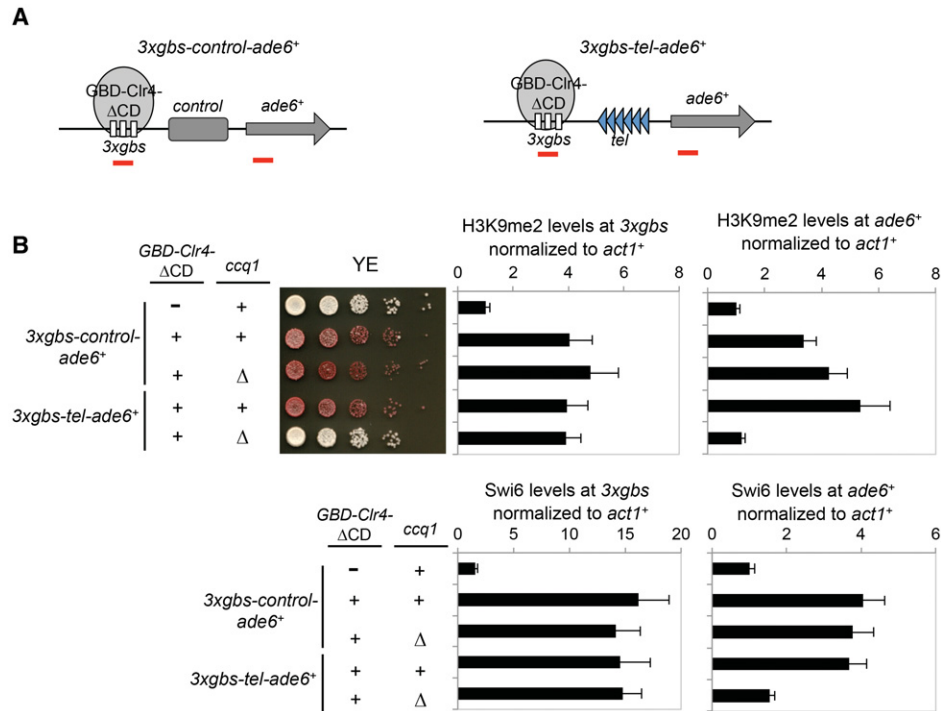


Figure 5. Telomeric repeats block heterochromatin spreading. (A) Schematic diagrams of the reporter genes used. Red bars indicate the positions of the PCR fragments used in ChIP analyses. (B, left) Tenfold serial dilution analyses of the indicated yeast strains grown on YE medium to measure the expression of *ade6+*. (Right) ChIP analyses of H3K9me2 and Swi6 levels at *3xgbs* and *ade6+*, normalized to *act1+*.

previous work showed that the Mst2 histone H3K14 acetyltransferase complex promotes histone turnover at heterochromatin regions, and *mst2Δ* results in increased heterochromatin spreading into subtelomeric regions (Reddy et al. 2011; Wang et al. 2012, 2015). We found that silencing of *TEL::ura4+* was significantly restored in *mst2Δ poz1Δ*, *mst2Δ poz1-W209A*, and *mst2Δ tpz1-I501R* double mutants (Fig. 6A; Supplemental Fig. S7A). In addition, H3K9me2 and Swi6 levels at *TEL::ura4+* were restored to near wild-type levels (Fig. 6A). Moreover, mutation of the active site of Mst2 (*mst2-G250E*) or other components of the Mst2 complex required for its acetyltransferase activity (*nto1Δ* and *ptf2Δ*) (Wang et al. 2012) also rescued *poz1Δ* in silencing of *TEL::ura4+*, consistent with the fact that the enzymatic activity of the Mst2 complex counteracts heterochromatin spreading (Supplemental Fig. S7B).

Co-IP analysis showed that Clr4 failed to interact with Rap1 in *mst2Δ poz1Δ* cells, similar to *poz1Δ* cells, suggesting that *mst2Δ* does not rescue CLRC-shelterin interaction in *poz1Δ* cells (Fig. 6B). Moreover, telomere length remains the same in *poz1Δ* and *mst2Δ poz1Δ* cells, suggesting that shelterin connections are not restored (Fig. 6C). However, ChIP analysis showed increased levels of Clr4 at *TAS1* and *TEL::ura4+* in *mst2Δ poz1Δ* cells (Fig. 6D; Supplemental Fig. S3B), consistent with the idea that the rescue of *TEL::ura4+* silencing in *mst2Δ poz1Δ* cells is due to increased spreading of CLRC from the telomere end to the reporter. We also observed restored levels

of Clr3 at *TAS1* in *mst2Δ poz1Δ* cells (Supplemental Fig. S1E), which is in line with the idea that Clr3 is recruited to the telomeres by CLRC-mediated heterochromatin formation. In addition, overexpression of Swi6 or *epe1Δ*, which promote heterochromatin spreading (Ayoub et al. 2003; Wang et al. 2013), also rescued telomeric heterochromatin defects in *poz1Δ* cells (Supplemental Fig. S7C). Furthermore, introducing a *clr4-W31G* mutation, which abolishes heterochromatin spreading from nucleation centers (Zhang et al. 2008), resulted in loss of silencing at *TEL::ura4+* in *mst2Δ poz1Δ* or *epe1Δ poz1Δ* cells (Fig. 6E; Supplemental S7D).

To rule out the possibility that the restoration of silencing at *TEL::ura4+* in *mst2Δ poz1Δ* cells is due to heterochromatin maintenance, we performed a heterochromatin re-establishment assay. We crossed *mst2Δ poz1Δ* cells with *mst2Δ poz1Δ clr4Δ* cells containing *TEL::ura4+* to obtain *mst2Δ poz1Δ TEL::ura4+* cells, in which the *TEL::ura4+* was inherited without pre-existing H3K9me. Dilution analyses showed that the resulting *mst2Δ poz1Δ* cells were able to establish heterochromatin at the reporter gene de novo, accompanied by the enrichment of H3K9me2 and Swi6 at the reporter (Supplemental Fig. S8).

Although we expect that Clr4 is recruited to ssDNA in *poz1Δ* cells through its interaction with Pot1 (Fig. 3F), we cannot directly measure the enrichment of Clr4 at the ssDNA given the identical sequences of the dsDNA and ssDNA portions of telomeric repeats. Nonetheless, given

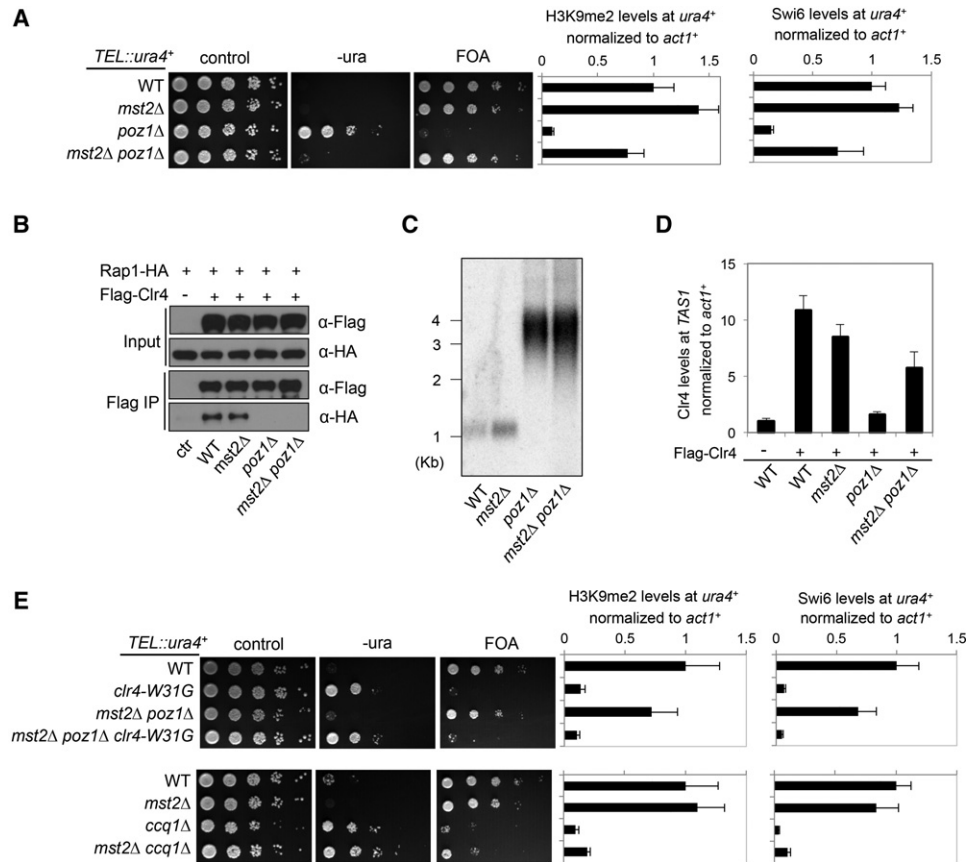


Figure 6. Increased heterochromatin spreading alleviates telomeric silencing defects caused by compromised shelterin connections. (A,E, left) Tenfold serial dilution analyses of the indicated yeast strains grown on the indicated media to measure the expression of *TEL::ura4⁺*. (Right) ChIP analyses of H3K9me2 and Swi6 levels at *TEL::ura4⁺*, normalized to *act1⁺*. (B) Co-IP analysis of Clr4–Rap1 interaction in different mutant backgrounds. (C) Southern blot analysis to measure telomere length. (D) ChIP analyses of Flag-Clr4 levels at *TAS1*, normalized to *act1⁺*.

that *ccq1*Δ resulted in complete loss of Clr4 from telomeres (Fig. 2G, since the fragment used for PCR amplification in ChIP analysis is <300 bp from the chromosome end, the result also reflects the binding of CLRC to telomeric repeats), the fact that *mst2*Δ *ccq1*Δ failed to silence *TEL::ura4⁺* suggests that Mst2 antagonizes telomeric silencing downstream from CLRC recruitment (Fig. 6E). Therefore, recruitment of CLRC to telomeric ssDNA is a prerequisite of *mst2*Δ to rescue the silencing defects associated with *poz1*Δ, although it is also possible that the failure of *mst2*Δ *ccq1*Δ cells to assemble telomeric heterochromatin is the result of the loss of both CLRC and SHREC from telomeres.

Discussion

In eukaryotic cells, telomeres are prominent sites of constitutive heterochromatin. However, the mechanism of telomeric heterochromatin assembly is poorly understood, making it difficult to dissect its biological functions. In fission yeast, shelterin is required for the establishment of subtelomeric heterochromatin, and it has been suggested that shelterin is responsible for the re-

cruitment of the histone H3K9 methyltransferase CLRC to telomeric repeats (Kanoh et al. 2005; Tadeo et al. 2013). However, mutations of individual shelterin components do not significantly affect the association of other shelterin components with telomeres due to independent shelterin recruitment pathways through both the ssDNA and the dsDNA portions of telomeric repeats (Miyoshi et al. 2008). Therefore, it is difficult to explain why many different shelterin mutants lead to subtelomeric heterochromatin assembly defects if the only function of shelterin is to recruit CLRC. Our results show that shelterin recruits the histone H3K9 methyltransferase CLRC through protein–protein interactions mediated by the Ccq1 subunit. Consistently, *ccq1*Δ results in defective recruitment of CLRC to telomeres. Interestingly, mutations other than *ccq1*Δ that affect subtelomeric heterochromatin still maintain the CLRC–Pot1 interaction, suggesting that shelterin plays other crucial roles in subtelomeric heterochromatin assembly aside from recruiting CLRC to chromosome ends. A common theme is that these mutations affect the association between CLRC and the dsDNA-binding portion of shelterin, and restoring such connections can rescue heterochromatin defects.

Heterochromatin has the propensity to spread from the site of initiation to neighboring regions generally in a sequence-independent manner (Wang et al. 2014a). However, the failure of CLRC to spread from the site of recruitment (the Tpz1–Pot1 subcomplex located at the ssDNA of telomeres) over telomeric repeats to more internal regions in mutations that disrupt interactions between shelterin subunits suggests that telomeric repeats are refractory to heterochromatin spreading. The proper connection of shelterin components overcomes this barrier by promoting direct interaction between CLRC and chromosomal regions internal to telomeres, allowing heterochromatin assembly (Fig. 7). Therefore, the ability of CLRC to regulate heterochromatin assembly depends on not only its recruitment but also the local chromatin context.

It is interesting that, without CLRC recruitment, a short stretch of repeats only a little more than one nucleosome length can function as a barrier to heterochromatin spreading at an ectopic site. Such a barrier function requires the binding of shelterin to form a physical block to CLRC spreading (Fig. 5). At telomeres, CLRC spreading from the chromosome ends to subtelomeric regions is also affected by *taz1Δ*, which compromises the binding of shelterin to double-stranded telomeric DNA and increases the distances between chromosome end-bound CLRC and subtelomeres. Therefore, telomeric DNA also contribute to barrier activity when they are of sufficient length.

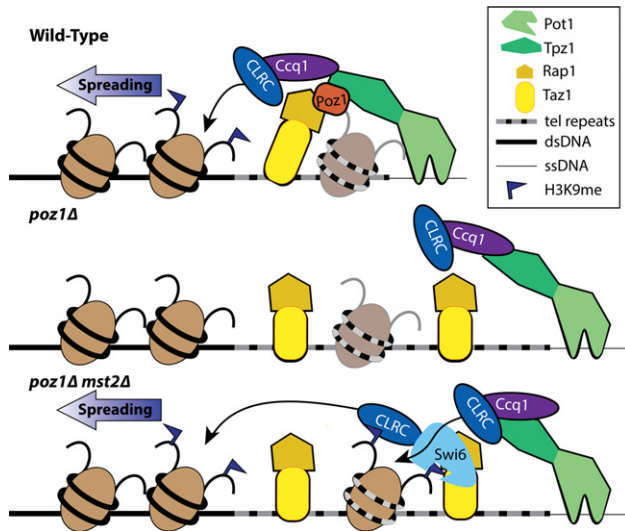


Figure 7. A model for telomeric heterochromatin assembly. In wild-type cells, the connection between shelterin allows CLRC to skip telomeric DNA to reach nucleosomes at subtelomeric regions to initiate histone H3K9me. In *poz1Δ* cells, although CLRC is still recruited to chromosome ends through interaction with Pot1–Tpz1–Ccq1, the paucity of nucleosomes on double-stranded telomeric DNA and the presence of shelterin components on these repeats prevent heterochromatin from spreading into internal chromosomal regions. In *mst2Δ poz1Δ* cells, the reduction of histone turnover allows H3K9me of the residual nucleosomes on telomeric DNA, which provides a platform for heterochromatin to spread.

It has been shown that telomeric DNA sequences from diverse species disfavor nucleosome assembly in vitro and in vivo (Chikashige et al. 1989; Wright et al. 1992; Cacchione et al. 1997; Ichikawa et al. 2014). The wrapping of DNA around a histone octamer requires sharp bending of DNA, which is facilitated by a 10-bp periodicity of AA/TT/TA dinucleotides (Segal et al. 2006). The 6- to 8-bp periodicity of telomeric repeats makes it difficult to maintain such phasing. Moreover, telomeric DNA can assemble G quadruplexes, which might also contribute to the formation of alternative chromatin structures at telomeres (Lipps and Rhodes 2009). Regardless of the mechanism, the nucleosome density at telomeres might be low enough to prevent the proximity-mediated positive feedback mechanism that facilitates spreading of heterochromatin, and the connections between shelterin components allow the heterochromatin machinery to skip telomeric repeats (Fig. 7). Although the telomeric chromatin structure, indicated by the MNase digestion pattern, is different from that of the chromosome arms, nucleosomes might still be present at fission yeast telomeres, as indicated by the detection of histone post-translational modifications (Carneiro et al. 2010). The fact that increased heterochromatin spreading overcomes silencing defects in the absence of shelterin connections further supports the existence of nucleosomes, most likely at lower levels, at telomeric DNA. However, these nucleosomes may be rapidly turned over or poorly positioned or possess post-translational modifications that prohibit heterochromatin assembly. The binding of shelterin to telomeric repeat DNA might further strengthen the barrier activity, as shelterin binding can directly impact the formation of nucleosomes over telomeric repeats. In addition, the binding of shelterin also creates a physical barrier for the spread of CLRC. Therefore, the effects of telomeric repeats in blocking CLRC spreading at native telomeres might be the combined action of telomeric chromatin structure and the binding of shelterin.

In budding yeast, the looping back of telomeres is thought to strengthen telomere position effect, a measure of heterochromatin integrity (Strahl-Bolsinger et al. 1997; de Bruin et al. 2000). However, such a telomeric chromatin organization differs from what we discovered in fission yeast. Budding yeast heterochromatin machinery is significantly divergent from that of fission yeast and is established at telomeres by RAPI1, which directly binds to the dsDNA portion of telomere repeats and recruits SIR proteins (Rusche et al. 2003). Therefore, even though telomeres lack nucleosome organization, looping at telomeres is not required to bypass the telomeric DNA, and it is likely that such a structure is required for other aspects of gene silencing.

In fission yeast, although loss of heterochromatin factors has no effect on telomere length control or chromosome end protection, heterochromatin does affect the mode of survival of cells without telomerase (Ekwall et al. 1996; Tuzon et al. 2004; Jain et al. 2010). However, as heterochromatin assembly factors are involved in heterochromatin formation genome-wide, it is unknown whether such phenotypes are the direct result of loss of

telomeric heterochromatin or an indirect effect of losing heterochromatin at nontelomeric locations. Our studies showing that most mutations of shelterin affect connections between shelterin subunits rather than shelterin-CLRC interactions suggest that these mutations are unlikely to be definitive in addressing the functions of telomeric heterochromatin. Nonetheless, our results do point out that Ccq1 is the critical subunit that mediates interaction between CLRC and shelterin. It is important to note that Ccq1 is a multifunctional protein involved in telomerase recruitment and DNA damage checkpoint suppression (Tomita and Cooper 2008). It is also part of the SHREC complex, in addition to its role in CLRC recruitment to telomeres (Sugiyama et al. 2007; Motamedi et al. 2008). Further isolation of separation-of-function mutations of Ccq1 holds great potential for genetically dissecting the function of telomeric heterochromatin in telomere length regulation and other aspects of chromosome biology.

It has long been known that telomeres form heterochromatin structures in diverse organisms (Blasco 2007). However, the function of telomeric heterochromatin is not well understood. In *Drosophila*, retrotransposons that form heterochromatin replace telomeric repeats, and heterochromatin proteins are directly involved in the recruitment of chromosome end protection factors (Mason et al. 2008). In fission yeast, the emergence of HAATI (heterochromatin amplification-mediated and telomerase-independent) survivors in response to telomerase loss also suggests a direct role of telomeric heterochromatin in the recruitment of telomere proteins (Jain et al. 2010), possibly mediated by direct protein-protein interactions between shelterin and CLRC. Moreover, heterochromatin is also involved in regulating transcription of telomeric noncoding RNA TERRA, which has been increasingly recognized to play important roles in telomere functions (Azzalin and Lingner 2015). Thus, a more complete understanding of the mechanism of telomeric heterochromatin assembly will allow a more definitive dissection of the functions of telomeric heterochromatin.

Materials and methods

Fission yeast strains and genetic analyses

Yeast strains containing Poz1-myc, Poz1-GBP, Poz1-W209A-GBP, Tpz1-GBP, Tpz1-myc, Tpz1-I501R-GBP, and Pot1-myc were generated by a PCR-based module method (Bahler et al. 1998). Due to the effect of C-terminal tags on the functionality of Tpz1 (Kallgren et al. 2014), the Tpz1-I501R and Tpz1-I200R used in this study were constructed without any tags unless otherwise noted. All strains containing *tpz1Δ* and *pot1Δ* were first constructed in diploid cells, followed by tetrad dissection to obtain desired haploid colonies. The *3xgbs-tel-ade6⁺* reporter was constructed by inserting 257-bp telomeric DNA sequences between *3xgbs* and the *ade6⁺* reporter gene, and the *3xgbs-control-ade6⁺* reporter was constructed by inserting 306-bp DNA from the *leu1⁺* locus (corresponding to amino acids 230–331) between *3xgbs* and the *ade6⁺* reporter gene. A list of the yeast strains used is in Supplemental Table 1. For serial dilution plating assays, 10-fold dilutions of a mid-log-phase culture were plated on the indicated media and grown for 3 d at 30°C.

Protein purification, co-IP, and mass spectrometry analyses

Exponentially growing yeast cells were harvested, washed with 2× HC buffer (300 mM HEPES-KOH at pH 7.6, 2 mM EDTA, 100 mM KCl, 20% glycerol, 2 mM DTT, protease inhibitor cocktail [Roche]), and frozen in liquid nitrogen. Crude cell extracts were prepared by vigorously blending frozen yeast cells with dry ice using a household blender, followed by incubation with 30 mL of 1× HC buffer containing 250 mM KCl for 30 min. The lysate was cleared by centrifugation at 82,700g for 3 h. The supernatants were precleared with protein A agarose, incubated with 200 μL of Flag-agarose overnight, and washed eight times with 1× HC containing 250 mM KCl. For mass spectrometry analysis, bound proteins were eluted with 200 μg/mL 3xFlag peptides followed by TCA precipitation. MudPIT (multidimensional protein identification technology) mass spectrometry analysis was performed as described previously (Wang et al. 2014b). For co-IP analysis, bound proteins were resolved by SDS-PAGE followed by Western blot analyses with Myc (Santa Cruz Biotechnology), Flag (Sigma), and HA (3F10; Roche) antibodies.

ChIP analyses

ChIP analyses were performed as described previously (Wang et al. 2015). The antibodies used were H3K9me2 (Abcam), Swi6 (Reddy et al. 2011), and Flag (Sigma). Quantitative real-time PCR (qPCR) was performed with Maxima SYBR Green qPCR master mix (Fermentas) in a StepOne Plus real-time PCR system (Applied Biosystems). DNA serial dilutions were used as templates to generate a standard curve of amplification for each pair of primers, and the relative concentration of target sequence was calculated accordingly. An *act1* fragment was used as reference to calculate the enrichment of ChIP over WCE for each target sequence. The numbers are the averages of three experiments, and error bars represent standard deviation. A list of the DNA oligos used is in Supplemental Table 2.

Yeast two-hybrid assay

Full-length individual CLRC subunits were cloned into the XmaI/BamHI site of pGBT9 (Clontech) to generate fusion proteins with the GAL4 DNA-binding domain. Full-length Ccq1 was cloned into the XmaI/BamHI site of pGAD424 (Clontech) to generate fusion proteins with the GAL4 activation domain. Both plasmids were transformed into the budding yeast strain pJ69-4A, and transformants were selected on medium lacking tryptophan and leucine to maintain both plasmids and were confirmed by PCR analyses. The interaction of the two proteins was indicated by the activation of a *HIS3* reporter, allowing growth on medium lacking histidine.

Telomere length analysis

Genomic DNA was isolated, digested with EcoRI, and separated on 1% agarose gel. Hybridization with a radiolabeled telomeric DNA probe was performed as previously described (Moser et al. 2011).

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