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Splicing Factors Facilitate RNAi-Directed Silencing in Fission Yeast

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Abstract

Heterochromatin formation at fission yeast centromeres is directed by RNA interference (RNAi). Noncoding transcripts derived from centromeric repeats are processed into small interfering RNAs (siRNAs) that direct the RNA-induced transcriptional silencing (RITS) effector complex to engage centromere transcripts, resulting in recruitment of the histone H3 lysine 9 methyltransferase Clr4, and hence silencing. We have found that defects in specific splicing factors, but not splicing itself, affect the generation of centromeric siRNAs and consequently centromeric heterochromatin integrity. Moreover, splicing factors physically associate with Cid12, a component of the RNAi machinery, and with centromeric chromatin, consistent with a direct role in RNAi. We propose that spliceosomal complexes provide a platform for siRNA generation and hence facilitate effective centromere repeat silencing.

RNA interference (RNAi) and related pathways regulate gene expression at both transcriptional and posttranscriptional levels. In fission yeast (*Schizosaccharomyces pombe*), RNAi directs the formation of heterochromatin (1, 2). Analogous to metazoan centromeres, fission yeast centromeres comprise a kinetochore domain flanked by outer repeat (otr) sequences that are assembled in heterochromatin. These otr regions are transcribed by RNA polymerase II (Pol II) and give rise to double-stranded RNA that is processed into small interfering RNAs (siRNAs) by Dicer (Dcr1). These siRNAs are loaded into Argonaute (Ago1), a component of the RNA-induced transcriptional silencing (RITS) effector complex (3). siRNAs target RITS to cognate nascent transcripts, resulting in recruitment of further factors including the RDRC complex (comprising Rdp1, Cid12, and Hrr1) (4), and ultimately Clr4, which methylates histone H3 on Lys⁹ (H3K9me2). H3K9me2 is bound by the HP1-related protein Swi6, which in turn recruits cohesin, critical for centromere function (5).

To further dissect the mechanism of RNAi-directed chromatin modification, we previously performed a screen that identified mutations at 12 loci termed csp (centromere: suppressor of position effect), which at 25°C alleviate silencing of marker genes inserted in the otr of centromere 1 (6). Several of the csp mutants are alleles of known RNAi components (7, 8). The csp4 and csp5 mutants are temperature-sensitive (ts) lethal alleles. Complementation

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and sequencing revealed that *csp4* and *csp5* are alleles of *cwf10* and *prp39*, respectively, both of which encode splicing factors. *csp4*, now denoted *cwf10-1*, creates a missense mutation (C323Y) in the guanosine triphosphate-binding domain of Cwf10. Cwf10 is the homolog of the *Saccharomyces cerevisiae* U5 small nuclear ribonucleoprotein Snu114 (and of human EFTUD2) that is required for U4/U6 small nuclear RNA (snRNA) unwinding (9). *csp5*, now denoted *prp39-1*, makes a nonsense mutation in Prp39 (W550stop). *S. cerevisiae* Prp39 (homologous to human PRPF39) is associated with U1 snRNA and is required for commitment to splicing of pre-mRNA (10). Thus, mutations in two distinct essential splicing factors affect silencing at centromeres.

To further investigate possible links between splicing and centromere silencing, we surveyed several additional ts lethal splicing mutants for silencing defects at the permissive temperature (11-14). Only particular splicing mutants affected silencing. Silencing of a centromeric *cen1:ade6⁺* marker gene (Fig. 1A) remained intact in the presence of *prp1* (Prp6^{Sc/Hs}), *prp2* (U2AF^{Hs}), *prp3* (Prp3^{Sc}/PRPF3^{Hs}), or *prp4* (PRPF4B^{Hs}) mutations (where the superscripts Sc and Hs denotes *S. cerevisiae* and human, respectively). In contrast, mutations in *prp5* (Prp46^{Sc}/PLRG1^{Hs}), *prp8* (Prp2^{Sc}/DHX16^{Hs}), *prp10* (Hsh155^{Sc}/SF3B1^{Hs}), and *prp12* (Rse1^{Sc}/SF3B2^{Hs}), like *cwf10* and *prp39*, alleviated *cen1:ade6⁺* silencing (Fig. 1B) and increased *cen1:ade6⁺* transcript accumulation (Fig. 1C, *ade6*). Moreover, mutants that alleviated *cen1:ade6⁺* silencing also displayed increased levels of noncoding centromeric *otr* transcripts and concomitant reductions in centromeric siRNA accumulation, with *prp10-1* showing the most severe silencing defects (Fig. 1C, *cen-dh* and *cen-dg*, and Fig. 1D). Thus, several specific splicing mutants affect processing of centromeric transcripts into siRNAs and impair centromere silencing.

A mundane explanation for these observations is that impaired splicing of mRNA encoding an RNAi component indirectly affects silencing. However, the silencing and splicing defects observed in these ts splicing mutants can be uncoupled. Reverse transcription polymerase chain reaction (RT-PCR) analysis of an mRNA (*tbp1*) that is highly sensitive to splicing defects (15) confirmed that these mutants accumulated increased levels of unspliced pre-mRNA at restrictive temperature (36°C). However, at the permissive temperature of 25°C (at which centromeric silencing was alleviated), splicing efficiency was similar to that in wild-type cells (Fig. 2A). Conversely, for mutants that did not alleviate silencing at 25°C, growth at semipermissive temperature impaired splicing of *tbp1* but did not induce increased accumulation of *cen1:ade6⁺* or *otr* (*cen-dh*) transcripts, as seen in *prp10-1* (Fig. 2B). Even after prolonged incubation at 36°C, which strongly inhibits splicing (Fig. 2A), centromeric siRNAs were still readily detected in *prp1-1*, *prp2-1*, *prp3-3*, and *prp4-73* (fig. S1). Thus, defective splicing does not inherently perturb the RNAi pathway.

The RNAi genes *ago1⁺* and *hrr1⁺* contain introns; if their splicing is particularly sensitive to defects, this could explain the observed phenotypes. We therefore constructed strains in which the endogenous *ago1⁺* and *hrr1⁺* genes were replaced by cDNAs. Even in these strains, the *prp10-1* mutation alleviated silencing as in wild-type cells (Fig. 2C, white colonies). Thus, the silencing defects in *prp10-1* do not result from inefficient splicing of these RNAi components. However, defective splicing of a gene encoding some other, unknown contributory factor cannot be excluded.

Because splicing is generally coupled to transcription (16), defective splicing might affect centromeric siRNA production by impairing *otr* transcription. However, in contrast to the *rpb7-G150D* mutation affecting RNA Pol II (7), splicing mutants did not affect the abundance or length of centromeric transcripts accumulating in a *dcr1* background (fig. S2). Thus, in these splicing mutants the defect in RNAi-directed silencing lies downstream of *otr* transcription.

Strains lacking RNAi exhibit reduced H3K9 methylation and Swi6 association at centromeric otr chromatin (17). Chromatin immunoprecipitation (ChIP) revealed that splicing mutants *cwf10-1* and *prp10-1* (but not *prp2-1*) exhibited only a modest decrease in levels of H3K9me2 associated with both centromere repeats and *cen1:ura4⁺* (Fig. 3A and fig. S3). Reductions in H3K9me2 in *cwf10-1* and *prp10-1* were greater on *cen1:ura4⁺* than on otr repeats, and greater still on *cen1:ade6⁺* (fig. S4); however, these mutants consistently retained more H3K9me2 than did *dcr1* cells. Levels of centromeric Swi6 were also moderately reduced in *cwf10-1* and *prp10-1* cells (Fig. 3B). Thus, *prp10-1* cells maintain relatively high levels of H3K9me2 and Swi6 at centromeres, even though siRNA generation is severely compromised. This is similar to cells lacking the polyA polymerase *Cid14* (18).

siRNAs are absolutely required for establishment, but not maintenance, of H3K9 methylation on centromeric repeats (19). To determine whether *cwf10-1* and *prp10-1* affect the establishment of H3K9me2 on naïve repeats, we transformed wild-type or mutant strains with a plasmid bearing part of a *cen1* otr (*dg*) repeat (20). ChIP analysis using plasmid-specific primers confirmed that H3K9me2 was established on the plasmid in wild-type but not in *dcr1* cells. In *prp10-1* cells, H3K9me2 was still established on the plasmid (Fig. 3C, pH-cc2), albeit at a lower level than in wild-type cells, consistent with the lower level of H3K9me2 seen on endogenous centromeric repeats (Fig. 3, A and C, *cen-dg*). This result suggests that *prp10-1* cells must retain a low level of siRNAs, and indeed we detected siRNAs at about 1% of wild-type levels in *prp10-1* cells (Fig. 1E). Thus, very low levels of siRNAs are sufficient to establish H3K9me2 heterochromatin, but high siRNA levels are required to maintain robust heterochromatin, and this siRNA amplification requires splicing factors such as *Prp10* and *Cwf10*.

Unlike at the centromeres, maintenance of silencing at the mating-type locus does not require RNAi components but does require *Cid14* (18) and chromatin components such as Swi6 (21). Analysis of a *ura4⁺* marker inserted within the mating-type locus revealed that, like *dcr1*, none of the tested splicing mutants alleviated mating-type silencing (Fig. 4A). We also tested the effect of the splicing mutants on a *ura4⁺* locus silenced by tethering of the RITS component *Tas3* to the *ura4* transcript (22). As with RNAi mutants (*dcr1*), both *prp10-1* and *cwf10-1* mutants alleviated silencing, whereas *prp2-1* did not (Fig. 4B). Together these observations confirm a specific role for *Cwf10* and *Prp10* in RNAi-mediated silencing that is downstream of RITS recruitment, consistent with a function related to amplification of the RNAi response.

The phenotypes of splicing mutants suggest that splicing factors may interact with the RNAi machinery. Indeed, in cells lacking *Rdp1*, spliceosome subunits have been reported to copurify with affinity-selected *Cid12* (4). To examine this more closely, we analyzed immunoprecipitates of FLAG epitope-tagged *Cid12* from wild-type cells by liquid chromatography-mass spectrometry (LC-MS)/MS. Many splicing factors were found to specifically associate with *Cid12*-FLAG. These included *Cwf10*, *Prp10*, *Prp5*, and *Prp12*, which were required for centromeric silencing, along with splicing factors such as *Prp3* that did not affect silencing (Fig. 4C). Splicing factors were rarely identified in immunoprecipitates of numerous other FLAG-tagged proteins, indicating that this interaction is specific. The association of *Cwf10* with *Cid12* was also verified by coimmunoprecipitation (Fig. 4D). Thus, *Cid12* may function in association with a large spliceosomal complex, with particular splicing factor mutants compromising its activity and thereby impairing RDRC-mediated siRNA amplification.

Because splicing factors interact with *Cid12*, they may (like other RNAi components) associate with centromeric repeats. To test this possibility, we performed ChIP with *Cwf10*, the splicing factor most strongly represented in *Cid12* immunoprecipitates. Hemagglutinin

epitope-tagged Cwf10 (Cwf10-HA) was found to be enriched on both dh and dg centromere repeats, relative to an unspliced control gene, act1 (Fig. 4E). Prp8-HA was also enriched on centromere repeats, and both Cwf10-HA and Prp8-HA were at least as strongly associated with cen-dh as was Cid12-HA (fig. S5). The association of Cwf10-HA with centromere repeats was essentially lost in dcr1 and prp10-1 cells, showing it to be linked to a functional RNAi pathway (Fig. 4F). We conclude that splicing factors associate with the RNAi machinery at the centromere to directly facilitate RNAi-mediated centromere silencing.

Processing of transcripts by both the splicing and RNAi machineries is thought to occur cotranscriptionally, implying that generation of siRNAs from centromere transcripts may occur in the context of larger RNA-processing “factories” (1, 16, 22). We propose that spliceosomal complexes provide a platform that promotes the processing of centromeric transcripts by RDRC, facilitating amplification of homologous siRNAs to high levels. The silencing and chromatin modification defects seen in particular splicing factor mutants are thus explained by disruption of this transcript-to-siRNA processing step, independently of splicing itself. Splicing factors have also been identified in screens for factors affecting RNAi in nematodes and plants (23, 24) and are required for processing of some microRNAs in nematodes, flies, and humans (25, 26). Thus, integration with other RNA processing events may be a conserved feature of RNAi-related pathways.

Supplementary Material

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Acknowledgments

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27. See supporting material on Science Online.

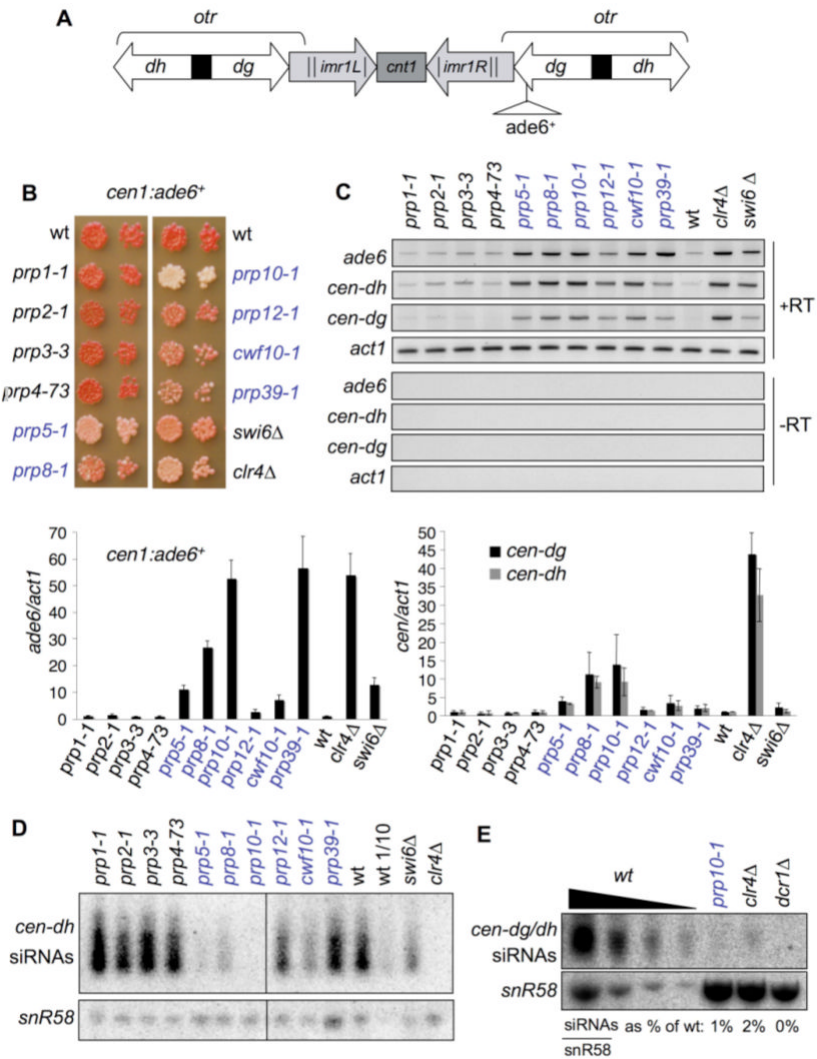


Fig. 1. Specific splicing mutants affect RNAi-directed silencing at centromeres (A) Schematic of fission yeast centromere 1, indicating site of integration of *cen1:ade6+* marker, outer repeat (*otr*) *dg* and *dh* elements, inner repeats (*imr*), and central core (*cnt*). (B) Silencing assay on strains bearing *cen1:ade6+* (red, silent; pink/white, alleviated). (C) RT-PCR and quantitative RT-PCR (qRT-PCR) analysis of transcripts from *ade6*, *cen-otr* (*dg* and *dh*), and *act1* control (-RT, no reverse transcriptase). Histograms show transcript levels relative to *act1*, normalized to the wild type. (D and E) Northern analysis of siRNAs corresponding to *cen-dh* or *cen-dg/dh*. *snoRNA58* (*snR58*) is a loading control.

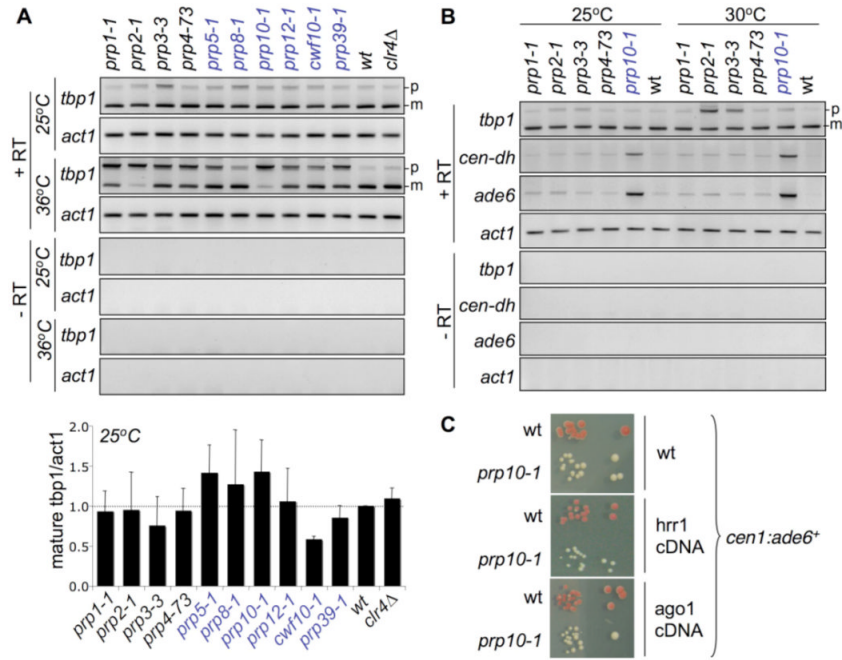


Fig. 2. Defective splicing does not cause defects in centromere silencing. **(A and B)** RT-PCR analysis of transcripts from *tbp1*, *ade6*, *cen-dh*, and *act1*. Spliced, mature (m), and unspliced, pre- (p) *tbp1*⁺ mRNA are indicated. Strains were grown at permissive (25°C) or semipermissive (30°C) temperatures or, for analysis at restrictive temperature, grown at 25°C and then shifted to 36°C for 6 hours. The histogram shows qRT-PCR analysis of *tbp1*⁺ mature transcript levels relative to *act1*, normalized to the wild type, at 25°C. **(C)** Silencing assay on strains bearing *cen1:ade6*⁺, with endogenous *ago1*⁺ or *hrr1*⁺ replaced by cDNAs.

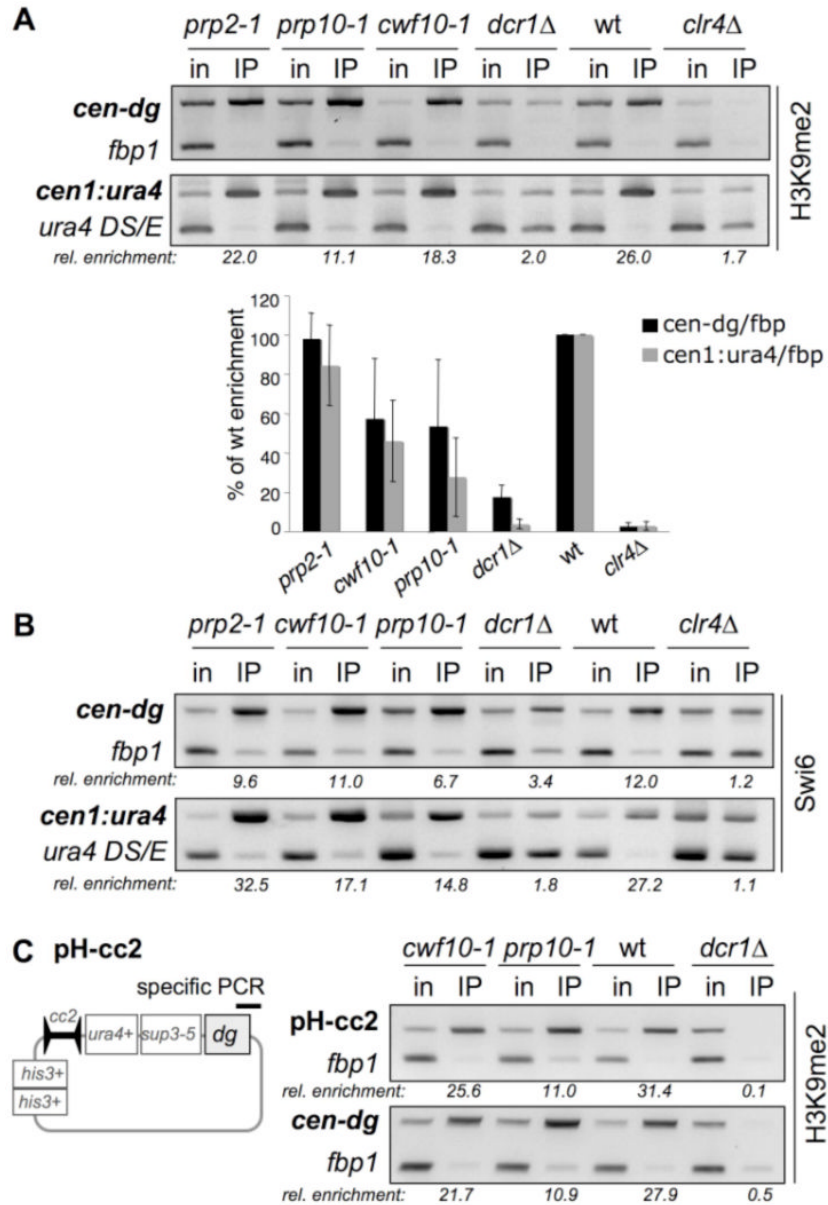


Fig. 3. Splicing mutants disrupt RNAi-dependent heterochromatin. (**A** and **B**) ChIP analysis of H3K9me2 and Swi6 at *cen-dg* or *cen1:ura4⁺* relative to a euchromatic control locus (*fbp1* or *ura4 DS/E*, respectively). Representative gels are shown. Relative enrichments were calculated as the ratio of product of interest to control product in immunoprecipitate (IP) relative to input (in). The histogram represents qPCR analysis of four independent experiments; relative enrichments (*cen-dg/fbp1* and *cen1:ura4/fbp1*) are shown as a percentage of the wild type. See also fig. S2. (**C**) ChIP analysis of H3K9me2 at *cen-dg* sequences on endogenous centromeres, or on plasmid pH-cc2 [see schematic and (20, 27)]. Plasmid was introduced by transformation and maintained under selection. Primers spanning the *dg*-plasmid backbone junction were used to specifically analyze H3K9me2 on the plasmid.

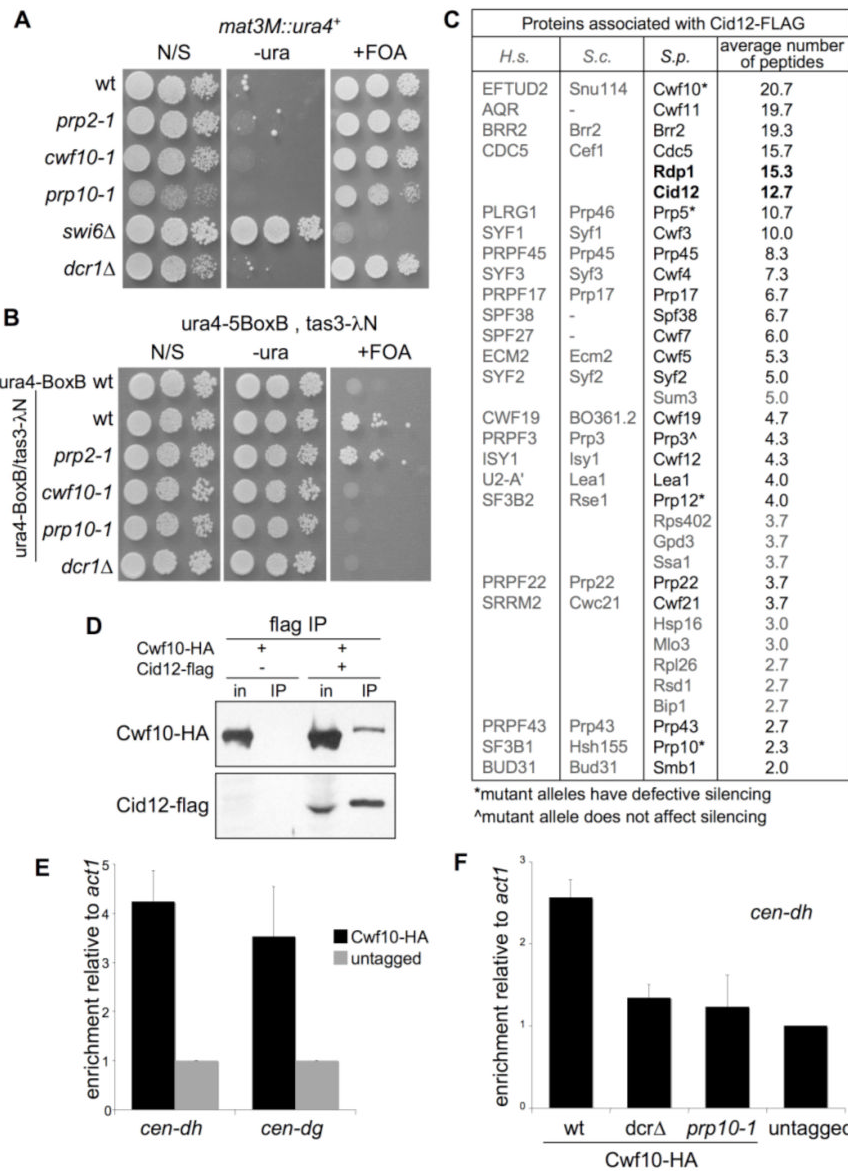


Fig. 4. Splicing factors are specifically required for RNAi-dependent silencing and physically associate with the RNAi machinery and centromere repeats. (**A** and **B**) Assays for silencing of *ura4⁺* at mating-type locus [*mat3-M::ura4⁺* (21) (**A**)] or with tethered Tas3 [*ura4-5BoxB/tas3-λN* (22) (**B**)]. Plates are nonselective (N/S), lacking uracil (-ura), or supplemented with FOA (+FOA). (**C**) Proteins specifically associated with Cid12-FLAG, analyzed by LC-MS/MS, as described (27). For splicing factors, human (H.s.) and budding yeast (S.c.) homologs are shown. (**D**) Coimmunoprecipitation of Cwf10-HA with Cid12-FLAG. (**E** and **F**) ChIP analysis of Cwf10 enrichment at *cen-dh* or *cen-dg* relative to a euchromatic, unspliced control locus *act1⁺*, normalized to an untagged control strain.