Reconstitution of human RNA interference in budding yeast

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ABSTRACT

Although RNA-mediated interference (RNAi) is a widely conserved process among eukaryotes, including many fungi, it is absent from the budding yeast *Saccharomyces cerevisiae*. Three human proteins, Ago2, Dicer and TRBP, are sufficient for reconstituting the RISC complex *in vitro*. To examine whether the introduction of human RNAi genes can reconstitute RNAi in *S. cerevisiae*, genes encoding these three human proteins were introduced into *S. cerevisiae*. We observed both siRNA and siRNA- and RISC-dependent silencing of the target gene *GFP*. Thus, human Ago2, Dicer and TRBP can functionally reconstitute human RNAi in *S. cerevisiae*, *in vivo*, enabling the study and use of the human RNAi pathway in a facile genetic model organism.

INTRODUCTION

The RNA interference (RNAi) pathway is an evolutionarily conserved mechanism of gene regulation (1). Discovered as a biological response to double-stranded RNA (dsRNA) in the nematode *Caenorhabditis elegans* (2), the RNAi pathway has been shown to be present in many eukaryotes, ranging from fission yeast to human.

The yeast *Saccharomyces cerevisiae* is a 'model' model organism with an impressive collection of resources for expression and mutational studies, and dynamic molecular and cell-biological phenotyping. Although analogous functions have been uncovered in closely related species (3), the budding yeast *S. cerevisiae* completely lacks any analog to the RNAi pathway (4), suggesting that it may provide an ideal test-bed for the molecular genetic characterization of the exogenous human RNAi pathway.

Gene silencing via RNAi requires the RNA-induced silencing complex (RISC), a large ribonucleoprotein complex composed of three proteins, Argonaute-2 (Ago2), Dicer and HIV-1 transactivating response (TAR) RNA-binding protein (TRBP) (5-6). The RISC complex cleaves pre-miRNA and selectively loads a guide miRNA onto Ago2. The three purified proteins Ago2, Dicer and TRBP were previously shown to successfully reconstitute RISC *in vitro* without any cofactors or chaperones (7–10). Together, these results raise the possibility that the RNAi pathway could be established in *S. cerevisiae* to enable detailed molecular genetic studies.

MATERIALS AND METHODS

Plasmid construction

(i) Human Ago2, Dicer and TRBP expression plasmids. Human Ago2, Dicer and TRBP cDNAs (11) individually cloned into Advanced Gateway Destination Vectors (12) by Gateway recombination cloning (Invitrogen) to produce the galactose-inducible expression plasmids pAG413Gal-Ago2, pAG416Gal-Dicer and pAG415Gal-

TRBP, respectively. All plasmids are CEN-based and under the control of GAL1 promoter with different auxotrophic markers. All constructs were sequence-verified. (ii) Silencing antisense constructs. GFP (S65T) and Ade2 of antisense orientation were cloned into pAG424Gal to produce silencing constructs pAG424Gal-AS-GFP or pAG424Gal-AS-Ade2. These are 2-micron plasmids under GAL1 promoter. The Ade2 antisense construct was used as a negative control.

Yeast strains and methods

LPY3498 (*MATa his3Δ200 leu2-3,112 trp1Δ1 ura3-52 ESA1*) (13) was used to create a GFP-expressing RNAi reporter strain. The GFP(S65T)-KanMX6 module from pFA6a was integrated at the endogenous *TDH3* locus in LPY3498 to create a wild-type strain (*WT*) used in this study [*MATa his3Δ200 leu2-3,112 trp1Δ1 ura3-52 ESA1 TDH3::GFP(S65T)-KanMX6*]. The *WT* strain was sequentially transformed with three plasmids (described above), each bearing one of the human RISC-encoding genes, and selected using auxotrophic markers to generate the '*ADT*' strain. All yeast strains were grown at 30°C according to the standard protocol. Transformation was carried out as previously described (14).

Reverse transcription–PCR

Total RNA was isolated using the hot phenol method. Reverse transcription (RT) reactions were performed with total RNA and oligo(dT) primer using Superscript III according to the manufacturer's instructions (Invitrogen). PCR reactions were assembled in 50 μl with 2 μl RT reaction and the following gene-specific primers: Ago2 forward, 5′-AGCGCCAGTGCACGGAA GTC; Ago2 reverse, 5′-GGTGCCGGAACATGGGCT CC; Dicer forward, 5′-CCAGCTGTGGGGAGAGGG CT; Dicer reverse, 5′-TCCCGAGTAGGGCACAGG GC; TRBP forward, 5′-CGCAGTTGCTCCCTGGGC TC; TRBP reverse, 5′-GCCGGCTGGGTGGACAGT TC; ACT1 forward, 5′-CGTTCCAATTTACGCTGG TT; ACT1 reverse, 5′-AGTTTGGTCAATACCGGC AG. After 30 cycles, an aliquot was removed and analyzed by a 1.5% agarose gel electrophoresis. DNA was visualized by EtBr staining.

Northern blot analysis

Small RNA blots were performed using 10–15 μg total RNA per lane and carbodiimide-mediated cross-linking to the membrane (15), with the following DNA probes: 5′-TATGCAGGGGAACTGCTGAT radio-labeled at their 5′ termini for *S. cerevisiae* U6 small nuclear RNA (SNR6; GenBank Accession No. X12565); a full-length GFP cDNA probe radiolabeled by random priming for *GFP* siRNA.

Flow cytometry

Each strain was inoculated in prototrophic selection media with 2% raffinose and grown overnight. Fresh cultures were then seeded from the overnight cultures and cells were grown to log-phase with either 2%

glucose (non-inducing) or 2% galactose (inducing). Cells were analyzed using FACSCalibur (BD Biosciences) and data were processed with CellQuest Pro (BD Biosciences).

RESULTS AND DISCUSSION

In an attempt to reconstitute the human RNAi system in budding yeast, three human RNAi genes were introduced into *S. cerevisiae*, together with the appropriate silencing and reporter constructs. Human Ago2, Dicer and TRBP cDNAs were individually cloned and placed under the control of the inducible *GAL1* promoter using the Gateway recombination cloning system. For use in silencing, antisense *GFP* was cloned under the *GAL1* promoter; antisense *Ade2* was used as a negative control (Figure 1A). A GFP-expressing reporter strain was created by integrating the *GFP(S65T)-KanMX6* module into the endogenous *TDH3* locus (Figure 1B). The GFP-expressing reporter strain was sequentially transformed with plasmids bearing each of the three human RNAi gene constructs, and the expression of each gene under galactose-induction conditions was confirmed by RT–PCR (Figure 2A). Expression of RISC genes was not observed when the yeast strains were not induced in galactose. This Ago2/Dicer/TRBP (ADT) strain was transformed with plasmids bearing the antisense GFP silencing constructs, followed by northern blot detection of GFP siRNA (Figure 2B). Introduction of human Ago2, Dicer and TRBP was sufficient to generate GFP siRNA in *S. cerevisiae*. Strains expressing all possible pairwise combinations of two RISC components (Ago2/TRBP, Ago2/Dicer and Dicer/TRBP) were also constructed. The GFP siRNA biogenesis was observed in the strain lacking Ago2 and the strain lacking TRBP, but not in the strain lacking Dicer, indicating that Dicer is necessary for siRNA
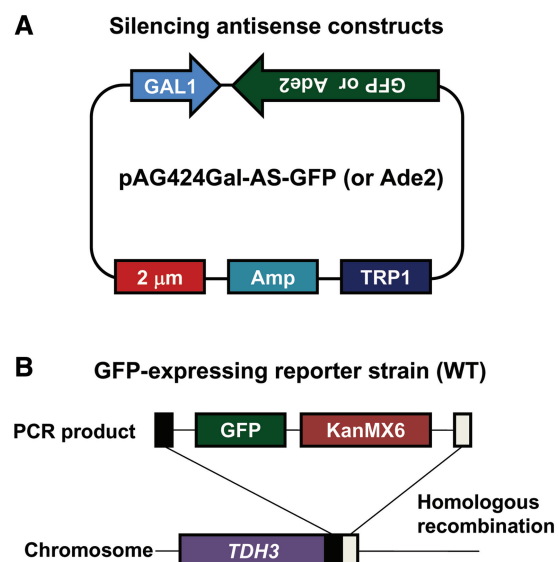

Figure 1. A schematic diagram showing the silencing antisense constructs and the GFP reporter strain. (**A**) GFP or Ade2 were each separately cloned under control of *GAL1* promoter in the antisense orientation to generate silencing antisense constructs. (**B**) GFP reporter strain was created by homologous recombination of GFP(S65T)-KanMX6 module into *TDH3* locus.
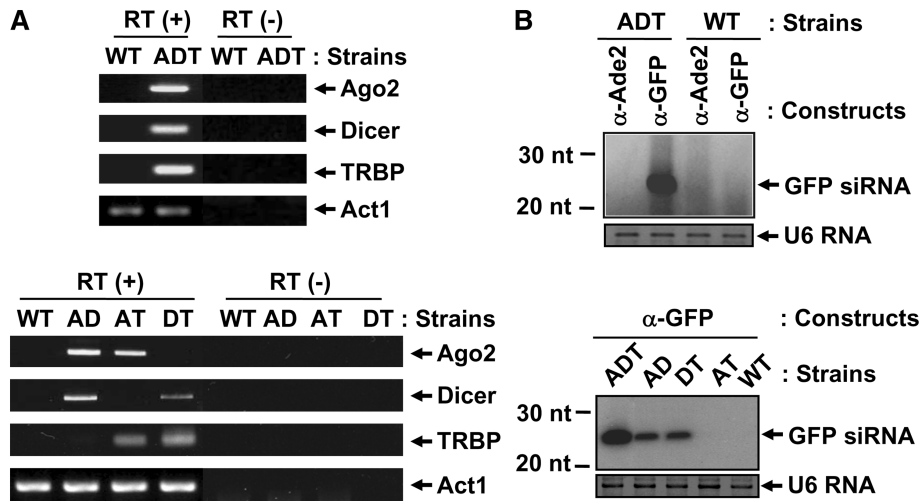
Figure 2. Expression of human RNAi genes and generation of siRNA in yeast. (**A**) Expression of human Ago2, Dicer and TRBP genes was assessed by RT–PCR in *S. cerevisiae* strains expressing either no human RNAi genes (WT) or in an isogenic strain carrying plasmid-borne copies of human Ago2/Dicer/TRBP genes (ADT) or pairwise combinations of the three human genes (AD, AT and DT). Act1 mRNA was detected to confirm the integrity of RNA samples. RT (−) indicates the reverse transcription reaction without reverse transcriptase. (**B**) Northern blots were performed to probe for siRNA antisense to *GFP* in galactose-induced *S. cerevisiae* strains expressing either no human RNAi genes (WT) or human Ago2/Dicer/TRBP genes (ADT), and either the negative-control antisense *ADE2* construct (α-Ade2) or antisense *GFP* construct (α-GFP). The blot was reprobed for U6 small nuclear RNA. GFP siRNA biogenesis was similarly determined by northern blot in the strains expressing pairwise combinations of the three human genes (AD, AT and DT).
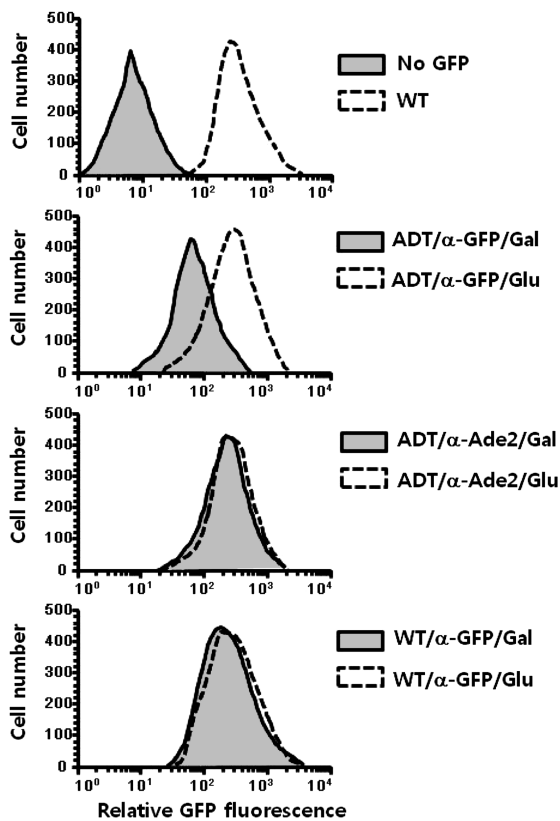

Figure 3. Gene silencing by human RNAi in yeast. GFP gene silencing was determined by flow cytometric analysis. Histograms show GFP fluorescence in the indicated *S. cerevisiae* strains (WT or ADT) expressing the indicated silencing constructs (α-*ADE2* or α-*GFP*) under either non-inducing (Glu) or inducing (Gal) condition. 'No GFP', strain LPY3498; 'WT', LPY3498 expressing GFP; 'ADT', WT with human Ago2/Dicer/TRBP; 'α-GFP', antisense *GFP* construct; 'α-Ade2', antisense *ADE2* construct.

biogenesis and that Ago2 and TRBP are not (Figure 2B). Flow cytometric analysis was next performed to assess whether the introduced human RNAi genes are capable of silencing GFP gene expression. When the ADT strain expressing antisense-GFP silencing construct was induced with galactose, a significant decrease in the GFP fluorescence intensity was observed, indicating that human RNAi system was successfully reconstituted in *S. cerevisiae* (Figure 3). Silencing effects were not observed either under the uninduced condition or when an antisense-*ADE2* construct was used in place of the antisense-*GFP* construct. Strains expressing all possible pairwise combinations of two RISC components (Ago2/TRBP, Ago2/Dicer and Dicer/TRBP) did not exhibit silencing effects (Table 1). Taken together, our results show that the three human genes are necessary and sufficient for reconstitution of the human RNAi system in *S. cerevisiae*.

In this study, we used antisense GFP as a silencing construct. Although hairpin sequences can also be used for this purpose, the GFP hairpin RNA transcribed under the *GAL1* promoter may lack a structure that is required for Dicer-mediated processing (16–18). Although RNA polymerase III promoters are often used for the transcription of shRNAs in mammalian cells (19–21), the yeast U6 RNA promoter is ill-defined (22). Antisense GFP RNA should hybridize with the endogenous GFP transcript, and thus generate substrate double-stranded RNA for Dicer-mediated cleavage. Indeed, GFP siRNA was detected by northern blot (Figure 2) as was target gene silencing (Figure 3). The silencing effect of antisense GFP alone (without the RNAi genes) was negligible (Figure 3, bottom).

Recently, Drinnenberg *et al.* (3) reported the reconstitution of RNAi in *S. cerevisiae* by introducing Dicer and

Table 1. Comparison of silencing effects of different combination of RISC components

RISC components	Glucose condition	Galactose condition
Ago2/Dicer/TRBP	455.2 ± 3.5	91.8 ± 0.8
Ago2/TRBP	430.1 ± 3.9	422.7 ± 4.7
Ago2/Dicer	389.6 ± 2.6	392.0 ± 3.6
Dicer/TRBP	456.5 ± 4.6	446.3 ± 4.1

The GFP-expressing *S. cerevisiae* strain was transformed with the indicated combination of RISC components and the GFP-silencing constructs. GFP gene silencing was determined by flow cytometric analysis under either non-inducing (glucose) or inducing (galactose) condition. Values indicate the mean fluorescence intensity $\pm$ SEM.

Argonaute of *Saccharomyces castellii*. They identified a novel class of Dicer protein present in *S. castellii* which—unlike other known Dicer genes in *Schizosaccharomyces pombe*, plants and animals—has two double-stranded RNA-binding domains (dsRBDs) but only a single RNaseIII domain and no helicase or PAZ domains. It has been proposed that *S. castellii* Dicer may act as a homodimer and may not require additional dsRBDs. They also showed that the two genes Dicer and Argonaute of *S. castellii* were sufficient to reconstitute the functional RNAi in *S. cerevisiae* (3). Here we showed that the introduction of the two human genes Dicer and Ago2 did not enable RNAi in *S. cerevisiae* without the concurrent presence of TRBP. The requirement for TRBP indicates a distinct domain and subunit architecture of RISC in human versus budding yeast. The reconstituted human RNAi system could facilitate functional dissection of human RNAi proteins and domains such as the helicase domain of Dicer and potential phosphorylation sites of TRBP.

In summary, we have shown that three human RNAi genes Ago2, Dicer and TRBP are sufficient to establish the human RNAi process in *S. cerevisiae*. The reconstituted human RNAi system in *S. cerevisiae* offers the potential for thorough molecular–genetic study of the regulation of human RNAi in an experimentally facile model eukaryote.

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