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Investigation

Dri1 mediates heterochromatin assembly via RNAi and histone deacetylation

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Abstract

Heterochromatin, a transcriptionally silenced chromatin domain, is important for genome stability and gene expression. Histone 3 lysine 9 methylation (H3K9me) and histone hypoacetylation are conserved epigenetic hallmarks of heterochromatin. In fission yeast, RNA interference (RNAi) plays a key role in H3K9 methylation and heterochromatin silencing. However, how RNAi machinery and histone deacetylases (HDACs) are coordinated to ensure proper heterochromatin assembly is still unclear. Previously, we showed that Dpb4, a conserved DNA polymerase epsilon subunit, plays a key role in the recruitment of HDACs to heterochromatin during S phase. Here, we identified a novel RNA-binding protein Dri1 that interacts with Dpb4. GFP-tagged Dri1 forms distinct foci mostly in the nucleus, showing a high degree of colocalization with Swi6/Heterochromatin Protein 1. Deletion of *dri1*$^{+}$ leads to defects in silencing, H3K9me, and heterochromatic siRNA generation. We also showed that Dri1 physically associates with heterochromatic transcripts, and is required for the recruitment of the RNA-induced transcriptional silencing (RITS) complex via interacting with the complex. Furthermore, loss of Dri1 decreases the association of the Sir2 HDAC with heterochromatin. We further demonstrated that the C-terminus of Dri1 that includes an intrinsically disordered (IDR) region and three zinc fingers is crucial for its role in silencing. Together, our evidences suggest that Dri1 facilitates heterochromatin assembly via the RNAi pathway and HDAC.

Keywords: heterochromatin; RNAi pathway; HDAC; *Schizosaccharomyces pombe*

Introduction

Heterochromatin is a transcriptionally repressed region in eukaryotic genome that plays an important role in genome stability and gene expression regulation (Allshire and Madhani 2018; Janssen *et al.* 2018). Two epigenetic marks that characterize heterochromatin are histone 3 lysine 9 methylation (H3K9me) and histone hypoacetylation. DNA replication poses a challenge to the inheritance of heterochromatin since DNA replication fork disrupts the chromatin structure (Probst *et al.* 2009; Budhavarapu *et al.* 2013; He *et al.* 2014). The mechanism behind how heterochromatin is assembled remains unclear.

In *Schizosaccharomyces pombe*, heterochromatin domains are found at pericentromeres, telomeres, and the mating type locus. The heterochromatic regions are enriched with H3K9me, which is bound by the human Heterochromatin Protein 1 (HP1) homolog, Swi6. RNA interference (RNAi) plays an important role in the initiation of heterochromatin formation by recruiting the H3K9 methyltransferase Clr4 to the nucleation sites (Volpe *et al.* 2002; Buscaino *et al.* 2013; Obersriebnig *et al.* 2016). Heterochromatin in fission yeast can be briefly transcribed during S phase (Chen *et al.* 2008; Kloc *et al.* 2008). The noncoding RNA transcripts are made into double stranded RNAs by the RNA-dependent RNA polymerase complex that are subsequently processed into small interference RNAs (siRNAs) by Dicer. The RNA-induced transcriptional silencing (RITS) complex consisting of Argonaute, the chromodomain protein Chp1 and Tas3 interacts with heterochromatin transcripts to mediate the recruitment of the Clr4 methyltransferase complex (CLRC) (Motamedi *et al.* 2004; Verdel *et al.* 2004; Sugiyama *et al.* 2005; Martienssen and Moazed 2015).

Histone deacetylases (HDACs) mediate the removal of the acetyl group from histones and are important for heterochromatin formation. One of the conserved HDACs in *S. pombe* is Sir2, which is NAD+-dependent deacetylase (Janke *et al.* 2015). Sir2 is important for the removal of several histone acetylation marks such as H3K4, H3K9 and H4K16. The Sir2-deleted cells show partial defects in pericentromeric heterochromatin integrity (Shankaranarayana *et al.* 2003; Freeman-Cook *et al.* 2005; Wiren *et al.* 2005; Alper *et al.* 2013). Like RNAi, it has been shown that Sir2 is involved in the initiation step of heterochromatin assembly (Alper *et al.* 2013; Buscaino *et al.* 2013). Sir2 also plays an important role in subsequent heterochromatin spreading and propagation (Buscaino *et al.* 2013). However, the mechanism for how the RNAi machinery and HDACs are mediated to ensure proper heterochromatin assembly during replication remains unclear.

Previously, we have shown that Cdc20, the catalytic subunit of the DNA polymerase (Pol) epsilon complex, interacts with the CLRC complex (Li *et al.* 2011; Gonzalez and Li 2012) while the histone

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fold subunit of the Pol epsilon Dpb4 associates with the Sir2 HDAC (He *et al.* 2017). Our data further suggest that these DNA replication components may serve as a platform for recruitment of histone modifiers to heterochromatin. Here we identified a novel protein Dri1 interacting with Dpb4. Cells without Dri1 show defects in transcriptional silencing and loss of small RNAs in heterochromatin. Dri1 physically interacts with heterochromatic transcripts, and is important for the association of the RITS complex with heterochromatin via interacting with Chp1, a subunit of RITS. Moreover, deletion of *dri1*$^+$ results in reduced Sir2 recruitment to heterochromatin and high level of histone acetylation within the region. Our data support a model that Dri1 mediates heterochromatin formation via HDAC and RNAi pathways, and provides new insights into how heterochromatin is assembled through cell cycle.

Materials and methods

Strains, media, and genetic analysis

Fission yeast strains used in this study are listed in Supplementary Table S1. Standard media and genetic analysis for fission yeast were used (Moreno *et al.* 1991). Yeast extract with supplements was used as a complete culture and PMG (Pombe glutamate medium) as a minimum media. For silencing assays, a series of 10-fold dilutions with a starting concentration of 2×10^7 cells/ml were spotted on the designated PMG media and incubated at 30°C for 3–4 days.

Small RNA northern blot

Total RNAs were extracted from exponentially growing cells by Trizol-isopropanol extraction. Small RNAs (<200 bp) were enriched by mirVana miRNA Isolation kit (ThermoFisher, AM1560) and separated by a 15% denaturing acrylamide gel. The RNAs were transferred to a charged nylon membrane (Hybond-N+, Amersham). RNA blots were cross-linked and hybridized with DNA probes specific for pericentromeric *dg* or *dh* region. tRNA was used as a loading control.

ChIP

Chromatin immunoprecipitation (ChIP) was performed as described (Dong *et al.* 2016). Briefly, 50 ml of a log-phase yeast culture was cross-linked by adding 37% formaldehyde for 30 min. Cells were collected and sonicated by an Ultrasonic Processor. 1 µl of antibody was used for immunoprecipitation. Immunoprecipitated DNA was purified using a PCR clean up column (Qiagen), and analyzed by PCR using primers listed in Supplementary Table S2. Antibodies used were anti-GFP (Abcam, ab290), anti-H3K9me (Abcam ab1220), anti-H3K9 acetylation (Abcam, ab4441), and anti-Myc (Abcam, ab32).

RNA immunoprecipitation

RNA immunoprecipitation (RIP) assays were carried out as described (Gilbert and Svejstrup 2006). Cell extracts were incubated with 1 µl of Myc (Abcam, ab32) or FLAG antibody (Sigma-Aldrich, M8823) for 1 hr. IgG magnetic beads (Sigma-Aldrich, 10003D) were then added and the incubation continued for another hour. After washes, immunoprecipitated RNAs were purified with Trizol and isopropanol precipitation followed by treatment with 2 µl of DNase for 2 hr at 37°C. The Input and IP samples were reversed transcribed by Takata RT-PCR kit and analyzed by qPCR.

Co-immunoprecipitation

Co-immunoprecipitation (co-IP) was performed as described previously (Dong and Li 2018). Briefly, cells were collected and resuspended in 100 µl of 1×lysis buffer with protease inhibitors prior to lysis by bead beating. Lysates were incubated with IgG sepharose (GE Healthcare, no. 17096901) at 4°C for 2 hr. After washing with lysis buffer, proteins were eluted in SDS loading buffer. Eluates were analyzed by western blotting using a commercial anti-GFP antibody (Abcam, ab290) or S-tag (ABR, MA1-981).

qPCR

Quantitative PCR (qPCR) was performed with SYBR Green on a Bio-Rad iCycler and analyzed with iCycler iQ Optical System Software. qPCR analysis primers are listed in Supplementary Table S2. Relative enrichments were calculated as the ratio of product of interest to control product (*act1*$^+$) in IP over input. Histograms represent three biological replicates; error bars represent one standard deviation (SD). Primers used in this study are listed in Supplementary Table S2.

Microscopic analysis

Fluorescence signal was captured by Olympus fluorescence microscopy or DeltaVision system (Applied Precision, Issaquah, WA). For DeltaVision imaging, images were taken as z-stacks of 0.2 µm increments with an oil immersion objective (×100).

Data availability

Strains and plasmids are available upon request. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables.

Supplementary Material is available at figshare: https://doi.org/10.25386/genetics.14132312.

Results

Dri1 interacts with Dpb4

In order to further study the role of Dpb4 in heterochromatin assembly, we performed a TAP-tag purification of Dpb4 followed by mass spectrometry analysis (He *et al.* 2017). We identified an uncharacterized protein (SPAC17H9.04c) precipitated along with Dpb4-TAP. The protein sequence analysis revealed that this protein contains one RNA recognition motif (RRM), and three zinc finger (ZF) motifs (Figure 1A). We additionally identified an intrinsically disordered region (IDR) using PONDR (Figure 1A and Supplementary Figure S1). We thus named the protein Dri1, an acronym for Dpb4-interacting, RRM, and IDR-containing factor. In order to confirm its interaction with Dpb4, we performed co-IP using cells carrying GFP-Dri1 and Dpb4-TAP. We reproducibly detected the band corresponding to GFP-Dri1 in the IP sample (Figure 1B), confirming that Dri1 interacts with Dpb4.

Dri1 is required for heterochromatin silencing

The heterochromatic pericentromeres in *S. pombe* is organized into outermost regions (*otr*), which consists of alternating *dg* and *dh* repeats, and innermost regions (*imr*). In order to gain insight into the role of Dri1 in heterochromatin, we examined silencing in pericentromeres in the Δ*dri1* mutant. We used a reporter strain that has the *ura4*$^+$ gene inserted at the second repeat within *otr* on chrIII (He *et al.* 2016; Yang and Li 2017). In wild type (WT), the *ura4*$^+$ reporter is transcriptionally silenced, which results in slow growth in the minimal medium depleted of uracil. We have previously shown that the reporter on chrIII is more strongly silenced than the one inserted in *otr* on chrI, and thus can allow us to detect subtle changes in silencing (He *et al.* 2016). We found that cells without *dri1*$^+$ showed much faster growth in the minimal

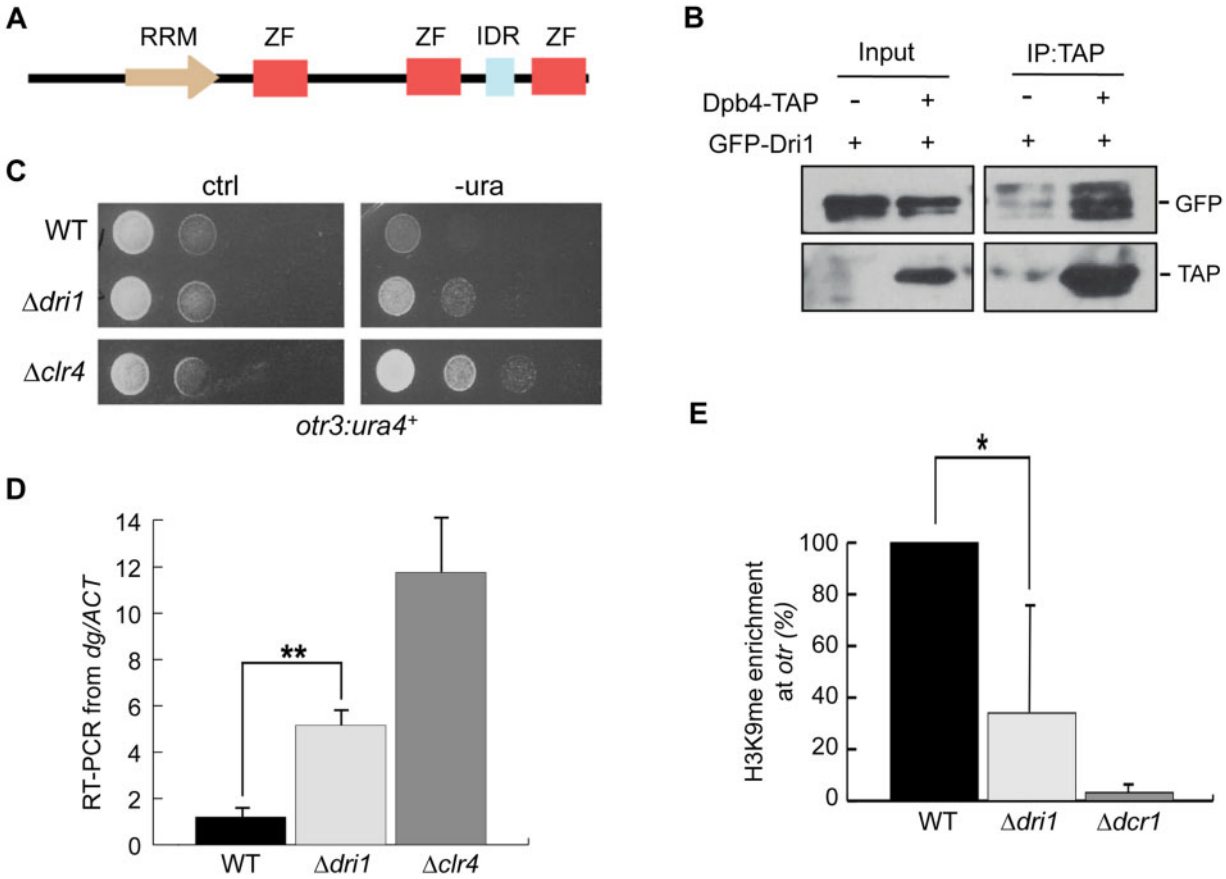

Figure 1 Dri1 interacts with Dpb4 and is important for heterochromatin silencing. (A) Schematic diagram showing the domain organization of Dri1. RRM, RNA recognition motif. ZF, zinc finger; IDR, Intrinsically disordered region. (B) Co-IP confirmed that Dri1 and Dpb4 interact. (C) Serial dilutions of Δ*dri1* mutant cells with *ura4*$^{+}$ in pericentromeric *otr3* repeat were spotted on the minimal medium without uracil (–ura) and incubated for 4 days. (D) The level of heterochromatic transcripts is increased in Δ*dri1*. Transcripts derived from *dh* peri-centromeric transcripts were analyzed by RT-qPCR. Actin was used as a control. $^{**}P < 0.01$. (E) ChIP-qPCR analysis of H3K9 methylation in an *otr* region in the indicated strains. Actin was used as a control. Four independent experiments were performed. The level of WT was set to 100%. Error bars indicate SD. $^{*}P < 0.05$.

medium, indicating that Dri1 is required for silencing at pericentromeres (Figure 1C). We also found that the double mutant of *dri1*Δ with *dpb4*Δ exhibited synthetic defects in heterochromatin silencing (Supplementary Figure S2), consistent with the idea that Dri1 functions together with Dpb4 to regulate pericentromeric heterochromatin. Furthermore, our RT-qPCR results showed that the level of RNA transcripts from *dg* repeats in Δ*dri1* increased approximately fivefold relative to WT, though is lower than that in the Δ*clr4* mutant (Figure 1D). On the other hand, Δ*dri1* did not show significant changes in transcription levels of key silencing factors (Supplementary Figure S3). We also tested heterochromatin silencing at the telomeres and the mating type locus using strains carrying *ura4*$^{+}$ in these regions. We did not observe defects in telomere silencing in Δ*dri1*, but the mating type locus displays minor loss of silencing (Supplementary Figure S4). We next examined how H3K9me2 at the *otr* region in Δ*dri1* is affected by ChIP-qPCR. We observed that the level of H3K9me2 decreased threefold in the mutant compared with WT (Figure 1E). This indicates that Dri1 is important for heterochromatin silencing, especially for pericentromeres.

Small RNA production is lost in the Δ*dri1* mutant

To determine whether Dri1 is involved in the RNAi pathway, we analyzed the level of small RNAs from pereicentromeric regions in the Δ*dri1* mutant. We performed small RNA Northern blot analyses using a probe against either *dg* or *dh* repeats. We observed that siRNAs from both *dg* or *dh* repeats were lost in Δ*dri1* (Figure 2A). The detected level of siRNAs in the mutant was comparable with that of the Δ*dcr1* mutant. Therefore, the desilencing phenotype of the *dri1* mutant is a result of defects in the RNAi pathway.

Recruitment of the RITS complex to heterochromatin depends on Dri1

Since the loss of *dri1*$^{+}$ led to the silencing defects and small RNA depletion similar to key players in the RNAi pathway, we next investigated whether the enrichment of the RITS complex at heterochromatin was affected in Δ*dri1*. We monitored the level of Chp1, a major component of the RITS complex, at the *dg* region by ChIP-qPCR. Our results indicated that Chp1-GFP was significantly reduced in Δ*dri1* (Figure 2B), indicating that Dri1 is required for the recruitment of Chp1 at pericentromere.

We then examined whether Dri1 physically interacts with Chp1 by co-IP using cells carrying GFP-Dri1 and Chp1-TAP. We reproducibly observed the interaction between Chp1-TAP and GFP-Dri1 (Figure 2C). We also added RNase to the samples during co-IP, and found that they still interacted, indicating that the interaction is not RNA-based (Supplementary Figure S5). These observations suggest that Dri1 interacts with RITS to promote the recruitment of the complex to heterochromatin.

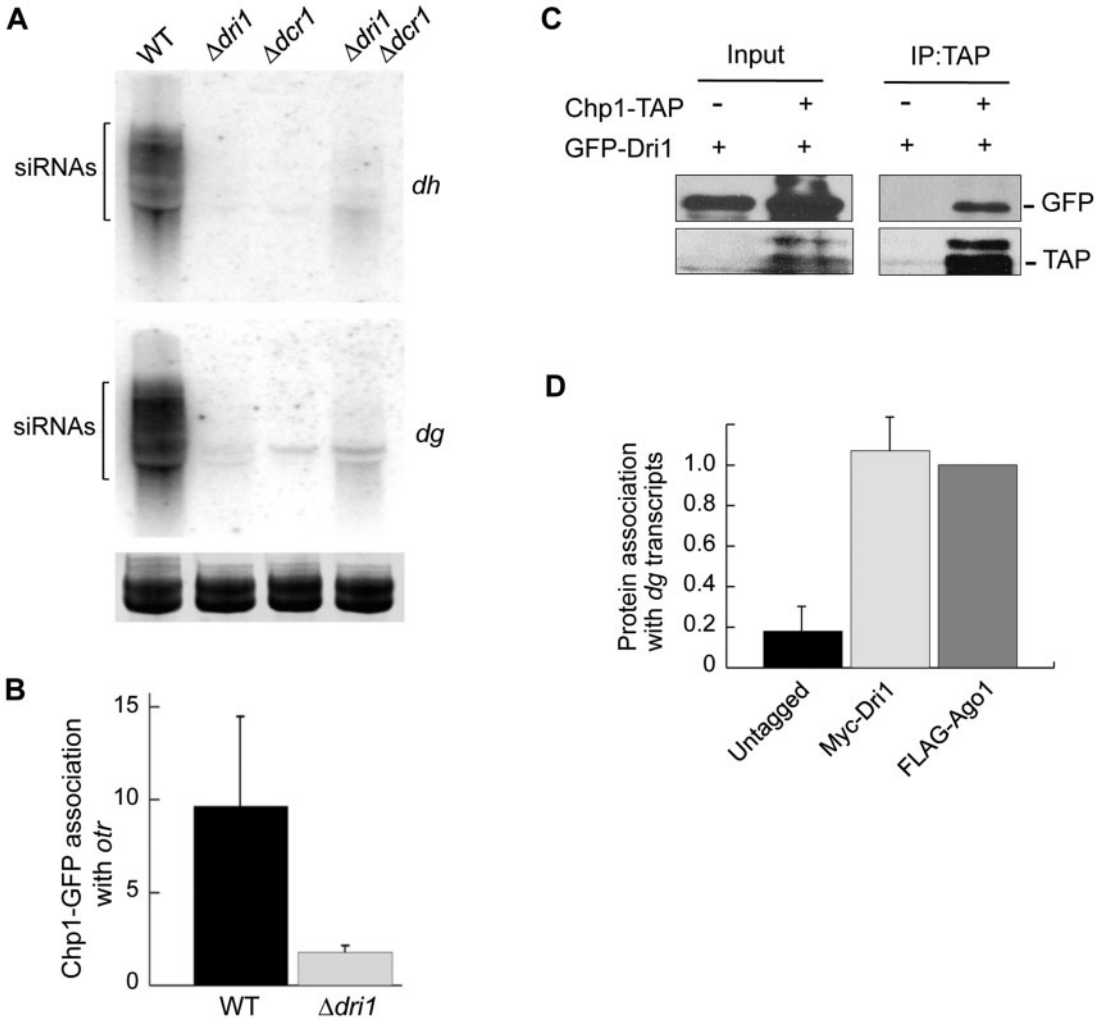

Figure 2 Dri1 mediates heterochromatic small RNA production via recruitment of the RITS complex. (A) Small RNA northern blot assay of the indicated strains with probes specific for *dh* and *dg* repeats. tRNA was used as a loading control. (B) ChIP analysis of Chp1-GFP in the *otr* region in the indicated strains. Error bars indicate SD. (C) Co-IP experiments indicate the interaction between GFP-Dri1 and Chp1-HA. (D) Dri1 associates with heterochromatic transcripts. RIP was performed from extracts of cells expressing Myc-Dri1 using an anti-Myc antibody. Untagged cells were used a negative control. Cells with FLAG-Ago1 were also analyzed using an FLAG antibody as a positive control. Immunoprecipitated RNAs were quantified by RT-qPCR using primers specific for the *dg* repeats. The *dg* level immunoprecipitated by FLAG-Ago1 RIP was set to 1. Experiments were performed in triplicate.

Dri1 interacts with the heterochromatic transcripts

The RITS complex physically interacts with the primary transcripts from heterochromatin (Motamedi *et al.* 2004). We then tested whether Dri1 also physically binds to the transcripts. We performed RIP using cells containing Myc-tagged Dri1 with an anti-Myc antibody. As a positive control, we also analyzed cells containing FLAG-Ago1 by RIP with a FLAG antibody. We observed that Myc-tagged Dri1 was copurified with heterochromatin transcripts at the level comparable to that of the transcripts copurified with FLAG-Ago1, but not with transcripts from rDNA and the subtelomeri *tlh1*$^{+}$ (Figure 2D and Supplementary Figure S6). This data further revealed that Dri1 acts in the RNAi-mediated heterochromatin pathway through interacting with pericentromeric heterochromatin transcripts.

Dri1 shows a high degree of colocalization with Swi6

We next investigated the localization of Dri1. We first created GFP-tagged Dri1 at its C-terminus, but found that it is not functional. We then constructed N-terminal GFP-tagged Dri1 under its endogenous promoter at its native locus, and confirmed that GFP-Dri1 maintained its functionality by silencing assay (Supplementary Figure S7). However, the signal of GFP-Dri1 is weak. To further analyze its localization, we constructed GFP-Dri1 under the thiamin-repressible *nmt1* promoter and lowly overexpressed GFP-Dri1 by treating cells with 0.05 μM thiamin for 18 hr. We observed that lowly overexpressed GFP-Dri1 formed foci mostly in the nucleus (Figure 3A and Supplementary Figure S8). We also noticed that the GFP foci were preferentially located at the nuclear periphery. In order to examine whether these GFP-Dri1 foci associate with heterochromatin, we used a strain carrying mCherry-Swi6, an H3K9me reader protein. We found that a major portion of GFP-Dri1 colocalized with mCherry-Swi6 (Figure 3B), indicating that GFP-Dri1 has affinity to associate with heterochromatin. Consistently with this, our ChIP analysis revealed that Dri1 was enriched in the *otr* region (Figure 3C). We further showed that the enrichment of Dri1 in heterochromatin is reduced in the Δ*clr4* and Δ*dpb4* mutants (Figure 3C), indicating that they are important for recruitment of Dri1 to heterochromatin. Interestingly, we found that strong overexpression of Dri1 by

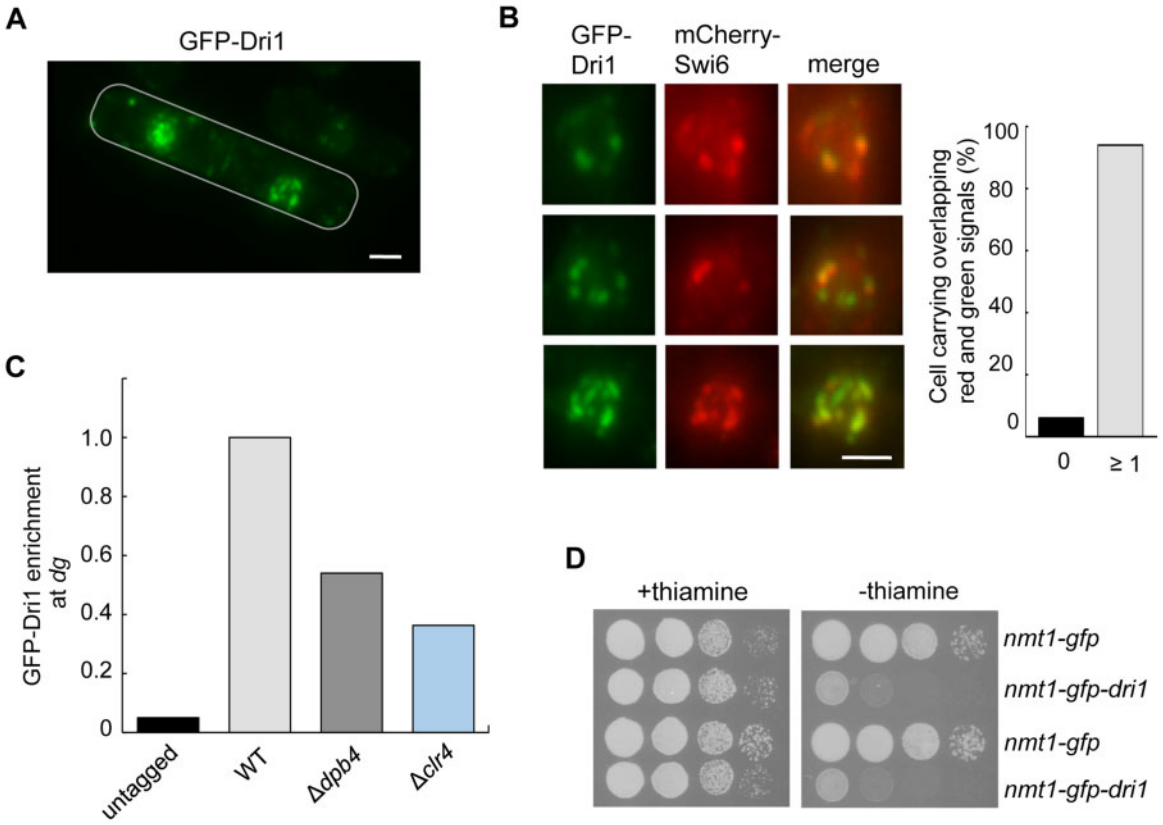

Figure 3 Dri1 associates with heterochromatin. (A) Mildly overexpressed GFP-Dri1 results in formation of distinct foci that are highly enriched in nucleus. Cells carrying GFP-Dri1 under the *nmt1* promoter were incubated in minimal medium supplemented with 0.05 μM thiamin for 18 hr. Scale bar, 2 μm. (B) Mild overexpression-induced GFP-Dri1 foci preferentially assemble near nuclear envelope, and often overlap with mCherry-Swi6 foci. Left, quantification of cells showing overlapping GFP-Dri1 and mCherry-Swi6 signals. 0, cells having no overlapping signal; ≥ 1, cells containing at least one overlapping spot of GFP and mCherry signals. Scale bar, 2 μm. (C) ChIP analysis of GFP-Dri1 in the *otr* region in the indicated strains. ChIP performed with an antibody specific for GFP. (D) Strong overexpression of Dri1 results in growth defects. Cells carrying GFP-Dri1 under the *nmt1* promoter was used. Experiments were performed in triplicate.

incubating cells carrying *nmt1-gfp-dri1*$^+$ in the minimal medium without thiamine for 48 hr results in growth defects (Figure 3D).

Dri1 is important for silencing at ectopic heterochromatin

Previously it has been shown that artificially tethering the chromodomain-deleted Clr4 (Clr4-*CD*) by the DNA binding domain of *Saccharomyces cerevisiae* Gal4 protein (GBD) to a euchromatic locus carrying the GAL4 binding sequences (*gbs*) can lead to silencing of the neighboring region of *gbs* (Kagansky *et al.* 2009). The ectopic heterochromatin assembly depends on CLRC and HDAC, but does not require the components of RNAi (Kagansky *et al.* 2009). We next decided to use the GBD-Clr4-*CD* assay to examine how the deletion of *dri1*$^+$ impacts the establishment of artificially induced heterochromatin. As readout for silencing, *ade6*$^+$ was inserted right next to the *gbs* (Figure 4A). Silencing of *ade6*$^+$ leads to the formation of red colonies in rich media with low adenine while active *ade6*$^+$ produces white colonies. We found that cells containing GBD-Clr4-*CD* in the Δ*dcr1* mutant produce 98% of red colonies on low adenine media, consistent with the previous report (Kagansky *et al.* 2009). However, Δ*dri1* only produces 46% of red colonies, similar to Δ*sir2* (Figs. 4B and 4C). These results indicate that Dri1 is important for heterochromatin on an ectopic site, in a manner similar to HDAC or CLRC.

Dri1 is important for the recruitment of Sir2 to heterochromatin

Dpb4 has been shown to recruit the HDAC Sir2 to heterochromatin (He *et al.* 2017). To determine whether the Dpb4-interacting protein Dri1 is important for histone deacetylation at heterochromatin, we analyzed the pericentromeric *otr* region in the Δ*dri1* mutant by ChIP-qPCR with an H3K9Ace antibody. We observed that the Δ*dri1* mutant showed approximately fourfold increase in H3K9Ace at pericentromere compared with WT (Figure 5A). To determine whether Dri1 is important for the recruitment of Sir2 to pericentromere, we performed ChIP-qPCR using Δ*dri1* cells that contain Sir2-Myc. Sir2-Myc was significantly reduced in the *otr* region in the mutant (Figure 5B). We also found that the level of Sir2-Myc was decreased in the mating-type region and telomeres in Δ*dri1* (Figure 5, C and D). This suggests that Dri1 may mediate histone deacetylation in heterochromatin by recruiting Sir2 to the regions. Together, our data also reveal that Dri1 may function as a link between Sir2- and RNAi-mediated silencing.

Domains of Dri1 contributes to its role in silencing

To further understand the function of Dri1, we investigated the role of different domains of Dri1 in heterochromatin silencing. We first deleted the RRM domain by homologous recombination (*dri1*-Δ*rrm*; Figure 6A and Supplementary Figure S9). Our growth assays showed that deletion of the domain had minimal effect on

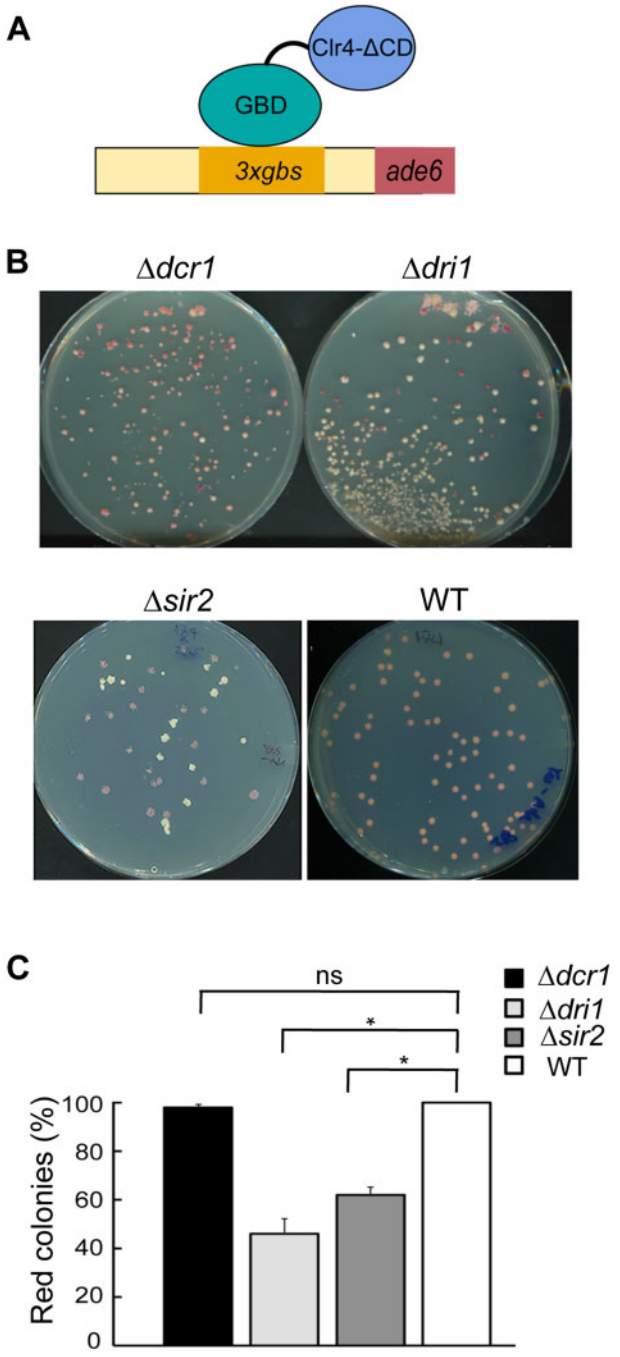

Figure 4 Dri1 is required for silencing at an ectopic heterochromatin. (A) Schematic representation of the system used to induce ectopic heterochromatin. The DNA binding domain of Gal4 protein (GBD) was fused with the chromodomain-deleted Clr4 (Clr4-ΔCD). GBD-Clr4-ΔCD was expressed in cells carrying the Gal4 binding site fused with *ade6*$^+$ (*gbs-ade6*$^+$) at a euchromatic region. (B) Cells carrying GBD-Clr4-ΔCD and *gbs-ade6*$^+$ in Δ*dcr1*, Δ*dri1*, Δ*sir2* and WT backgrounds were plated on the rich medium without adenine. (C) The percentage of red colonies produced by the indicated strains. Experiments were performed in triplicate. Error bars indicate SD. *$P < 0.05$. ns, no significant differences.

silencing in pericentromeric region (Figure 6B). To determine how each ZF contributes to silencing, we created the first and second ZF mutants (*dri1-zf1** and *dri1-zf2**) by changing four cysteines with each domain to alanine. We also created a third ZF-deleted mutant (*dri1-*Δ*zf3*; Figure 6A). We found that *dri1-zf2** and *dri1-zf3*Δ showed partial loss of silencing while *dri1-zf1** had no obvious silencing defects (Figure 6C). In addition, we made IDR-deleted version of Dri1 (*dri1-*Δ*idr*; Figure 6A). The *dri1-*Δ*idr* mutant also displays partial loss of silencing (Figure 6C). We next deleted the C-terminus of Dri1, including three ZFs and IDR domain (*dri1-*ΔC; Figure 6A) and tested the silencing by growth assays. The *dri1-*ΔC mutant strain showed strong loss of silencing, almost comparable to the desilencing phenotype in Δ*dri1* (Figure 6D). In addition, we found that the deletion of the C-terminus of Dri1 significantly decreased its interaction with Dpb4 by co-IP (Figure 6E). We also observed reduced association of C-terminus-deleted Dri1, although mild, with heterochromatin (Supplementary Figure S10). To determine the role of the RRM domain and the C-terminus in recruitment of Chp1 and Sir2, we analyzed the domain-deletion mutants by ChIP. Our results showed that deletion of the C-terminus of Dri1 resulted in significant loss of Chp1 in pericentromeric heterochromatin, whereas deletion of the RRM domain had little impact on Chp1 in the region (Figure 6F). These indicate that the C-terminus is important for recruiting Chp1. We also found that the level of Sir2-Myc in heterochromatin was not significantly affected in the *dri1-*Δ*rrm* and *dri1-*ΔC mutants (Supplementary Figure S11), suggesting that the two domains may function together to recruit Sir2. Together, these data indicate that different domains of Dri1 contribute to its function in heterochromatin assembly.

Discussion

Both RNAi and HADC play an important role in heterochromatin silencing, but how RNAi and HDAC are coordinated to mediate heterochromatin assembly remains unclear. Here, we provide evidences suggesting that Dri1, an unstudied RNA-binding protein, modulates the recruitment of the RITS complex and Sir2. Our studies point to Dri1 as a physical and functional link between RNAi and HDAC pathway, suggesting a plausible mechanism for how the two processes functions together to regulate heterochromatin formation.

It has been previously shown that Cdc20, the POL epsilon catalytic subunit, is important for recruitment of CLRC to heterochromatin during S phase (Li *et al.* 2011). Dpb4, a histone fold subunit of the complex, interacts with Sir2 to remove acetylation from histones at heterochromatin (He *et al.* 2017). Here using mass spectrometry and co-IP, we demonstrated that Dri1 interacts with the Pol epsilon subunit Dpb4. Dri1 is a highly conserved protein, containing the RRM domain and three ZFs. We also identified an IDR region at its C-terminus. The function of its budding yeast ortholog is still unknown. Its human orthologs are predicted to be TEX13A and TEX13B. We found that GFP-tagged Dri1 in fission yeast forms distinct foci in the nucleus, showing a high degree of colocalization with Swi6. This observation differs from a previous global study of protein localization (Matsuyama *et al.* 2006). The difference possibly lies in the fact that tagging a fluorescent protein at the C-terminus of Dri1 results in nonfunctional Dri1.

We presented multiple lines of the evidence suggesting that Dri1 plays a direct role in the RNAi pathway in fission yeast. Cells without *dri1*$^+$ showed significant silencing defects and compromised H3K9me in heterochromatin. We also found that heterochromatic small RNAs are lost in Δ*dri1*. Moreover, we found that Dri1 interacts with the heterochromatic transcripts similar to the RITS complex. Interestingly, deletion of the RRM domain only has mild effect on heterochromatin silencing. The ZFs also have been shown to interact with RNAs (Nguyen *et al.* 2011). It is possible that Dri1 associates with heterochromatic

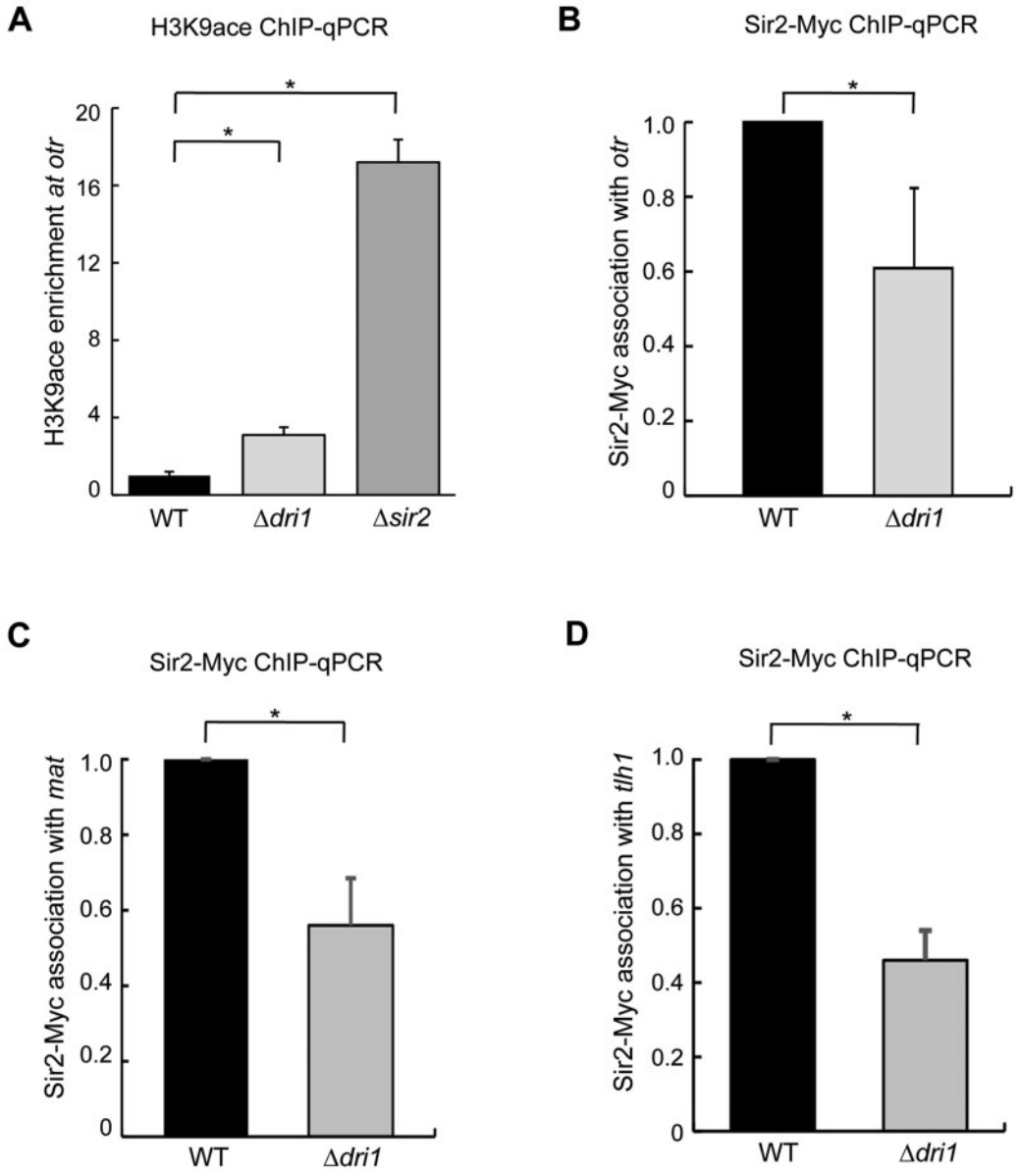

Figure 5 Dri1 is required for recruitment of Sir2 to heterochromatin. (A) ChIP analysis of H3K9 acetylation in the *otr* region in the indicated strains. (B–D) ChIP analysis of Sir2-Myc in the indicated strains in *otr*, the mating type region (*mat*), and telomere (*tlh1*), respectively. Experiments were performed in triplicate. Error bars indicate SD. $^*P < 0.05$.

RNAs via both the ZFs and the RRM domain. Consistent with this idea, ZF mutants of Dri1 show defects in heterochromatin silencing. We also found that Dri1 interacts with the RITS subunit Chp1, and regulates the association of the complex with heterochromatin via its C-terminal domain. We speculate that Dri1 may interact with the Pol epsilon complex at replication fork and help restore the RITS complex to heterochromatin after replication. However, the loss of silencing of *dri1* mutant is weaker than that of *ago1* or *dcr1*. This may imply that there are redundant molecular components that recruit RITS complex other than Dri1. Moreover, the low level of siRNAs was sufficient to nucleate heterochromatin (Buhler *et al.* 2007). We cannot exclude the possibility that residual level of siRNAs is still produced in the Δ*dri1* mutant.

HDACs also play a crucial role in heterochromatin organization. Sir2 is a conserved NAD+-dependent HDAC (Shankaranarayana *et al.* 2003; Freeman-Cook *et al.* 2005; Wiren *et al.* 2005; Alper *et al.* 2013; Janke *et al.* 2015). In fission yeast, similar to RNAi, Sir2 participates in the initiation step of heterochromatin formation (Alper *et al.* 2013; Buscaino *et al.* 2013). Sir2 also facilitates H3K9me spreading to the neighboring regions (Buscaino *et al.* 2013). In this study, we found that Dri1 is important for the recruitment of Sir2 to all heterochromatic regions. Consistent with this, cells without $dri1^+$ show a higher level of histone acetylation in heterochromatin. Nevertheless, we found that silencing is strongly lost in pericentromeres, but only mildly affected in telomeres and mating-type locus. This may be due to redundant pathways in telomeres and mating-type locus used for heterochromatin silencing. In addition, it has been shown that artificially induced heterochromatin depends on Sir2 and Clr4 but not RNAi (Kagansky *et al.* 2009). Our data showed that Dri1 is required for the ectopic heterochromatin formation, in agreement with

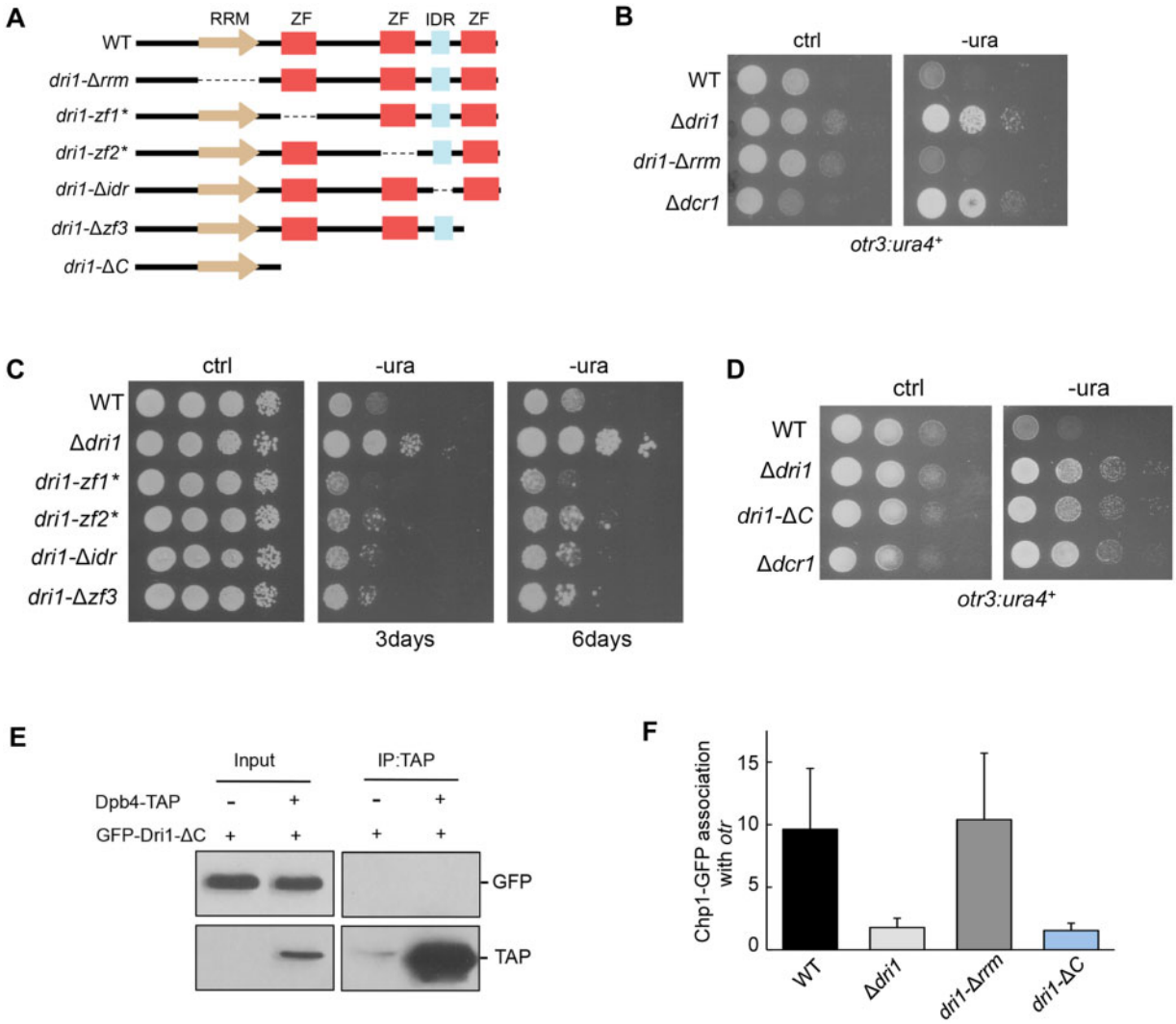

Figure 6 Domains of Dri1 contribute to its function in heterochromatin silencing. (A) Schematic representation of domains of Dri1 and structures of domain deletion strains. (B–D) Serial dilutions of the indicated strains carrying with *ura4*$^{+}$ in *otr3* were spotted on minimal medium without uracil. The plates were incubated at 30°C in –ura medium for three days. For (C), longer incubation (6 days) in –ura medium is also shown. (E) Co-IP experiments show that deletion of the C-terminus of Dri1 abolished its interaction with Dpb4. (F) ChIP analysis of Chp1-GFP in the *otr* region in the indicated strains. Error bars indicate SD. Experiments were performed in triplicate.

the idea that Dri1 is also involved in the Sir2-mediated pathway. This study thus establishes a plausible link between RNAi and HDAC. We speculate that Dri1 may coordinate RNAi and HDAC at the replication fork to promote heterochromatin reassembly.

Proteins that have disordered structures often display phase separation property: they are sufficient to drive the "demixing" from their surroundings to form their own phase. Protein phase separation has been implicated in a variety of processes, such as RNA binding and transcriptional regulation (Shin and Brangwynne 2017; Maharana *et al.* 2018; Sabari *et al.* 2018). HP1 in *Drosophila* and human cells has been shown to have a phase separation property (Larson *et al.* 2017; Strom *et al.* 2017). Dri1 also contains an intrinsically disordered region. In addition, Dri1 has a RRM motif and ZF domains that are known to help phase separation properties (Schwartz *et al.* 2013; Maharana *et al.* 2018). Similar to other phase separation proteins (Bolognesi *et al.* 2016), overexpressed Dri1 resulted in severe growth defect. Further studies are needed to determine whether Dri1 has a phase separation property and its role in heterochromatin silencing.

Author contributions

H.B. and F.L. designed the experiments and wrote the article with input from Y.C., Y.H.C., and W.S. H.B. conducted the experiments.

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Conflicts of interest

None declared.

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