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# Elimination of shelterin components bypasses RNAi for pericentric heterochromatin assembly

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**The RNAi pathway is required for heterochromatin assembly at repetitive DNA elements in diverse organisms. In fission yeast, loss of RNAi causes pericentric heterochromatin defects, compromising gene silencing and chromosome segregation. Here we show that deletion of telomere shelterin components restores pericentric heterochromatin and its functions in RNAi mutants. We further isolated a separation-of-function mutant of Poz1 and revealed that defective telomere silencing, but not telomere length control, is critical for bypassing RNAi. Further analyses demonstrated that compromising shelterin-mediated heterochromatin assembly in RNAi mutants releases heterochromatin protein Swi6, which is redistributed to pericentric regions through RNAi-independent heterochromatin assembly pathways. Given the high mobility of Swi6 protein and that increased levels of Swi6 facilitates heterochromatin spreading as well as ectopic heterochromatin assembly, our results suggest that constitutive heterochromatin domains use multiple pathways to form high-affinity platforms to restrain Swi6, thus limiting its availability and avoiding promiscuous heterochromatin formation.**

[*Keywords*: RNAi; heterochromatin assembly; telomere; shelterin; centromere]

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Eukaryotic genomes possess large stretches of repetitive DNA elements that form compact chromatin structures termed heterochromatin. These regions are relatively gene-poor but occupy crucial chromosome regions, such as centromeres and telomeres, which play critical roles in chromosome segregation and three-dimensional genome organization. Heterochromatin also inhibits transcription and recombination of the underlying DNA repeats, rendering it indispensable for maintaining genome integrity (Grewal and Jia 2007; Almouzni and Probst 2011).

Genomic DNA is folded with histones to form chromatin. Each chromosomal region is associated with distinct profiles of histone post-translational modifications, which play essential roles in regulating chromatin structure and function. Histones at heterochromatic regions are generally devoid of acetylation and are methylated at H3 Lys9 (H3K9me). H3K9me allows for the binding of heterochromatin protein 1 (HP1), which compacts chromatin through its self-association and the recruitment of other chromatin-modifying activities (Grewal and Jia 2007). Although heterochromatin has traditionally been considered silent, stable, and static, recent biochemical and imaging studies show that the binding of HP1 proteins to heterochromatin regions is surprisingly dynamic (Cheutin et al. 2003, 2004; Festenstein et al. 2003; Sadaie et al. 2008). Moreover, although heterochromatin normally excludes the transcriptional machinery, the DNA repeats within heterochromatin are transcribed, and the transcripts are processed by the RNAi pathway into siRNAs, which target histone-modifying complexes to repetitive DNA elements (Moazed, 2009; Lejeune and Allshire 2011; Castel and Martienssen 2013).

RNAi-dependent heterochromatin assembly is best studied in the fission yeast *Schizosaccharomyces pombe* (for review, see Moazed 2009; Lejeune and Allshire, 2011; Goto and Nakayama 2012; Castel and Martienssen 2013). In this organism, constitutive heterochromatin is localized at the centromeres, telomeres, and the silent mating type region, all of which share similar repetitive DNA elements composed of *dg* and *dh* repeats. The DNA repeats

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are transcribed by RNA polymerase II (Pol II) during the S phase of the cell cycle, producing dsRNAs with the help of a RNA-dependent RNA polymerase complex (RDRC). The ribonuclease Dicer (Dcr1) processes these dsRNAs into siRNAs, which are loaded onto the Argonaute siRNA chaperone complex (ARC) and then transferred to RNAi-induced transcriptional silencing complex (RITS). The Argonaute protein (Ago1) within RITS binds siRNAs and targets RITS to nascent RNA transcripts from repeat regions. RITS then recruits the CLRC complex, which contains the H3K9 methyltransferase Clr4. H3K9me recruits chromodomain proteins Swi6 and Chp2 together with their associated histone deacetylases and chromatin remodeling activities to achieve transcriptional silencing. H3K9me also stabilizes the binding of RITS to chromatin through the chromodomain protein Chp1, which in turn recruits RDRC to generate more dsRNAs and siRNAs, thus forming a self-reinforcing loop between siRNA production and heterochromatin assembly.

In addition to RNAi-based mechanisms, DNA-based mechanisms also function at repeat regions to recruit CLRC. For example, ATF/CREB family proteins Atf1 and Pcr1 cooperate with RNAi to establish heterochromatin at the silent mating type region (Jia et al. 2004; Kim et al. 2004). Similarly, telomere shelterin component Taz1 and the telomere-associated sequence (TAS) function together with RNAi to establish heterochromatin at telomeres (Kanoh et al. 2005). As a result, loss of RNAi severely affects pericentric heterochromatin functions but has little effect on heterochromatin structures at the silent mating type region or telomeres. However, even at pericentric regions, H3K9me and Swi6 are still present in RNAi mutants, albeit at lower levels, suggesting the existence of RNAi-independent mechanisms to establish heterochromatin (Sadaie et al. 2004). These pathways involve the Clr3 and Sir2 histone deacetylases since H3K9me levels are further reduced from pericentric regions in *clr3Δ dcr1Δ* and *sir2Δ dcr1Δ* cells, although Sir2 seems to function in a separate pathway from Clr3 (Yamada et al. 2005; Alper et al. 2013; Buscaino et al. 2013; Marina et al. 2013).

Interestingly, elimination of the Mst2 histone H3K14 acetyltransferase complex activity, the JmjC domain protein Epe1, or RNA quality control factor Mlo3 bypasses the requirement of RNAi for pericentric heterochromatin assembly (Trewick et al. 2007; Reddy et al. 2011; Reyes-Turcu et al. 2011). Although the mechanisms by which these mutants regulate heterochromatin assembly in the absence of RNAi differ, these results nonetheless indicate that RNAi is not obligatory for pericentric heterochromatin formation under certain conditions. To further understand heterochromatin assembly pathways at pericentric regions, we performed a high-throughput screen of the fission yeast deletion library for mutants that support pericentric heterochromatin assembly in the absence of RNAi. We discovered that loss of the telomere shelterin components involved in proper telomere maintenance also bypassed RNAi for pericentric heterochromatin formation. We further isolated a mutant of shelterin component Poz1 that specifically affected telomeric heterochromatin assembly without affecting telomere length and showed that loss of shelterin in an RNAi mutant background resulted in the redistribution of heterochromatin protein Swi6 from telomeric heterochromatin to pericentric regions through RNAi-independent heterochromatin assembly pathways involving Clr3 and Sir2. Furthermore, we showed that increasing overall Swi6 levels was sufficient to bypass RNAi for pericentric heterochromatin assembly. Our results demonstrate a dynamic equilibrium of different heterochromatin domains through the high mobility of Swi6 and suggest that constitutive heterochromatin regions, such as centromeres and telomeres, play important roles in balancing the availability of silencing factors to maintain the overall epigenetic landscape of the genome.

## Results

### *A high-throughput screen for mutants that bypass the requirement of the RNAi machinery for pericentric heterochromatin assembly*

In order to understand the mechanism of RNAi and heterochromatin assembly, we performed a screen of the fission yeast deletion library for mutants that allow restoration of pericentric heterochromatin in the absence of RNAi function. We used a query strain containing a *ura4*$^+$ reporter gene inserted at the outer repeat of centromere I (*otr::ura4*$^+$) (Supplemental Fig. S1A). In wild-type cells, this reporter gene is silenced due to the formation of heterochromatin. As a result, there is little growth on medium lacking uracil but robust growth on counter-selective medium containing 5-fluoroorotic acid (FOA), which is toxic to cells expressing *ura4*$^+$ (Fig. 1A). Mutations that affect heterochromatin structure at pericentric regions, such as *dcr1Δ*, resulted in strong cell growth on medium lacking uracil but little growth on medium containing FOA (Fig. 1A). Through two successive crosses, we introduced the *otr::ura4*$^+$ reporter and *dcr1Δ* into the deletion library and obtained haploid cells, each containing *otr::ura4*$^+$, *dcr1Δ*, and a single gene deletion (Supplemental Fig. S1B). The resulting cells were grown on medium containing FOA to measure cell growth (Supplemental Fig. S1C; Supplemental Table S1). Our screen identified mutants known to bypass RNAi for pericentric heterochromatin assembly, such as Mst2 complex components *mst2Δ*, *nto1Δ*, and *ptf2Δ* (Reddy et al. 2011; Wang et al. 2012), validating the effectiveness of our screen. Our screen also identified *poz1Δ*, a telomere shelterin component involved in telomere length control (Miyoshi et al. 2008), as showing the strongest rescue of heterochromatic silencing (Supplemental Fig. S1C; Supplemental Table S1). We also performed similar screens with an *otr::ade6*$^+$ reporter (Supplemental Fig. S2A). This reporter gene is silenced in wild-type cells, resulting in the formation of red colonies on low-adenine medium due to the accumulation of metabolic intermediates. In RNAi mutants, such as *dcr1Δ*, loss of pericentric heterochromatin resulted in the expression of Ade6, giving rise to white colonies (Supplemental Fig. S2A). We also identified *poz1Δ* as one

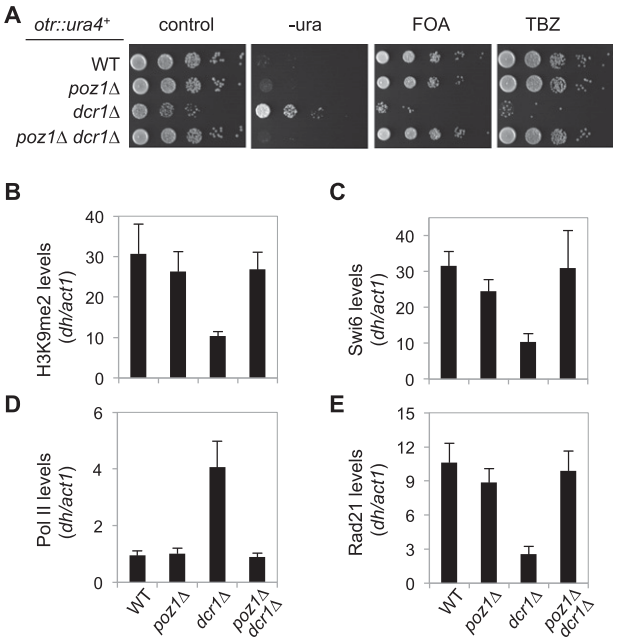


**Figure 1.** Elimination of Poz1 bypasses the requirement of Dicer for pericentric heterochromatin assembly and function. (*A*) Tenfold serial dilution analyses of the indicated yeast strains grown on the indicated medium to measure the expression of *otr::ura4*$^+$ and sensitivity to TBZ. (*B–E*) ChIP analysis of H3K9me2, Swi6, Pol II (Rpb1), and Rad21-Flag levels at pericentromeric *dh* repeats, normalized to *act1*. The numbers are averages of three experiments, and error bars represent standard deviation.

of the strongest suppressors of *dcr1Δ* and *ago1Δ* with the *otr::ade6*$^+$ reporter (Supplemental Fig. S2B,C; Supplemental Table S1). Altogether, we identified a total of 33 gene deletions that bypassed RNAi in both *otr::ura4*$^+$ and *otr::ade6*$^+$ reporter screens.

### *Elimination of Poz1 bypasses RNAi for pericentric heterochromatin functions*

To confirm that loss of Poz1 can indeed restore pericentric heterochromatin in RNAi mutants, we constructed a *poz1Δ dcr1Δ* double mutant strain containing *otr::ura4*$^+$. Serial dilution analyses confirmed that *poz1Δ dcr1Δ* grew weakly on medium without uracil but robustly on medium containing FOA (Fig. 1A). Chromatin immunoprecipitation (ChIP) analyses showed that in *poz1Δ dcr1Δ* cells, heterochromatin hallmarks at pericentric *dh* repeats, such as H3K9me2 and Swi6, were restored to near wild-type levels (Fig. 1B,C). Moreover, ChIP analyses showed that Pol II was strongly excluded from pericentric repeats (Fig. 1D), suggesting that heterochromatin formed in *poz1Δ dcr1Δ* cells is capable of transcriptional gene silencing.

Pericentric heterochromatin is required for the accumulation of high levels of cohesins, which are critical for chromosome segregation during mitosis (Bernard et al. 2001; Nonaka et al. 2002; Yamagishi et al. 2008). When heterochromatin is compromised, such as in *dcr1Δ*, loss of cohesin results in defective attachment of centromeres by microtubules for biorientation. As a result, cells are very sensitive to the microtubule poison thiabendazole (TBZ) (Hall et al. 2003; Volpe et al. 2003). In *poz1Δ dcr1Δ* cells, both cohesin localization, as measured by ChIP analyses of cohesin subunit Rad21-Flag, and TBZ sensitivity were significantly rescued (Fig. 1A,E). Taken together, these results indicate that pericentric heterochromatin formed in *poz1Δ dcr1Δ* cells is functional in regulating chromosome segregation.

### *Bypassing RNAi requires RNAi-independent heterochromatin assembly pathways at pericentric regions*

In fission yeast, the RNAi pathway involves several different complexes in addition to Dicer (Goto and Nakayama 2012). We also found that *poz1Δ* rescued silencing defects at *otr::ura4*$^+$ and TBZ sensitivity of all RNAi mutants tested, such as those in RITS (*ago1Δ* and *chp1Δ*) and RDRC (*rdp1Δ*) (Fig. 2A). However, *poz1Δ* could not rescue silencing defects and TBZ sensitivity of heterochromatin mutants involved in histone modifications or their recognition, such as *clr4Δ*, *swi6Δ*, or *clr3Δ* (Fig. 2B), suggesting that histone modifications are still essential for pericentric heterochromatin assembly in *poz1Δ dcr1Δ* cells.

RNAi is required for both the establishment and maintenance of heterochromatin at pericentric regions (Sadaie et al. 2004). To distinguish whether *poz1Δ dcr1Δ* rescues heterochromatin maintenance or establishment, we introduced *otr::ura4*$^+$ in either a silenced state (from wild-type cells) or a desilenced state (from *clr4Δ* cells) into

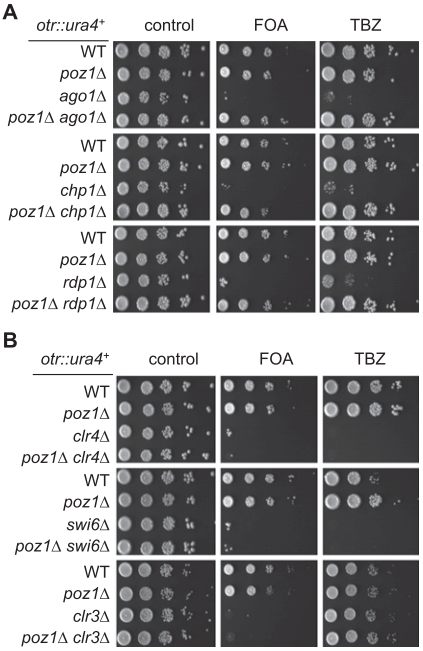


**Figure 2.** Elimination of Poz1 bypasses the entire RNAi pathway, but not histone modifications, for pericentric heterochromatin assembly and function. (*A*,*B*) Tenfold serial dilution analyses of the indicated yeast strains grown on the indicated medium to measure the expression of *otr::ura4*$^+$ and sensitivity to TBZ.

*poz1Δ dcr1Δ* cells by genetic crosses (Fig. 3A). Interestingly, *poz1Δ dcr1Δ* cells efficiently maintained silencing of *otr::ura4*$^+$ when it was inherited from a wild-type cell but failed to establish silencing when it was inherited from a *clr4Δ* cell (Fig. 3B). ChIP analyses of H3K9me2 also confirmed this conclusion (Fig. 3B).

In RNAi mutants, although silencing of reporter genes is completely lost, significant amounts of H3K9me are still retained at pericentric repeats (Fig. 3C; Sadaie et al. 2004). The histone deacetylases Clr3 and Sir2 function cooperatively with RNAi to establish heterochromatin at pericentric repeats, and H3K9me2 is completely abolished in *clr3Δ dcr1Δ* and *sir2Δ dcr1Δ* cells (Yamada et al. 2005; Alper et al. 2013; Buscaino et al. 2013; Marina et al. 2013). The results that *poz1Δ dcr1Δ* cells maintained preexisting heterochromatin but failed to establish heterochromatin de novo suggest that the residual H3K9me in *dcr1Δ* cells is necessary for *poz1Δ dcr1Δ* cells to maintain pericentric heterochromatin. Indeed, neither *poz1Δ dcr1Δ clr3Δ* nor *poz1Δ dcr1Δ sir2Δ* cells can maintain silencing of *otr::ura4*$^+$ or H3K9me levels at pericentric repeats (Fig. 3C), suggesting that the RNAi-independent pathway is critical for the ability of *poz1Δ dcr1Δ* cells to maintain pericentric heterochromatin.

### *Elimination of other shelterin components also bypasses RNAi for pericentric heterochromatin assembly*

Poz1 is a member of the telomere-associated shelterin complex (Miyoshi et al. 2008) that protects chromosome ends from being recognized as DNA damage sites and prevents DNA loss during replication (Palm and de Lange 2008; Jain and Cooper 2010). Telomeric DNA consists of short tandem DNA repeats followed by a single-stranded overhang (Fig. 4A). In fission yeast, Taz1 (homolog of mammalian TRF1/2) binds to the dsDNA repeats (Cooper et al. 1997), and the Pot1–Tpz1 complex (homologous to mammalian POT1–TPP1) binds to the ssDNA overhang (Baumann and Cech 2001; Miyoshi et al. 2008). Rap1 and Poz1 form a bridge that connects Taz1 and Pot1–Tpz1 complexes (Miyoshi et al. 2008). Pot1–Tpz1 associates with Ccq1 to recruit telomerase Trt1, which uses associated RNA as a template to elongate the telomere overhang (Nakamura et al. 1997; Miyoshi et al. 2008; Tomita and Cooper 2008; Moser et al. 2011; Yamazaki et al. 2012). By ChIP analyses, we found that Poz1 was enriched at telomeres, as expected, but not detected at pericentric repeats, even in *dcr1Δ* cells (Fig. 4B). Furthermore, Northern blot analyses showed that no siRNAs derived from pericentric repeats were detected in *poz1Δ dcr1Δ* cells (Fig. 4C), suggesting that Poz1 regulates pericentric heterochromatin assembly indirectly through RNAi-independent mechanisms.

Since our screens of mutants that bypass RNAi also consistently identified *rap1Δ* (Supplemental Table S1), we tested whether other shelterin mutants behaved similarly. We found that *rap1Δ*, *taz1Δ*, and *ccq1Δ* also rescued RNAi mutants' defects in *otr::ura4*$^+$ silencing and TBZ sensitivity (Fig. 4D; Supplemental Fig. S3). Similarly, *rap1Δ dcr1Δ clr3Δ* and *taz1Δ dcr1Δ clr3Δ* failed to maintain pericentric heterochromatin silencing (Supplemental Fig. S4), indicating the requirement of RNAi-independent pericentric heterochromatin pathways. In contrast, *trt1Δ* and a *ccq1-T93A* mutant, which attenuated recruitment of Trt1 to telomeres (Moser et al. 2011; Yamazaki et al. 2012), had no effect on this process (Fig. 4D). We reasoned that the different behavior of *ccq1Δ* and *ccq1-T93A* is because Ccq1 is also part of the SHREC complex involved in telomere silencing (Sugiyama et al. 2007; Motamedi

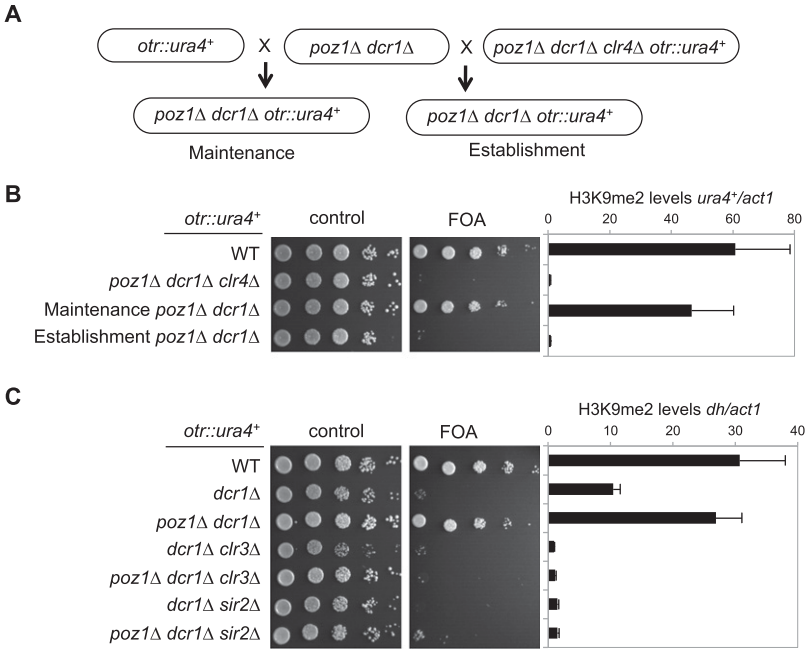


**Figure 3.** Elimination of Poz1 bypasses RNAi for pericentric heterochromatin maintenance but not for its establishment. (*A*) Schematic diagram of the experimental design to examine heterochromatin maintenance and establishment. (*B*,*C*, *left*) Tenfold serial dilution analyses of the indicated yeast strains to measure the expression of *otr::ura4*$^+$. (*Right*) ChIP analysis of H3K9me2 levels at *ura4*$^+$ or pericentric *dh* repeats, normalized to *act1*. The numbers are averages of three experiments, and error bars represent standard deviation.

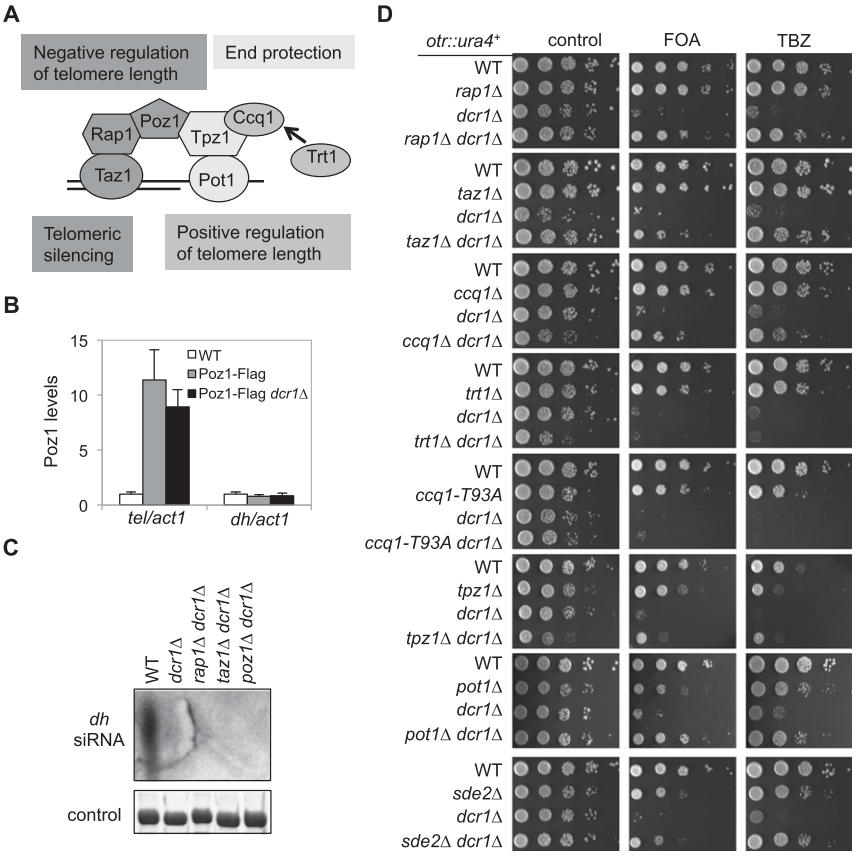


**Figure 4.** Elimination of other shelterin components also bypasses RNAi for pericentric heterochromatin assembly. (*A*) Schematic diagram of the telomere complex. (*B*) ChIP analysis of Poz1-Flag levels at subtelomeric and pericentric regions. The numbers are averages of three experiments, and error bars represent standard deviation. (*C*) Northern blot analyses of siRNAs derived from pericentric *dh* repeats. (*D*) Tenfold serial dilutions of the indicated yeast strains were grown on the indicated medium to measure the expression of *otr::ura4*$^+$ and sensitivity to TBZ. The *trt1Δ dcr1Δ* and *ccq1-T93A dcr1Δ* cells were freshly germinated to avoid senescence-associated telomere rearrangement.

et al. 2008), which is not affected by the *ccq1-T93A* mutation (Moser et al. 2011; Yamazaki et al. 2012). Consistent with this idea, we also found that *sde2Δ*, which resulted in selective loss of telomere silencing (Sugioka-Sugiyama and Sugiyama 2011), was able to bypass RNAi for pericentric heterochromatin formation (Supplemental Table S1; Fig. 4D). Pot1 and Tpz1 are required for telomere end protection. As a result, *pot1Δ* or *tpz1Δ* cells lose telomeres quickly and can only survive by circularizing all three chromosomes (Baumann and Cech 2001; Miyoshi et al. 2008). Nonetheless, pericentric heterochromatin was efficiently maintained in *pot1Δ dcr1Δ* and *tpz1Δ dcr1Δ* cells, as indicated by *otr::ura4*$^+$ silencing and TBZ sensitivity (Fig. 4D).

### *The ability of shelterin to regulate telomeric heterochromatin is key to bypassing RNAi*

One of the common phenotypes of the telomere-related mutants that bypass RNAi is that they all affect telomere silencing (Cooper et al. 1997; Nimmo et al. 1998; Kanoh and Ishikawa 2001; Sugiyama et al. 2007; Moser et al. 2009). However, the mechanism by which shelterin nucleates heterochromatin assembly is unknown. Because Taz1 and Pot1 contribute independently to shelterin recruitment, it is difficult to examine the sequence by which these factors are recruited at native telomeres. We constructed Rap1 fused with a Gal4 DNA-binding domain (Rap1-GBD) at its endogenous chromosome location. Rap1-GBD is sufficient to induce ectopic heterochromatin assembly at a reporter gene with adjacent Gal4-binding sites (*3xgbs-ade6*$^+$) under the condition of mild Swi6 overexpression (Supplemental Fig. S5). Silencing depends on Poz1 but not Taz1 (Fig. 5A), suggesting that Poz1 functions downstream from Taz1 and Rap1 in telomeric heterochromatin assembly.

To distinguish the function of telomere length control and telomeric silencing in bypassing RNAi, we mutated seven conserved residues of Poz1 individually at the endogenous *poz1* locus and screened for mutations that specifically affected telomeric silencing without affecting telomere length homeostasis (Supplemental Fig. S6). In the end, we isolated a W209A mutation that had no effect on Poz1 protein levels, as indicated by Western blot analysis (Fig. 5B), and had little effect on telomere length homeostasis, as indicated by Southern blot analyses (Fig. 5C). However, it caused defects in Rap1-GBD-mediated heterochromatin assembly, like in *poz1Δ* cells (Fig. 5A). We also tested the effect of *poz1-W209A* on silencing near telomeres. Since multiple pathways cooperatively regulate telomeric heterochromatin assembly, including shelterin, RNAi (through a repeat sequence within the *tlh1*$^+$ gene), and an unknown mechanism at TAS (Kanoh et al. 2005), we used a *ura4*$^+$ reporter inserted near telomeric repeats on a minichromosome (*TEL::ura4*$^+$) (Nimmo et al. 1994) that lacks the *tlh1*$^+$ gene and TAS, making the role of shelterin on silencing more apparent (Supplemental Fig. S7). Indeed, *poz1-W209A* resulted in a strong loss of

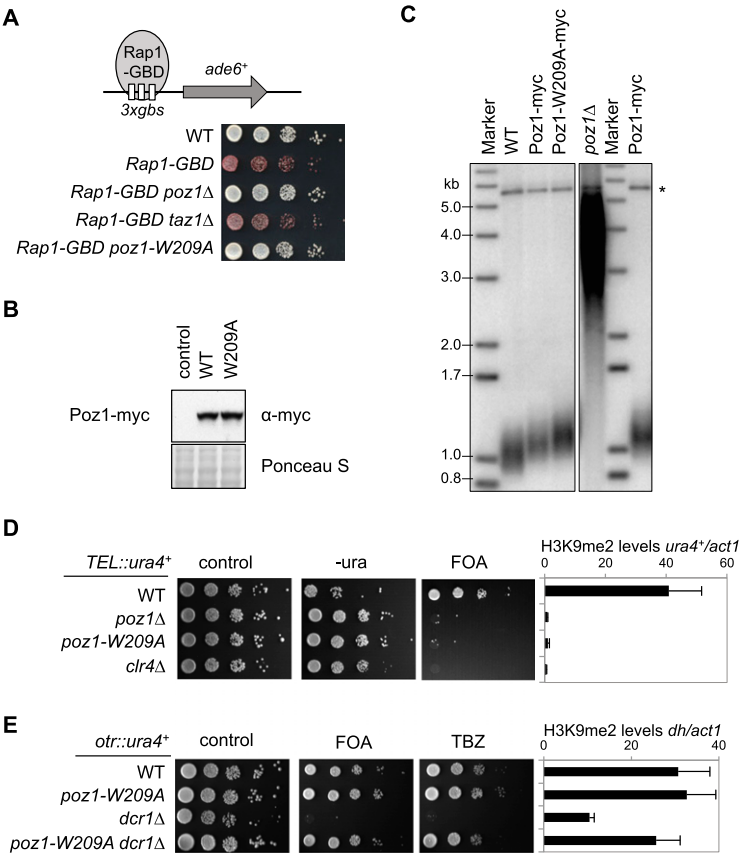


**Figure 5.** Loss of shelterin-mediated silencing at telomeres is required for bypassing RNAi for pericentric heterochromatin assembly. (*A*) Tenfold serial dilutions of the indicated yeast strains were grown on a low-adenine medium to measure the expression of *3xgbs-ade6*$^+$. (*B*) Western blot analyses were performed to measure Poz1-myc protein levels. (*C*) Southern blot analyses were performed to measure telomere length. The genomic DNA was digested with EcoRI, and the telomeric repeat sequence was used as a probe. (*) Control *pol1* fragment. (*D*,*E*) Tenfold serial dilutions of the indicated yeast strains were grown on the indicated medium to measure the expression of *TEL::ura4*$^+$ and ChIP analysis of H3K9me2 levels at *ura4*$^+$ or pericentric *dh* repeat, normalized to *act1*. The numbers are averages of three experiments, and error bars represent standard deviation.

silencing at this reporter, concomitant with the loss of H3K9me at this location, to a degree similar to *poz1Δ* and *clr4Δ* cells (Fig. 5D). Remarkably, *poz1-W209A* also rescued pericentric heterochromatin assembly in *dcr1Δ* cells (Fig. 5E), as indicated by the restoration of silencing at the *otr::ura4*$^+$ reporter and H3K9me at pericentric *dh* repeats. Thus, compromised telomeric silencing is the reason why loss of shelterin rescues pericentric heterochromatin assembly defects associated with RNAi mutants.

### *Shelterin and RNAi modulate the distribution of Swi6 between pericentric regions and telomeres*

We then performed ChIP–chip analysis of Swi6 with an Agilent microarray. We designed additional probes that provide high coverage of centromere I, which was originally absent in the array due to its repetitive nature. In *dcr1Δ* cells, Swi6 levels were lower, but not abolished, at the entire pericentric region, as expected (Fig. 6A). Unexpectedly, Swi6 levels increased significantly across the subtelomeric heterochromatin domain, with significant spreading into euchromatic regions (Fig. 6A). In *poz1-W209A dcr1Δ* cells, Swi6 is not only restored to wild-type levels at pericentric regions but also reduced at the subtelomere regions compared with *dcr1Δ*. The total levels of Swi6 remain constant in these cells (Fig. 6B), suggesting that the differences in Swi6 localization are the result of Swi6 redistribution at these regions.

To examine whether this redistribution of Swi6 has any physiological consequences, we measured RNA transcript levels derived from the pericentric *dh* repeat and subtelomeric *tlh1*. In *dcr1Δ* cells, high levels of *dh* repeat transcripts were detected, and in *poz1-W209A dcr1Δ* cells, *dh* transcript levels decreased significantly, consistent with our results that these cells form normal pericentric heterochromatin. In contrast, *tlh1* transcripts levels were high in *poz1-W209A* cells but significantly reduced in *poz1-W209A dcr1Δ* cells (Fig. 6C). Thus, *poz1-W209A* and *dcr1Δ* mutually suppressed the other's phenotypes.

If mobilization of silencing proteins from telomeres is the reason that shelterin mutants bypass RNAi for pericentric heterochromatin assembly, we expect that simply increasing the concentration of silencing proteins is sufficient to bypass RNAi. As expected, introducing the *OE-swi6*$^+$ transgene, which resulted in about fourfold excess Swi6 protein in the cells (Supplemental Fig. S5), was sufficient to rescue silencing defects and TBZ sensitivity of all RNAi mutants tested, such as *dcr1Δ*, *ago1Δ*, and *rdp1Δ* (Fig. 6D; Supplemental Fig. S8A). The *OE-swi6*$^+$ transgene also complemented *swi6Δ* but could not rescue the silencing phenotype and TBZ sensitivity of *clr4Δ* (Supplemental Fig. S8A). Moreover, *OE-swi6*$^+$ *dcr1Δ clr3Δ* and *OE-swi6*$^+$ *dcr1Δ sir2Δ* failed to maintain pericentric silencing (Supplemental Fig. S4), suggesting that the ability of Swi6 overexpression to rescue RNAi defects also depends on RNAi-independent heterochromatin assembly

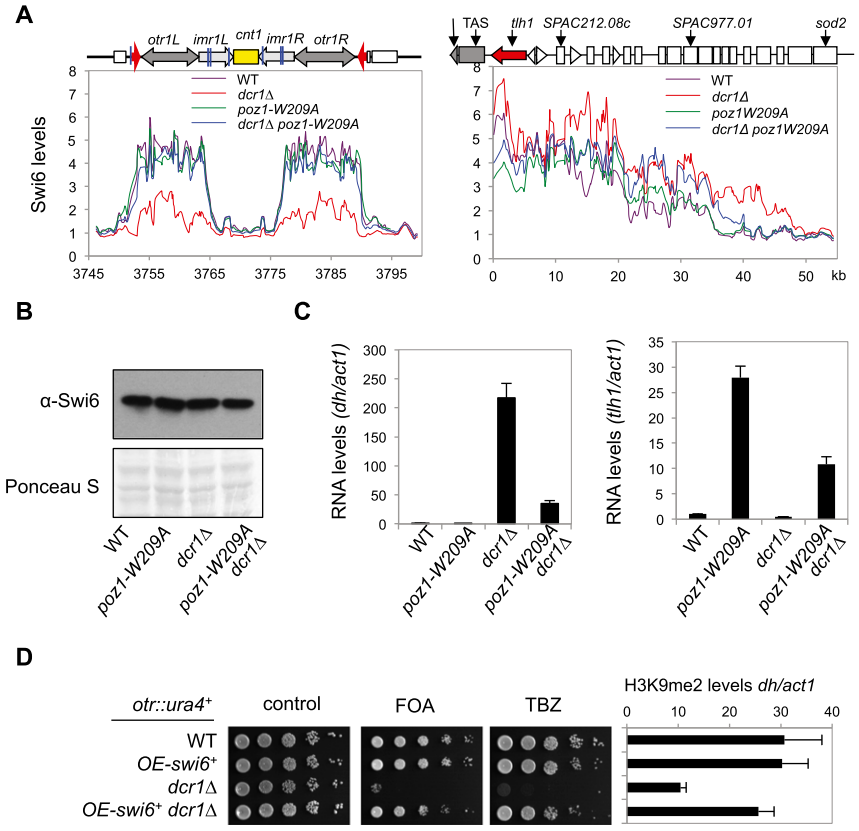


**Figure 6.** Shelterin and RNAi affect the distribution of Swi6 between pericentric and telomeric regions. (*A*) ChIP–chip analyses of Swi6 levels at centromere I (*left* panel) and the left side of telomere I (*right* panel). (*B*) Western blot analyses were performed to measure Swi6 protein levels. (*C*) RT–PCR analyses were performed to measure pericentric *dh* (*left*) or telomeric *tlh1* (*right*) transcript levels, normalized to *act1*. The numbers are averages of three experiments, and error bars represent standard deviation. (*D*) Tenfold serial dilutions of the indicated yeast strains were grown on the indicated medium to measure the expression of *otr::ura4*$^+$ and ChIP analysis of H3K9me2 levels at *ura4*$^+$ or pericentric *dh* repeat, normalized to *act1*. The numbers are averages of three experiments, and error bars represent standard deviation. The *OE-swi6*$^+$ strains also contain a wild-type copy of *swi6*$^+$.

pathways at pericentric regions. We obtained similar results with cells containing one additional copy of Swi6 inserted at the *ars1* locus driven under the endogenous *swi6* promoter, which is expected to increase Swi6 levels by twofold (Supplemental Fig. S8B; Sadaie et al. 2008).

We also tested whether overexpression of other heterochromatin factors could also bypass RNAi. Chp2 is another HP1 homolog and is enriched at all heterochromatin domains (Thon and Verhein-Hansen 2000; Sadaie et al. 2004; Motamedi et al. 2008; Fischer et al. 2009). However, incorporating an additional copy of *chp2*$^+$ under the control of its endogenous promoter into the *ars1* locus (Sadaie et al. 2008) could not rescue *dcr1Δ* (Supplemental Fig. S8B). Moreover, introducing Chp2 on a multicopy plasmid driven under an inducible *nmt1* promoter could not rescue *dcr1Δ* either (Supplemental Fig. S8C). Similar results were obtained with overexpression of Clr4 or Clr3 (Supplemental Fig. S8C). However, we caution that both Clr4 and Clr3 are present in multisubunit complexes, so their overexpression might not result in significant up-regulation of their enzymatic activities in vivo. Nonetheless, the different results obtained with HP1 homologs suggest that Swi6, but not Chp2, plays a major role in balancing the strength of different heterochromatin domains.

## Discussion

RNAi plays essential roles in heterochromatin assembly in diverse organisms. In fission yeast, loss of RNAi selectively impairs heterochromatin assembly at pericentric regions. We found that elimination of telomere shelterin components bypassed the requirement of RNAi for pericentric heterochromatin assembly. The separation-of-function mutant of Poz1 allowed us to demonstrate that loss of shelterin and RNAi results in the release from telomeres of silencing factors such as Swi6, which are then competitively recruited to pericentric regions through RNAi-independent heterochromatin assembly pathways (Fig. 7). In wild-type cells, the histone deacetylase Clr3/Sir2 functions cooperatively with RNAi to regulate pericentric heterochromatin assembly, whereas shelterin and TAS

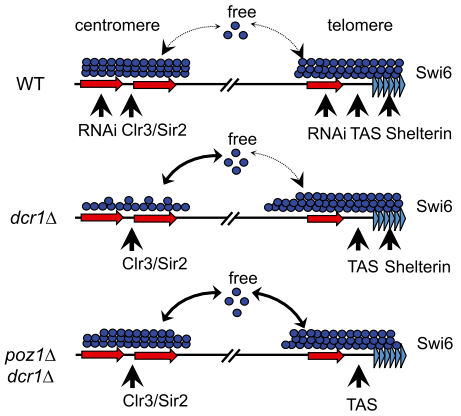


**Figure 7.** A model for shelterin and RNAi in regulating heterochromatin assembly.

sequences function together with RNAi for telomeric heterochromatin assembly. These parallel pathways form high-affinity platforms for the recruitment of heterochromatin protein Swi6. In *dcr1Δ* cells, pericentric heterochromatin is severely compromised, and the Clr3/Sir2 pathway recruits Swi6 at a lower efficiency. The relatively higher affinity of telomeric heterochromatin, which is due to the presence of shelterin and TAS-mediated heterochromatin assembly pathways, absorbs the extra Swi6, resulting in higher Swi6 levels at telomeres and the spreading of Swi6 into euchromatin at subtelomeric regions. In *poz1Δ dcr1Δ* cells, telomeric heterochromatin assembly pathways are further compromised, and the Clr3/Sir2 pathway at pericentric regions is able to compete with TAS sequences for Swi6. Increased Swi6 might facilitate the recruitment of CLRC and the restoration of H3K9me at pericentric heterochromatin. The subtelomere region's lower affinity for Swi6 in *poz1Δ dcr1Δ* cells results in a reduction of Swi6 levels and heterochromatin spreading compared with *dcr1Δ* cells. We suspect that the ability of *pot1Δ* and *tpz1Δ* to bypass *dcr1Δ* is due to the loss of telomere and subtelomere sequences, which consequently affects silencing at telomeres. Such a model is consistent with recent imaging and biochemical studies showing that HP1/Swi6 proteins are highly mobile on chromatin (Cheutin et al. 2003, 2004; Festenstein et al. 2003; Sadaie et al. 2008).

Our results that increasing Swi6 levels bypasses RNAi for pericentric heterochromatin assembly suggest that the availability of heterochromatin proteins in the nucleus, such as Swi6, is limiting. Sustaining low Swi6 availability is essential for maintaining the epigenetic landscape of the genome, as higher levels of Swi6 have been associated with increased efficiency of heterochromatin assembly at endogenous and ectopic sites. For example, artificial targeting of Clr4 to DNA fails to induce heterochromatin assembly unless Swi6 is overexpressed (Supplemental Fig. S5B) or endogenous heterochromatin structures are compromised to release silencing proteins (Kagansky et al. 2009). In addition, exogenously introduced siRNAs fail to establish heterochromatin unless Swi6 is overexpressed (Iida et al. 2008). Moreover, overexpression of Swi6 increases the conversion rate of a less stable heterochromatin domain at the mating type region (Nakayama et al. 2000) and enhances the spreading of heterochromatin when chromatin boundaries are compromised (Noma et al. 2001; Wang et al. 2013). Thus, the multiple pathways that regulate heterochromatin assembly at constitutive heterochromatin regions, such as telomeric, pericentric, and the silent mating type regions, might be key to the formation of higher-affinity platforms for heterochromatin proteins, thus balancing the need for proper heterochromatin assembly at these regions and preventing heterochromatin assembly at cryptic sites. When any of these mechanisms are compromised, Swi6 redistributes according to the strength of heterochromatin assembly pathways at different regions and is more likely to be recruited to sites that otherwise cannot form heterochromatin.

Heterochromatin is mainly present at pericentric and subtelomeric regions in diverse organisms. Although the role of pericentric heterochromatin in regulating chromosome segregation has been well studied, the functions of telomeric heterochromatin are less clearly defined. In certain organisms such as *Drosophila*, telomeres are composed of retrotransposons instead of telomeric repeats, which are packaged into heterochromatin to recruit end protection factors (Mason et al. 2008). In fission yeast, amplification of subtelomere heterochromatin is also associated with cell survival in the absence of telomerase (Jain et al. 2010). Certain heterochromatin factors, such as Clr4 and Rik1, also regulate telomere clustering during meiosis (Tuzon et al. 2004). However, heterochromatin factors have no effect on telomere length control and do not protect against telomere end fusions (Tuzon et al. 2004). Compared with other heterochromatin regions, the telomere and subtelomere regions are unique in buffering the changes in heterochromatin levels because there are no defined heterochromatin boundaries, and a large transition zone is formed, with Swi6 levels gradually declining (Fig. 6A). In contrast, at centromeres and the silent mating type region, silencing factor concentrations are high, and well-defined heterochromatin boundaries prevent spreading (Cam et al. 2005). Thus, these regions might already be saturated by silencing factors, and the buffering capacity is limited. This is exemplified in *dcr1Δ*, in which Swi6 protein levels were significantly increased across the entire subtelomeric region, with significant amounts of spreading into euchromatic regions, whereas in *poz1Δ* cells, there were little changes of Swi6 levels at pericentric regions but enhanced heterochromatin assembly by GBD-Clr4 (Fig. 6A; Supplemental S5B). Conversely, cells containing the minichromosome Ch10 (Niwa et al. 1989), which is mainly composed of pericentric repeats and centromere and telomere sequences, show reduced levels of Swi6 selectively at subtelomeric regions (Chikashige et al. 2007). It is worth noting that the budding yeast, which uses Sir2/3/4 proteins to assemble heterochromatin instead of the H3K9me-HP1 system, also uses telomeres to limit silencing factors, and releasing these factors from telomeres similarly increases silencing at internal sites (Maillet et al. 1996; Marcand et al. 1996; Taddei et al. 2009). Thus, the sequestration of silencing factors through constitutive heterochromatin domains might be especially important for genomes that are highly active to prevent ectopic heterochromatin assembly in order to maintain the epigenetic landscape.

## Materials and methods

### *Fission yeast strains and genetic analyses*

Yeast strains containing *poz1Δ*, *rap1Δ*, and *trt1Δ* were derived from the Bioneer fission yeast deletion library, verified via PCR, and backcrossed. The strain containing *OE-swi6*$^+$ was constructed by integrating a PCR fragment containing the *ade6* promoter, *swi6* coding region, and 3′ untranslated region and a *hphMX6* cassette into the endogenous *ade6*$^+$ locus. Poz1 mutants were constructed by integrating a PCR fragment containing the mutations, a myc tag, and a *KanMX6* cassette into the endogenous *poz1*$^+$ locus. Rap1-Flag and *taz1Δ* were constructed by a PCR-based module method. Genetic crosses were used to construct all other strains. Strains containing *tpz1Δ* or *pot1Δ* were

first generated in diploid cells, followed by tetrad dissection to obtain desired genotypes. For serial dilution plating assays, 10-fold dilutions of a log-phase culture were plated on the indicated medium and grown for 3 d at 30°C.

### *Screens for mutations that suppress the silencing defects of RNAi mutants*

Query strains were mated with the fission yeast deletion library with the aid of the Singer RoToR HDA pinning robot as previously described (Roguev et al. 2007). The fission yeast deletion library was constructed with a *KanMX4* cassette that confers resistance to geneticin (Bioneer). Two reporter strains (*otr::ura4*$^+$ or *otr::ade6*$^+$) were constructed by inserting a *NatMX6* cassette, which confers resistance to nourseothricin, ~400 base pairs (bp) to the right side boundary of centromere I heterochromatin. The *dcr1Δ* and *ago1Δ* query strains were constructed with an *hphMX6* cassette, conferring resistance to hygromycin. The *otr::ura4*$^+$ or *otr::ade6*$^+$ reporter and *dcr1Δ* were sequentially introduced into the deletion library, and the resulting haploid cells were selected and pinned to selective medium to measure cell growth or colony color.

### *ChIP analysis*

ChIP analyses were performed as described previously (Hou et al. 2010). Antibodies used were H3K9me2 (Abcam), Swi6 (Reddy et al. 2011), Pol II (Covance), myc (Covance), and Flag (Sigma). Quantitative real-time PCR (qPCR) was performed with Maxima SYBR Green qPCR master mix (Fermentas) in an ABI 7300 Real-Time PCR System. 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 a reference to calculate the enrichment of ChIP over whole-cell extract for each target sequence. ChIP–chip analysis was performed according to the Agilent Yeast ChIP-on-chip analysis protocol. The microarray used was an Agilent *S. pombe* Whole-Genome ChIP-on-chip microarray (G4810A) with additional probes that encompass centromeres, which were originally absent from the array due to the repetitive nature of these DNA sequences.

### *RNA analyses*

Total cellular RNA was isolated from log-phase cells using a MasterPure Yeast RNA purification kit (Epicentre) according to the manufacturer's protocol. Quantification with real-time RT–PCR was performed with Power SYBR Green RNA-to-CT One-Step kit (Applied Biosystems). RNA serial dilutions were used as template to generate the standard curve of amplification for each pair of primers, and the relative concentration of target sequence was calculated accordingly. An *act1* fragment served as a reference to normalize the concentration of samples. The concentration of each target gene in wild type was arbitrarily set to 1 and served as reference for other samples. Northern blot of siRNAs was performed as described previously (Reddy et al. 2011).

### *Telomere length analysis*

For *poz1* mutant and *poz1Δ* strains, a single colony from the first restreak after the mutation or deletion was grown overnight. Genomic DNA was isolated, digested with EcoRI, and separated on a 1% agarose gel. Hybridization with radiolabeled telomeric DNA and *pol1* (control) probes was performed as previously described (Jun et al. 2013).

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