Xrn1p acts at multiple steps in the budding-yeast RNAi pathway to enhance the efficiency of silencing

Matthew A. Getz, David E. Weinberg, Ines A. Drinnenberg, Gerald R. Fink, and David P. Bartel

Abstract

RNA interference (RNAi) is a gene-silencing pathway that can play roles in viral defense, transposon silencing, heterochromatin formation and post-transcriptional gene silencing. Although absent from Saccharomyces cerevisiae, RNAi is present in other budding-yeast species, including Naumovozyma castellii, which have an unusual Dicer and a conventional Argonaute that are both required for gene silencing. To identify other factors that act in the budding-yeast pathway, we performed an unbiased genetic selection. This selection identified Xrn1p, the cytoplasmic 5′-to-3′ exoribonuclease, as a cofactor of RNAi in budding yeast. Deletion of XRN1 impaired gene silencing in N. castellii, and this impaired silencing was attributable to multiple functions of Xrn1p, including affecting the composition of siRNA species in the cell, influencing the efficiency of siRNA loading into Argonaute, degradation of cleaved passenger strand and degradation of sliced target RNA.

Introduction

RNA interference (RNAi), a gene-silencing pathway triggered by double-stranded RNAs (dsRNAs), can play important roles in viral defense, transposon silencing and host gene regulation (1,2). In this pathway, dsRNAs originating from viruses, transposons, or cellular loci are initially processed by the ribonuclease (RNase) III enzyme Dicer into ~21–23-nucleotide (nt) small interfering RNAs (siRNAs) that are paired to each other with 2-nt 3′ overhangs characteristic of RNase III-mediated cleavage. The siRNA duplexes are then loaded into the RNAi effector endonuclease Argonaute, after which one siRNA strand, designated the passenger strand, is cleaved by Argonaute and discarded (or is occasionally discarded without cleavage), thereby generating the mature RNA-induced silencing complex (RISC). Within RISC, the remaining strand, designated the guide strand, pairs with single-stranded RNAs (ssRNAs) to direct the Argonaute-catalyzed slicing of these target transcripts (1,3–4).

Although conserved in most eukaryotes, RNAi is absent from the model budding-yeast species, Saccharomyces cerevisiae (5,6). Indeed, the loss of RNAi has allowed S. cerevisiae and some other fungal species the opportunity to harbor Killer, a dsRNA element that encodes a toxin that kills neighboring yeast cells that lack Killer (7). RNAi is present in related budding-yeast species, including Naumovozyma castellii and Vanderwaltozyma polyspora (formerly Saccharomyces castellii and Kluyveromyces polysporus, respectively), which consequently do not have Killer dsRNA (7,8). Naumovozyma castellii produces siRNAs that map to hundreds of endogenous loci, a majority of which correspond to transposable elements and Y′ subtelomeric repeats (8). Naumovozyma castellii and other budding yeast thought to have retained RNAi possess a non-canonical Dicer gene (DCR1) and a canonical Argonaute gene (AGO1), which are, respectively, essential for the production and function of siRNAs in N. castellii (8). These two genes are also sufficient for the reconstitution of RNAi in S. cerevisiae (8).

In species outside the budding-yeast lineage, factors in addition to Dicer and Argonaute support RNAi pathways. For example, RNA-dependent RNA polymerases (RdRPs) are required for RNAi and related pathways in nematodes, some fungi, and plants (9–15). These enzymes synthesize complementary RNAs either to initiate or to amplify the RNAi response (9,16). Other factors are involved in the loading and maturation of RISC. In Drosophila, R2D2 (named for its two dsRNA-binding domains and association with Dicer-2) associates with Dicer-2, and this heterodimer binds to, recognizes the thermodynamic asym-
mety of, and facilitates, with the help of Hsc70/Hsp90 chaperone machinery, the loading of siRNA duplexes into Ago2 (17–23). HSP90 has also been implicated in siRNA loading into AGO1 in plants (24). In human cells, TRBP (HIV transactivating response element binding protein), like R2D2 in flies, can help to recruit siRNA-containing Dicer to AGO2 (18,25–26). In the filamentous fungus Neurospora crassa, the QIP exonuclease removes the passenger strand during RISC activation (27) and in Drosophila and human, the C3PO endonuclease is reported to have a similar activity that degrades the cleaved passenger strand during RISC maturation (28).

Another factor that can enhance the efficiency of RNAi is the Xrn1 5'-to-3' exonuclease. Xrn1 is a member of a family of enzymes broadly involved in eukaryotic transcription and RNA metabolism (e.g. processing and degradation) (29). Xrn1 is primarily cytosolic and degrades RNA species possessing a 5' monophosphate, including mRNA-decay intermediates. Xrn1 orthologs in Arabidopsis and Drosophila enhance RNAi by degrading the 3' slicing products of RISC (30,31). Likewise, in human cells, XRN1 degrades the 3' slicing fragments and acts together with the exosome to degrade the 5' slicing fragments of mRNAs targeted by siRNAs (32). By degrading the slicing products of RISC, Xrn1 orthologs likely relieve product inhibition and facilitate multiple turnover of the enzyme. In Caenorhabditis elegans, xrn-1 has also been implicated as being involved in RNAi (33), and its knockdown is associated with the accumulation of certain passenger strands in the microRNA (miRNA) pathway (34), an RNA-silencing pathway that derives from the more basal RNAi pathway (35).

Although the only known protein components of the budding-yeast RNAi pathway were Dicer and Argonaute (Dcr1p and Ago1p), we reasoned that, as observed in animals, plants and other fungi, one or more additional factors might be important for either siRNA production, duplex loading, RISC maturation, or silencing efficiency in yeast. If such a factor did exist, the ability to reconstitute RNAi in S. cerevisiae by adding only DCR1 and AGO1 indicated that the additional factor either was not essential for RNAi or had other functions in budding yeast, leading to its retention in S. cerevisiae after loss of the RNAi pathway. Based on the success of genetic screens and selections in other systems, including Arabidopsis, C. elegans and Drosophila, which provided early insight into the core components of the RNAi pathway and identified additional factors that influence RNAi efficacy (36–46), we implemented a genetic selection in N. castellii to identify mutants with reduced RNAi activity. This selection identified mutants in the gene encoding Xrn1p. Employing RNA sequencing and biochemical assays, we found Xrn1p acts at multiple steps of the pathway to enhance the efficiency of RNAi in budding yeast. These steps included affecting siRNA populations and loading of siRNA duplexes into Argonaute—steps for which it had not been previously reported to play a role in any species.

**MATERIALS AND METHODS**

**Growth conditions and genetic manipulations**

*Naumovozyma castellii* was grown at 30°C on standard *S. cerevisiae* liquid and solid media (YPD and SC), unless otherwise indicated. Transformations were performed as described (8), with the following modifications: The transformation mix (cells + DNA + PEG + lithium acetate) was incubated at 23°C for 30 min with rotation and without dimethyl sulfoxide. The mixture was then incubated at 37°C for 20 min, resuspended and plated on selective media.

**Plasmid and strain construction**

Plasmid and strain construction is detailed in the supplementary materials, and lists of plasmids and strains generated in this study are provided (Supplementary Tables S5 and S6).

**EMS mutagenesis and selection**

Mutagenesis of DPB537, the *N. castellii* genetic-selection strain, with ethyl methane sulfate (EMS) was carried out as described (47), except scaled-up 10-fold. An overnight YPD culture of *N. castellii* was used to inoculate a large YPD (150 ml) culture that was grown until OD₆₀₀ ≈ 2, split equally into aliquots that contained ~0.5 ml cultures (8), and the one which was incubated with 300 µl EMS and the other which was incubated with 300 µl sodium phosphate buffer. The OD₆₀₀ of each culture was measured and was used to calculate the dilutions needed to plate 100 mutagenized or control cells on separate YPD plates to determine the death rate of cells in the mutagenesis. Mutagenized cells were split into four equal aliquots and plated on SGal – His, – Leu, – Ura (drop-out media); SD – His, – Leu, – Ura (drop-out media); SM (synthetic minimal) + 2% galactose (SMG) (drop-in media); and SM (synthetic minimal) + 2% glucose + Lys (SD + Lys) (drop-in media). These plates were incubated at 25°C until colonies formed.

**Flow cytometry and mating of RNAi mutant strains**

A culture of each mutant strain that had grown on selective media was inoculated in 200 µl inducing SC media (2% galactose) in a 96-well plate and grown to saturation. Fresh cultures were then inoculated at OD₆₀₀ 0.2 with cells from the saturated cultures and were grown until all strains were in log phase (~6 h). Cells were analyzed using FACSCalibur (BD Biosciences), and data were processed with CellQuest Pro (BD Biosciences) and FlowJo (Tree Star). For each experiment, a gate for eGFP fluorescence was defined using the wild-type (WT) genetic-selection strain (DPB537) as a negative control to define the boundaries of the gate. Strains for which > 0.5% of the population was in the eGFP-positive gate were carried forward for complementation analysis.

Matings of candidate *N. castellii* RNAi-mutant strains with WT (DPB079), Δago1 (DPB325), Δdcr1 (DPB534) and Δxrn1 (DPB535) strains were performed by mixing equal volumes (100 µl) of saturated YPD cultures in a round-bottom 96-well plate and incubating at 30°C for 24 h. Diploid cells were then selected by plating these mixed saturated cultures on SD – Ade, – Lys agar plates (for RNAi mutants × DPB325) or SD – Met, – Lys agar plates (for all other matings). Flow cytometry was performed as with
the haploid mutant strains, except with no gating for eGFP positive cells. Diploid strains presented in Figure 1D were created from matings of the WT genetic-selection strain (DPB537) and its derived RNAi-mutant strains with a WT strain (DBP079) or a Δxrn1 strain (DPB535) of the opposite mating type. The diploid XRN1-knockout strain was created by mating strain DPB541 with strain DPB510.

Genome sequencing and analysis

Genomic DNA was isolated as described (48) from saturated YPD cultures of the N. castellii genetic-selection strain (DPB537) and RNAi-mutant strains. DNA concentration was calculated using the Qubit dsDNA HS (High Sensitivity) Assay Kit (Invitrogen Q32854) with an Invitrogen Qubit Fluorometer. Genomic DNA libraries were prepared with either the Nextera DNA Library Preparation Kit (Illumina) or the QIAseq FX DNA Library Kit (QIAGEN 180475) and sequenced using Illumina SBS. Single-end sequencing was performed on the HiSeq platform, and paired-end sequencing was performed on the HiSeq or NextSeq platforms. A complete description of the genome sequencing analysis is detailed in the supplementary materials.

Ag01p-Xrn1p co-immunoprecipitation

Overnight YPD cultures of haploid strains DPB215, DPB220, DPB221 were grown, diluted to OD_{600} 0.2 in 150 ml YPD, and then grown until OD_{600} 0.8. Cultures were harvested by centrifugation, washed with 25 ml cold phosphate-buffered saline (PBS), transferred to an Eppendorf tube in 750 μl cold PBS and resuspended in one volume IP buffer (50 mM HEPES pH 7.6, 300 mM NaCl, 5 mM MgCl₂, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM EGTA, 5% glycerol, 0% NP-40, PMSF (Sigma E6779) was added to lysates from strain DPB215 ± RNase and DPB221 or 20 μl of EZview Red Anti-HA Affinity Gel (Sigma F2426) was added to lysates from strain DBP215 ± RNase and DPB220. The lysates were incubated at 90°C for 5 min, and then were centrifuged at room temperature for five min at 10,000 × g. All samples were run on a 4–12% Bis-Tris gel in MOPS buffer. The proteins were transferred from the gel to a PVDF membrane (Invitrogen LC2002), each blot was probed with anti-HA7 (Sigma H3663) and anti-Flag BioM2 (1:2000) (Sigma F9291) overnight at 4°C, and probed with the secondary antibody anti-mouse IgG HRP (1:5000) (Amersham NXA391) for 1–2 h at room temperature. The blots were stripped with 100 mM bME, 2% sodium dodecyl sulphate, 62.5 mM Tris pH 6.8 at 50°C for 30 min, shaking every 10 min. For the RNA-degradation analysis, samples containing 100 μl of supernatant were phenol extracted, precipitated and resuspended in 45 μl water. A total of 1 μl of the RNA sample was added to 2 μl water, and then 3 μl 2× glyoxal loading dye was added. Samples were then incubated at 50°C for 30 min to denature the RNA and then placed on ice. Samples were resolved on a 1% agarose gel run in NorthernMax–Gly Gel Prep/Running Buffer (Invitrogen AM8678) and visualized with a Chemi-Doc MP Visualization System (Biorad).

Small-RNA sequencing, RNA-seq and analysis

Total RNA was isolated from N. castellii strains DPB220, DBP228, DBP622, DPB537 and DPB541. For small-RNA sequencing, 0.5 nM of four small RNA oligonucleotides (str miR-427, 5′-GAAAGUGCUUUCUGUUUGGCG; dme miR-14, 5′-GGGAGCGAGACGGGGACTCACT; Synthetic_sRNA_L_Guide, 5′-UUGGAGAGGGUAGUUUUUGU; Synthetic_sRNA_L_Passenger, 5′-CAAAAUAACCUGCUACUAUA) were added to 10 μg of total RNA for use as internal standards. Small-RNA cDNA libraries were prepared as described (50), except 9–26-nt RNAs were isolated and sequenced, using 9-nt and 26-nt RNA radiolabeled standards (5′-AAACACUGC and 5′-AGCGUAGUGCUACUUGAGGAA) to follow ligations and purifications.

For RNA-seq, 0.5 ng of two mRNAs (chloramphenicol and firefly luciferase) were added to the samples using Ribo-Zero Gold rRNA Removal Kit (Yeast) (Illumina, MRZY1324). RNA cDNA libraries was washed four times with IP buffer (each wash, 800 μl centrifuged 10,000 × g for 5 min), and the residual buffer was removed with a needle. A total of 100 μl 1× Laemmli buffer (Bio-Rad 1610737) + 2.5% beta-mercaptoethanol (bME) was added to the affinity agarose for each sample. A total of 10 μl 2× Laemmli buffer + 2.5% bME and 80 μl 1× Laemmli buffer + 2.5% bME was added to the input and supernatant samples. All samples were incubated at 90°C for 5 min, and then were centrifuged at room temperature for five min at 10,000 × g. All samples were run on a 4–12% Bis-Tris gel in MOPS buffer. The proteins were transferred from the gel to a PVDF membrane (Invitrogen LC2002), each blot was probed with anti-HA7 (Sigma H3663) and anti-Flag BioM2 (1:2000) (Sigma F9291) overnight at 4°C, and probed with the secondary antibody anti-mouse IgG HRP (1:5000) (Amersham NXA391) for 1–2 h at room temperature. The blots were stripped with 100 mM bME, 2% sodium dodecyl sulphate, 62.5 mM Tris pH 6.8 at 50°C for 30 min, shaking every 10 min. For the RNA-degradation analysis, samples containing 100 μl of supernatant were phenol extracted, precipitated and resuspended in 45 μl water. A total of 1 μl of the RNA sample was added to 2 μl water, and then 3 μl 2× glyoxal loading dye was added. Samples were then incubated at 50°C for 30 min to denature the RNA and then placed on ice. Samples were resolved on a 1% agarose gel run in NorthernMax–Gly Gel Prep/Running Buffer (Invitrogen AM8678) and visualized with a Chemi-Doc MP Visualization System (Biorad).
were prepared using NEXTflex rapid directional mRNA-seq kit (Bio Scientific, NOVA-5138-10). Libraries were sequenced using the Illumina SBS platform. Reads were mapped to the *N. castellii* genome, and those that mapped to small-RNA clusters (Supplementary Table S4) were fractionally counted. A complete description of the small-RNA sequencing and RNA-seq analysis is detailed in the Supplementary Materials.

**RNA and protein purification**

A complete description of the purification of RNAs and proteins used in *in vitro* assays is detailed in the Supplementary Materials.

**siRNA-binding assay**

Proteins and RNAs were diluted in siRNA-binding buffer (50 mM Hepes pH 7.6, 1 mM EGTA, 1 mM EDTA, 5 mM magnesium acetate, 10% glycerol, 300 mM potassium glutamate, 10 mM NaCl, 0.01% NP-40, 5 mM DTT added fresh). About 10× RNA mix (10 nM miR-20a siRNA duplex with a 5′-radiolabeled guide strand, 100 nM unlabeled miR-20a siRNA duplex, 1 μM capped target RNA, 1 μM tRNA from baker’s yeast (Sigma-Aldrich R5636), 1 μL SUPERase-In RNase Inhibitor (Invitrogen AM2696) per 20 μL RNA mix) was made, and 1 μL of this mix was added to a 1.5 mL G-tube siliconized microcentrifuge tube (BIO PLAS Inc., 4165SL). 1.1× protein mixes contained either no protein, 222 nM WT Ago1p and 222 nM WT Xrn1p, or 11.1 nM WT Ago1p-RISC and 111.1 nM WT Xrn1p. Each protein mix was pre-incubated at 30°C for 5 min before adding 9.9 μL of the protein mix to the RNA mix. After incubation at 30°C for the indicated time, 2 μL of each reaction was removed and quenched as for the combined assay. For the Ago1p-RISC target-slicing assay in Figure 6D–F, the reactions were carried out as above, except the target was 3′-cordycepin-labeled.

To assess cleavage and slicing, RNAs were resolved on denaturing (7.5 M urea) 20% polyacrylamide gels, and radiolabeled cleavage products were visualized and quantified by phosphorimaging as above. At each time point, the fraction product was measured as $F_p = \text{product}/(\text{product} + \text{substrate})$. For Figure 6F, the fraction product was measured as $F_p = \text{product}/(\text{product} + \text{substrate} + \text{degradation intermediate})$. For Figure 6C, the multiple-turnover slicing data were fit in Prism 8 (GraphPad) to the burst and steady-state equation (52,53),

$$F(t) = E \times \frac{a^2}{(a + b)^3} \left(1 - e^{-(a+b)t}\right) + E \times \frac{ab}{a + b} t,$$

Where $F(t)$ is target cleaved over time, $E$ is the enzyme concentration, and $a$ and $b$ are rate constants (reported as $k_1$ and $k_2$, respectively) according to the following scheme,

$E + S \xrightarrow{a} E \cdot P \xrightarrow{b} E + P$.

**RESULTS**

**XRN1 is a component of the budding-yeast RNAi pathway**

A genetic selection was designed to discover novel components of the RNAi pathway in *N. castellii*. This selection centered on an *N. castellii* strain that expressed RNA hairpins that were processed by Dicer to produce siRNAs targeting *HIS3, URA3* and *GFP* mRNAs expressed from endogenous genes that had been integrated into the genome (Figure 1A). The hairpin and *GFP* genes were under the control of the *S. cerevisiae* GAL1 and *URA3* promoters, respectively, and the *HIS3 and URA3* genes, together with their promoters, were obtained from *S. cerevisiae* and replaced their *N. castellii* counterparts in the genome of the selection strain. Because this parental strain was engineered to deploy the RNAi pathway to silence *HIS3* and *URA3*, it did not grow on media lacking histidine and uracil. This strain
Figure 1. Genetic selection identifying XRN1 as an enhancer of the RNAi pathway in budding yeast. (A) Schematic of the genetic selection. At the left are the six genomic insertions of the parental selection strain. Two of these insertions provided an additional copy of the AGO1 and the DCR1 genes (black and red boxes, respectively), each added under control of their native promoters. Three of the insertions provided silencing constructs against HIS3, URA3 and GFP genes (purple, blue and green convergent arrows, which indicate regions giving rise to palindromic transcripts that form hairpins) under control of the Saccharomyces cerevisiae GAL1 promoter (P_GAL1). These three insertions also provided S. cerevisiae LEU2, HIS3 and URA3 genes (orange, purple and blue boxes, respectively) under control of their S. cerevisiae promoters, which replaced their endogenous Naumovozyma castellii orthologs. The sixth insertion provided an exogenous GFP gene (green box) under control of the S. cerevisiae URA3 promoter. The haploid selection strain was mutated with EMS, and mutants able to grow on media that lacked Leu, His and Ura were isolated and screened by flow cytometry for their ability to silence GFP. Mutant strains able to silence GFP were subjected to a complementation analysis in which they were mated with N. castellii WT, Δago1 and Δdcr1 strains, and the resulting diploid strains were screened by flow cytometry for their ability to silence GFP. Strains for which the ability to silence GFP was restored in each of the three matings were carried forward as RNAi mutant strains, with the complementation analysis indicating that these each had one or more recessive mutations that reduced silencing and fell outside of AGO1 and DCR1. (B) Growth of RNAi mutant strains but not the parental strain on selective media. Representative RNAi mutant strains were serially diluted, plated on either non-selective media (SC (2% galactose)) or selective media (SGal– His, – Leu, – Ura), and allowed to grow for 12 days. For each strain, 10⁶ cells were plated in the leftmost spot, and dilutions were each 10-fold. (C) Ability of mutations in both XRN1 and SRB8 to confer growth of the selection strain on selective media. Assays compared the growth of single mutant strains (Δxrn1 or Δsrb8) to that of the double-mutant strain (Δsrb8 Δxrn1), which were each constructed in the background of the selection strain. Cells were allowed to grow for 10 days; otherwise, as in B. (D) Results of complementation analysis of RNAi mutant strains that had mutations in XRN1, shown as histograms of GFP fluorescence measured by flow cytometry in WT × WT (gray), Δxrn1 × Δxrn1 (green), RNAi mutant × WT (orange) and RNAi mutant × Δxrn1 (blue) diploid strains. All strains were induced. (E) An intermediate effect on GFP silencing observed upon disruption of XRN1. Shown are histograms of GFP fluorescence measured by flow cytometry in WT, Δago1, Δdcr1, Δxrn1, Δdcp2 and Δski3 haploid strains that either lacked the GFP gene (gray), contained GFP but had no silencing construct (green), or contained both GFP and a GFP-silencing construct (blue). All strains were induced.
was mutagenized with ethyl methane sulfonate (EMS), and the cells were plated on media lacking histidine and uracil with the goal of identifying mutants that had a His+ and Ura+ phenotype because they no longer efficiently silenced HIS3 and URA3. As a secondary screen, mutants were examined by flow cytometry for their ability to silence GFP, and those that had impaired GFP silencing were carried forward for complementation analysis (Figure 1A).

Analysis of mutants isolated in pilot experiments illustrated the ability of our selection to find mutants in the RNAi pathway. Mutant strains from these pilot selections were mated to each of three strains: WT, Δago1 and Δdcr1, and the resulting diploids were assessed for their ability to silence GFP (Figure 1A). If a new mutation was recessive and in the AGO1 gene, then the diploid formed with Δago1 would fail to silence GFP, and likewise, if a new mutation was recessive and in the DCR1 gene, then the diploid formed with Δdcr1 would fail to silence GFP. Indeed, these complementation analyses indicated that all of the strains tested had mutations in either AGO1 or DCR1, which showed that the selection scheme worked. To identify additional genes involved in RNAi without also having to contend with a large number of ago1 and dcr1 mutants, we modified the selection strain to contain an extra copy of AGO1 and DCR1, each under control of its native promoter, integrated into the genome at an ectopic location.

Mutations that lost silencing were selected in the new parental selection strain (DPB537) with duplicated AGO1 and DCR1 genes. As expected, isolated mutant strains but not the parental strain grew on selective media, as assessed by a serial-dilution growth assay (Figure 1B). Each of the isolated strains carried recessive mutations, as assessed from analyses of diploid products of mating with a WT strain. Moreover, as illustrated for strain 3 (Figure 1A), 19 strains retained both Ago1p and Dcr1p activities, as indicated by fully restored GFP silencing in diploid products of both Δago1 and Δdcr1 matings. These 19 strains were carried forward as potentially having mutations in genes encoding another protein needed for efficient RNAi.

To identify mutated genes, we sequenced the genomes of isolated strains (Supplementary Table S1). Of these six each derived from a mutant strain with a nonsense mutation in AGO1, whereas the one with efficient GFP silencing derived from the strain with a missense mutation in AGO1 (RNAi mutant 5). These results indicated that the nonsense mutation probably did not impair Xrn1p activity but that the XRN1 nonsense mutations were each loss-of-function mutations and were at least partially responsible for the reduced GFP silencing observed in the haploid mutant strains. Indeed, the Δxrn1/Δxrn1 diploid that we constructed, which had no other confounding mutations, also had reduced silencing.

We also examined the consequence of inactivating XRN1 in an RNAi-reporter strain containing an integrated GFP gene and an integrated gene expressing stem-loop transcripts that can be processed into siRNAs that target GFP. Compared to deleting AGO1 or DCR1, deleting XRN1 produced an intermediate GFP-silencing defect (Figure 1E), which depended on the presence of the silencing construct (Supplementary Figure S1). The observation that silencing was reduced but not completely abolished supported the conclusion that Xrn1p functions in the budding-yeast RNAi pathway but, unlike Dcr1p and Ago1p, is not an essential component of the pathway. In contrast, disrupting other genes involved in cytoplasmic mRNA decay, including DCP2 or SK1, which code for proteins important for decapping and 3′-to-5′ degradation, respectively, had minimal effect on silencing GFP (Figure 1E), indicating that their respective decay activities are not required for efficient RNAi in budding yeast.

Xrn1p and Ago1p physically interact and colocalize

As an orthogonal approach to identify components of the budding-yeast RNAi pathway, we expressed epitope-tagged Ago1p in N. castellii and used mass spectrometry to iden-
XRNI affects small RNA populations in budding yeast

To learn whether Xrn1p impacts the levels of siRNAs in the cell, we sequenced small RNAs from WT and Δxrn1 N. castellii strains after adding known quantities of synthetic standards to each sample, which enabled quantitative comparisons between samples. Mapping of the 22–23-nt reads to previously identified siRNA-producing loci (Supplementary Table S4) (8) and normalizing to the synthetic standard showed that siRNA levels from the most prolific loci (Supplementary Table S4) (8) and normalizing to the synthetic standard showed that siRNA levels from the most prolific loci were reduced upon XRN1 disruption (Figure 3A). Many of these prolific loci were inverted repeats (i.e. palindromic loci), which produce transcripts with a hairpin (stem-loop) structure. For nearly all of the palindromic loci, fewer siRNAs accumulated in the Δxrn1 strain (median change, 3.5-fold) (Figure 3A). In contrast, siRNAs mapping to Y′ subtelomeric elements and other non-palindromic loci, in which convergent overlapping transcription produces transcripts that base pair to form bimolecular siRNA precursor duplexes, typically increased in the Δxrn1 strain (median change, 4.1-fold). For example, siRNAs from the Y′ subtelomeric elements increased by over 5-fold in the XRNI-disrupted strain (Figure 3A and Supplementary Figure S2B). Subtelomeric siRNAs also accumulate in S. pombe upon the deletion of its XRNI ortholog, exo2 (61). When classifying the 22–23-nt reads as originating from either palindromic or non-palindromic loci, the proportion of siRNA reads originating from palindromic loci decreased by half in the XRNI-disrupted strain (from 74.1 to 37.0%) and the siRNA reads that mapped to non-palindromic loci increased (from 21.5 to 60.8%) (Figure 3B). The disparate changes for the different types of siRNAs observed by sequencing were also observed by probing an RNA blot analyzing small RNAs from the same strains (Figure 3C). This blot showed that an siRNA deriving from a palindromic region decreased by > 2-fold in the Δxrn1 strain, whereas a Y′ subtelomeric siRNA increased in expression by > 4-fold. The Y′ subtelomeric siRNA also increased in Δdep2 and Δski3 strains but not to the same degree as in the Δxrn1 strain, whereas the palindromic siRNA increased in the Δski3 strain and did not substantially change in the Δdep2 strain (Supplementary Figure S2A).

Xrn1p enhances RNAi in vitro

To study the ability of Xrn1p to enhance RNAi, we purified WT N. castellii Ago1p and WT and catalytically impaired versions of N. castellii Xrn1p (each from S. cere-
visiae overexpression strains that lacked XRN1) and examined the influence of Xrn1p activity on passenger-strand cleavage and target slicing in vitro. The in vitro assay used an siRNA duplex with a 5′-labeled passenger strand and a cap-labeled target RNA to follow both passenger-strand cleavage and target slicing in multiple-turnover conditions (Figure 4A). In the presence of WT but not catalytically impaired (mut.) Xrn1p, the amount of passenger-strand cleavage fragment substantially increased at early time points and then dropped at later time points (Figure 4B and C). The reduced passenger-strand fragment observed at later time points indicated that Xrn1p degraded this fragment, either as it exited RISC or after it dissociated from RISC—in either case, potentially enhancing RNAi efficiency by facilitating RISC maturation or preventing cleaved passenger strand from inhibiting RISC activity, respectively. An siRNA duplex with a 3′-labeled passenger strand showed a similar trend as the siRNA duplex with a 5′-labeled passenger strand; in the reaction with WT Xrn1p, the 3′-cleavage fragment of the passenger strand accumulated to a greater extent at early time points and then was degraded at later time points (Supplementary Figure S3A–C). These results indicated that Xrn1p degrades both cleavage fragments of the passenger strand in vitro—an observation consistent with the known preference of Xrn1p for substrates with a 5′ monophosphate.

WT Xrn1p also increased the rate of target slicing (Figure 4D). The increased rates of both passenger-strand cleavage and target slicing suggested that Xrn1p increased the amount of Ago1p that could be loaded with an siRNA duplex, perhaps by degrading non-specifically bound cellular RNAs that might be occluding siRNA loading. To assess whether Xrn1p activity increased the amount of Ago1p that could be loaded with a siRNA duplex, we performed filter-binding assays to measure the amount of siRNA bound to protein after incubating guide-labeled siRNA duplex with the purified proteins (Figure 4E). Preincubation of WT Xrn1p with Ago1p enhanced the amount of siRNA bound to protein, whereas preincubation with mutant Xrn1p did not have this effect (Figure 4E). These results supported the idea that the exonuclease activity of Xrn1p might help eliminate non-siRNA species that spuriously bind Ago1p and competitively inhibit siRNA duplex binding.

**XRN1 affects the stability of the passenger strand in vivo**

Because *N. castellii* Xrn1p degraded the cleaved passenger strand in vitro, we asked if it had this effect in vivo. We identified perfectly paired guide–passenger duplexes for which 22–23-nt reads were observed among small-RNA reads from WT, Δxrn1 and AGO1 slicing-impaired (*ago1-D1247R*) *N. castellii* strains. For each of these duplexes, the strand with more reads in the WT strain was designated as the guide, the other strand was designated as the passenger and duplexes for which the passenger-to-guide ratio increased in the *ago1* mutant strain were carried forward as experimentally supported siRNA duplexes. Twelve-nucleotide reads that perfectly matched the 3′ end of the passenger strand were designated as 3′-cleavage fragments of passenger strands, and depending on whether the duplex
**Figure 3.** The effect of Xrn1p on the abundance of small RNAs and their precursors in *Naumovozyma castellii.* (A) The effect of Xrn1p on the abundance of small RNAs originating from siRNA-producing loci yielding at least 1 RPM (read per million) in WT cells. The scatter plot shows the fold change (log2) of 22–23-nt small RNAs deriving from each locus in the Δxrn1 versus WT strain (after normalizing to the recovery of internal standards) as a function of the abundance of 22–23-nt small RNAs deriving from the locus in the WT strain. The Y′ consensus locus is represented by the filled red circle, and other non-palindromic loci are represented by empty gray circles. The two palindromic loci probed in C are represented by the filled yellow circles, and the other palindromic loci are represented by the filled cyan circles. For each category, the number of loci is indicated in parentheses. Sequencing was from strains in which the endogenous Ago1p had been FLAG tagged. (B) Overall effect of Xrn1p on the fraction of palindrome-derived and non-palindrome-derived small-RNA populations in *N. castellii.* The 22–23-nt reads that mapped to siRNA-producing loci were designated as deriving from either palindromic loci (cyan), non-palindromic loci (red), or both types of loci (purple) in the same strains as A. (C) Effect of Xrn1p on the expression levels of both a palindrome-derived siRNA and a non-palindrome-derived siRNA in *N. castellii.* At the left are phosphorimages of a small-RNA blot successively probed for siRNAs originating from either palindromic or non-palindromic precursor transcripts in the same WT and Δxrn1 strains as A. The migration of markers is indicated on the left. For a loading control, the blots were re-probed for the U6 snRNA. To the right of the blots is a bar graph showing the effect of deleting *XRN1* on these siRNAs, as measured by RNA blots and by sequencing in A. (D) Effect of Xrn1p on the abundance of small-RNA precursor transcripts in *N. castellii,* as measured using RNA-seq. Shown for each precursor transcript expressed at ≥1 transcript per million (TPM) in WT cells is its fold change (log2) in the Δxrn1 versus WT strains (after normalizing to the recovery of internal standards) as a function of its abundance in WT cells. Strains were as in A, and precursor transcripts are classified as in A. (E) Relationship between changes in small RNAs and changes in siRNA precursors observed after deleting *XRN1.* Shown for each precursor transcript expressed at ≥1 TPM in WT cells and that produced small RNAs at ≥1 RPM is the fold change (log2) in 22–23-nt small RNA reads observed in A as a function of the fold change (log2) in precursor transcripts observed in D. Representation of each type of locus as in A. The number of loci and Spearman’s rank correlation coefficient (R_s) for each category is indicated in parentheses.
Figure 4. The impact of Xrn1p on siRNA loading, passenger-strand degradation and target slicing. (A) Experimental scheme of a combined assay monitoring both passenger-strand cleavage and target slicing. An siRNA duplex is loaded into Ago1p, which cleaves and discards the passenger strand before beginning to catalyze multiple-turnover slicing of the target RNA. Initial concentrations of siRNA duplex and target RNA were 10 and 100 nM, respectively. In the duplex, the red star indicates radiolabeled monophosphate on the 5′-end of the passenger strand. On the target, the red-filled circle indicates a 5′-radiolabeled cap. (B) The effect of Xrn1p on Ago1p-catalyzed passenger-strand cleavage and target slicing, in the assay schematized in A. Shown is a representative denaturing gel that resolved cap-labeled target, cap-labeled target fragment, and radiolabeled guide RNA with Ago1p after incubating components of the combined assay of passenger-strand cleavage and target slicing. Each assay included siRNA duplex, unlabeled target RNA and the other components that bound to the nitrocellulose membrane by that of the RNA that bound to the nylon and nitrocellulose membranes combined.

was comprised of 22- or 23-nt species, 10- or 11-nt reads that perfectly matched the 5′ end of the passenger strand were respectively designated as 5′-cleavage fragments of passenger strands. For most duplexes, disrupting XRN1 increased the ratio of full-length passenger strand to guide strand, as would be expected if Xrn1p facilitates RISC loading (Figure 5A, median increase, 2.3-fold; P < 0.001, Wilcoxon signed-rank test). Disrupting XRN1 also increased the ratio of cleaved passenger strand to guide strand modestly (Figure 5B, median increase, 1.3-fold; P < 0.001).

We also examined the length distributions and the first nucleotide composition of the genomically mapping 9–26-nt reads in the libraries of each of these strains, excluding reads that mapped exclusively to rRNAs or tRNAs (Supplementary Figure S4). There were no substantial differences in the size distributions of the 9–26-nt reads in the Δxrn1 or AGO1 slicing-impaired strains compared with the WT strain, although there were slightly more 15–20-nt reads in the Δxrn1 strain (Supplementary Figure S4). Similar to previous studies, most 23-nt RNAs began with U, which suggested that most siRNA guide strands begin with this nucleotide (8,62). The Δxrn1 and AGO1 slicing-impaired strains had a higher fraction of 23-nt reads with A in the third to last position (24.8 and 36.0%, respectively, compared to 19.3% for the WT strain), which would be expected of full-length passenger strands that could pair with guide strands that begin with U. These results indicated that Xrn1p affects the stability of full-length and cleaved passenger strands in vivo, as it does in vitro.

Xrn1p enhances target slicing and eliminates the 3′ slicing fragment

To learn whether Xrn1p enhances the efficiency of any steps following duplex loading, RISC maturation and passenger-strand degradation, we purified mature N. castellii RISC programmed with a specific guide RNA (63) and examined the ability of N. castellii Xrn1p to enhance slicing of cap-
labeled target in vitro. Under single-turnover conditions, in which the RISC enzyme was in excess over target, Xrn1p activity had no detectable influence on slicing rates (Supplementary Figure S5A and B). Likewise, Xrn1p activity had no detectable influence on the initial burst phase of a multiple-turnover slicing reaction (Figure 6A), in which target was in 10-fold excess over the RISC enzyme (Figure 6B and C). However, after the slicing product reached the concentration of RISC (10 nM) and the reaction entered a slower phase characterized by rate-limiting product release, WT Xrn1p but not the catalytically impaired Xrn1p increased the rate of target slicing (Figure 6B and C). Enhancement of this second phase of target slicing increased after boosting the Xrn1p concentration from 10 to 30 nM but did not increase with even more Xrn1p (Figure 6C).

These results suggested that Xrn1p enhances release of sliced product, presumably by degrading the 3′ product of slicing, which contains the 5′-monophosphosphate characteristic of Xrn1p substrates. To examine whether Xrn1p has this function, we performed a slicing reaction with a 5′-capped, 3′-labeled target RNA in multiple turnover conditions (Figure 6D). In reactions lacking Xrn1p or containing catalytically impaired Xrn1p, the 3′ slicing product accumulated rapidly during the burst phase and then more slowly during the part of the reaction characterized by rate-limiting product release, as observed for the 5′ product in reactions with cap-labeled target (Figure 6E and F). In reactions containing WT Xrn1p, the 3′ slicing product similarly accumulated at an early time point but then began to decrease as it was degraded by Xrn1p (Figure 6E and F). This rate of degradation increased as the concentration of Xrn1p increased from 10 to 30 nM but did not significantly increase with additional Xrn1p (Figure 6F). The elimination of this 3′ product of slicing, which pairs to the guide-RNA seed region, which in turn is critical for target recognition and binding (52,64–65), would prevent this 3′ slicing product from re-binding to the guide and would thereby allow RISC to bind and slice another target RNA.

In support of the proposal that Xrn1p degrades 3′ products of slicing, comparison of RNA-seq data from the selection strain with and without XRN1 showed that, in the absence of Xrn1p, RNA preferentially accumulated in the mRNA regions downstream of HIS3, URA3 and GFP slicing (Supplementary Figure S5C). In contrast, endogenous genes that were not targets of RNAi in the selection strain showed a uniform increase in RNA sequencing reads mapping across the gene in the XRN1-knockout strain, and showed an increased proportion of reads mapping to their 3′ ends in both WT and XRN1-deficient backgrounds (Supplementary Figure S5D). Thus, as observed in other species (30–32), XRN1 is responsible for eliminating the 3′ products of slicing in budding yeast.
Figure 6. The impact of Xrn1p on multiple-turnover slicing and on stability of the 3′ target fragment. (A) Experimental scheme of the assay for multiple-turnover slicing using a cap-labeled target. The red-filled circle on the target RNA indicates a radiolabeled 5′ cap. The initial concentration of target was 100 nM. (B) The effect of Xrn1p on multiple-turnover slicing in the assay schematized in A. Shown is a representative denaturing gel resolving target from its sliced product after incubation with or without purified RISC–miR-20a (10 nM), with the indicated concentration of Xrn1p (WT or mut.; 0, 10, 30 or 100 nM) for the indicated amount of time. (C) Quantification of the 5′ product of target slicing. Results are shown for six independent experiments (circles, squares, triangles, diamonds, hexagons and exes) of reactions with either no protein (black), RISC–miR-20a only (red), RISC–miR-20a with Xrn1p mut. (blue), or RISC–miR-20a with Xrn1p WT (purple). The fraction of 5′ target fragment was calculated by dividing the signal of product + product + substrate. The lines indicate the best fit to the burst equation. (D) Experimental scheme of the assay for multiple-turnover slicing with a 5′-capped, 3′-radiolabeled target. The black-filled circle on the target indicates a 5′-cap, and the red A indicates a radiolabeled cordycepin at the 3′-end of the target. The initial concentration of target was 100 nM. (E) The effect of Xrn1p on multiple-turnover slicing in the assay schematized in D. The cross symbol (†) indicates degradation of the target after adding Xrn1p, which was observed at late time points and presumed to be due to a contaminant in the Xrn1p preparation. Otherwise, as in B. (F) Quantification of the 3′ product of target slicing. Results are shown for three independent experiments (circles, squares, triangles). The lines, shown for clarity, connect mean values and are not fit to an equation. Otherwise, as in C.
Figure 7. Schematic depicting the roles of Xrn1p in budding yeast RNAi. See main text for description.
DISCUSSION

Our results show that Xrn1p influences most steps of the RNAi pathway in budding yeast (Figure 7). Xrn1p increases the levels of siRNAs that accumulate from palindromic loci, presumably by reducing the amount of non-palindromic siRNA-precursor transcripts, thereby reducing competition for entry into the pathway from siRNAs that would otherwise be produced from these non-palindromic precursors. Once the siRNA duplexes are produced, Xrn1p facilitates their loading into Argonaute, perhaps by eliminating other RNA species that spursively bind to Argonaute and thereby inhibit duplex loading. After duplexes bind to Argonaute and passenger strands are cleaved, Xrn1p helps degrade the cleavage fragments, which prevents inhibition of RISC and might facilitate its maturation. Finally, Xrn1p facilitates multiple turnover of RISC by eliminating the 3′ products of slicing, thereby preventing product inhibition and perhaps helping to regenerate unbound RISC. We also detected a physical interaction between Ago1p and Xrn1p, which might play a role in one or more of these functions.

Even with these new-found roles of Xrn1p, the RNAi pathway of budding yeast appears to be the most streamlined of any characterized to date, with only three known components: Dcr1p, Ago1p and Xrn1p. Although we cannot rule out participation of other factors that might have been missed by our genetic and pulldown approaches, the notion that RNAi in budding yeast relies on only three proteins seems plausible. For example, the most notable difference between budding-yeast Ago1p and Argonaute proteins characterized from other lineages is that purified Ago1p can autonomously load an siRNA duplex and then cleave and remove the passenger strand of this duplex to form active RISC capable of slicing target RNA (66), whereas other Argonaute proteins require the Hsc70/Hsp90 chaperone and adenosine triphosphate to assume a conformation capable of duplex loading and RISC maturation (19–21, 23–24). That Ago1p can autonomously perform these functions in vitro, with some enhancement by Xrn1p, suggests that other factors might not be required for duplex loading and RISC maturation in budding yeast.

The unusual domain structure and activity of budding-yeast Dcr1p might also obviate the need for other accessory factors needed for siRNA loading into Argonaute in other systems. Budding-yeast Dcr1p possesses only one RNase III domain, whereas canonical Dicers possess two RNase III domains (67). Consistent with RNase III domains always functioning in pairs (68), budding-yeast Dicer forms a homodimer to cleave both strands of the dsRNA (67). Whereas canonical Dicers measure from the end of the dsRNA to determine the site of cleavage (69, 70), multiple Dcr1p homodimers bind cooperatively within the dsRNA, with the distance between active sites of adjacent homodimers determining the length of the siRNA duplexes (67). Each unit of the budding-yeast homodimer possesses two dsRNA-binding domains (dsRBDs), which are both required for accumulation of siRNAs to normal levels in vivo, although the second dsRBD is dispensable for producing siRNAs in vitro (67). One proposal is that in cells this second dsRBD acts after siRNA production, in tandem with the second dsRBD contributed by the other Dcr1p of the homodimer, to perform functions normally provided by dsRBD-containing Dicer accessory factors, such as helping to transfer the siRNA duplex from Dicer to Argonaute, thereby obviating the need for these cofactors (67).

Further supporting the existence of a streamlined pathway in budding yeast, N. castellii lacks orthologs of other factors that participate in RNAi and related pathways in other species. For example, N. castellii and all other RNAi-possessing budding-yeast species lack recognizable homologs of RdRPs (8). Budding yeast also lacks a discernible ortholog of HEN1, which methylates the 2′ oxygen of the 3′-terminal nucleotide of plant and metazoan siRNAs, plant miRNAs, as well as metazoan Pwi-interacting RNAs (piRNAs), thereby protecting these small RNAs from 3′-end uridylation and degradation (71–81). Indeed, we found that N. castellii siRNAs are susceptible to periodate oxidation and beta-elimination, thereby confirming that they are not methylated (Supplementary Figure S6A).

\emph{Naumovozyma castellii} does have orthologs of several other factors thought to participate in RNAi in other species, including the QIP exonuclease, which removes the cleaved passenger strand in \emph{Neurospora crassa} (27) and the autoantigen La RNA-binding protein, which is reported to enhance the multiple-turnover of RISC in Drosophila RNAi (82). However, disruption of the orthologs of the genes encoding QIP (GFD2 and its paralog, \emph{N. castellii} RNAi-reporter strain had little effect on \emph{GFP} silencing (Supplementary Figure S6B). Taken together, our results suggest that Xrn1p helps degrade the cleaved passenger strand and enhances the multiple turnover of RISC in budding yeast, thereby obviating the need for other proteins to perform these functions.

The general eukaryotic mRNA decay factors \emph{DCP2} and \emph{SK1} have also been implicated in small-RNA–mediated gene-silencing pathways. \emph{DCP2}, which encodes the mRNA decapping enzyme, is involved in miRNA-mediated silencing but not RNAi in Drosophila (83). \emph{SK1}, which encodes a component of the Ski complex, enables the cytoplasmic exosome to degrade 5′ target-slicing fragments in Drosophila (31). Although disrupting \emph{DCP2} and \emph{SK1} affected the abundances of siRNAs in the cell (Supplementary Figure S2A), we found that the deletions of these genes in an \emph{N. castellii} RNAi-reporter strain had little-to-no effect on \emph{GFP} silencing (Figure 1E). These results suggest that each protein is either not involved in budding-yeast RNAi or has effects on the pathway that are undetectable in the \emph{GFP}-silencing assay.

Xrn1p satisfies the expected criteria of a cofactor participating in the budding-yeast RNAi pathway. It is present in all known eukaryotes, playing important roles in RNA metabolism critical for cellular fitness, which explains why it was not lost in \emph{S. cerevisiae} and other species that have lost RNAi. Although not thought to be an essential component of RNAi in any species, Xrn1 has been shown to function in RNAi and related pathways in diverse species, and our observations in budding yeast expand this repertoire, both with respect to its evolutionary reach and with respect to its functions within a single species. Indeed, studies in other species have focused on the role of Xrn1 on a particular step
of the pathway, and thus it will be interesting to learn the degree to which Xrn1 has multiple functions in RNA-silencing pathways of other eukaryotes or whether these other lineages have evolved cofactors that assume these more specialized roles in more elaborated pathways.

**DATA AVAILABILITY**

The *N. castellii* whole-genome sequencing data are available at the NCBI Sequence Read Archive (SRA) database under accession number PRJNA601242. The small-RNA and RNA sequencing data are available at the NCBI Gene Expression Omnibus (GEO) under accession number GSE143548.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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**Author contributions**: M.A.G. and I.A.D. made the strains for the genetic selection. I.A.D. carried out the pilot genetic selection with the help from M.A.G., and M.A.G. performed the genetic selection with modified strains and analyzed the genomic sequencing data. D.E.W. made the tagged Ago1p constructs and carried out the immunoprecipitations and protein blots, the microscopy and RNA blots. M.A.G. purified *N. castellii* Ago1p and Xrn1p and did all of the biochemistry experiments and analyses. All authors contributed to experimental design. M.A.G. and D.P.B. wrote the manuscript, and all authors edited the manuscript.

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