Roles of mRNA Fate Modulators Dhh1 and Pat1 in TNRC6-dependent Gene Silencing Recapitulated in Yeast*

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Background: Animal microRNAs silence their target mRNAs by promoting mRNA degradation and inhibiting translation via GW182/TNRC6.

Results: TNRC6 induces silencing effects in S. cerevisiae via CCR4-NOT complex and Dhh1-Pat1 when tethered to reporter mRNAs.

Conclusion: TNRC6 utilizes the conserved mRNA fate modulators for gene silencing in yeast.

Significance: Yeast genetic tools are now available to study intricate actions of TNRC6.

The CCR4-NOT complex, the major deadenylase in eukaryotes, plays crucial roles in gene expression at the levels of transcription, mRNA decay, and protein degradation. GW182/TNRC6 proteins, which are core components of the microRNA-induced silencing complex in animals, stimulate deadenylation and repress translation via recruitment of the CCR4-NOT complex. Here we report a heterologous experimental system that recapitulates the recruitment of CCR4-NOT complex by TNRC6 in S. cerevisiae. Using this system, we characterize conserved functions of the CCR4-NOT complex. The complex stimulates degradation of mRNA from the 5’ end by Xrn1, in a manner independent of both translation and deadenylation. This degradation pathway is probably conserved in mRNA-mediated gene silencing in zebrafish. Furthermore, the mRNA fate modulators Dhh1 and Pat1 redundantly stimulate mRNA decay, but both factors are required for poly(A) tail-independent translation repression by tethered TNRC6A. Our tethering-based reconstitution system reveals that the conserved architecture of Not1/CNOT1 provides a binding surface for TNRC6C, thereby connecting microRNA-induced silencing complex to the decapping machinery as well as the translation apparatus.

The CCR4-NOT complex is a multisubunit complex involved in many aspects of mRNA metabolism (1–3). Its conserved functions include deadenylase catalyzed by the two deadenylase subunits CAF1/POB (CNOT7/8 in vertebrates) and CCR4 (CNOT6 in vertebrates). These two enzymes are incorporated into the complex via a direct interaction between CAF1 and the scaffold protein CNOT1 (4, 5). Thus, recruitment of the CCR4-NOT complex to mRNAs promotes deadenylation, which is usually followed by decapping and 5’-to-3’ degradation by Xrn1 (6, 7). In addition, recent studies have shown that the CCR4-NOT complex provides a link to the decapping machinery. For example, in Saccharomyces cerevisiae, the CCR4-NOT complex associates with Dhh1, a decapping activator (8). Similarly, in Drosophila and mammals, the CCR4-NOT complex interacts with the Dhh1 homolog Me31B/DDX6/RC1/k54 and the Pat1 homolog HPat/PatL1 (9–12). HPat/PatL1 in turn associates with the decapping enzyme Dcp2 and its activators Dcp1 and Edc3, thereby organizing assembly of the decapping machinery (9, 13, 14). Moreover, Dhh1 and Pat1 also function in translation repression. In S. cerevisiae, Dhh1 or Pat1 is required for translation repression under glucose deprivation, and both Dhh1 and Pat1 repress translation initiation in vitro (15). Drosophila Me31B and vertebrate DDX6 also act as translation repressors (11, 12, 16–19). These observations imply that the CCR4-NOT complex coordinates multiple processes of mRNA degradation and translation repression rather than merely promoting deadenylation.

MicroRNAs (miRNAs)4 are small non-coding RNAs that negatively regulate gene expression by inducing translational repression, mRNA degradation, and deadenylation (20–29). miRNAs regulate their target mRNAs by associating with specific protein factors to form the miRNA-induced silencing complex (miRISC). Argonaute (Ago), a core component of miRISC, directly incorporates miRNAs (30). Drosophila GW182 and its vertebrate ortholog TNRC6A-C (trinucleotide repeat-containing 6 A-C) interact with Ago via their N-terminal glycine and tryptophan (GW) repeats, whereas their C-terminal silencing domains provide a platform for interactions with RNA reg

4 The abbreviations used are: miRNA, microRNA; DIG, digoxigenin; qRT-PCR and qPCR, quantitative RT-PCR and PCR, respectively; MO, morpholino oligomer; miRISC, miRNA-induced silencing complex; PABP, poly(A)-binding protein; cRACE, circularized rapid amplification of cDNA ends; DN, dominant negative.
miRNAs. Previously, the Bartel and Roth laboratories (46, 47) reported that the CCR4-NOT complex is involved in sequence-specific post-transcriptional regulation independent of the emergence of miRNAs. However, the basic machinery for controlling mRNA stability and translation, including the CCR4-NOT complex, decapping factors, and translation initiation factors, is highly conserved (6, 7). Notably, the yeast Pumilio-like protein Puf5/Mpt5 binds to the CCR4-NOT complex to silence and deadenylate specific mRNAs (44, 45), suggesting that the CCR4-NOT complex is involved in sequence-specific post-transcriptional regulation independent of the emergence of miRNAs. Previously, the Bartel and Roth laboratories (46, 47) showed that gene silencing by siRNA could be reconstituted in *S. cerevisiae* by expressing either Saccharomyces castellii Ago1 and Dicer1 or human Ago2, Dicer, and TRBP. Do not hallucinate.}

### mRNA Fate Modulators in TNRC6-dependent Gene Silencing

**TABLE 1**

| Strains/Plasmids | Genotype/plasmid | Source |
|------------------|------------------|--------|
| S. cerevisiae    |                  |        |
| YIT2007          |                  |        |
| YIT2013          |                  |        |
| YIT2030          |                  |        |
| YIT2031          |                  |        |
| YIT2032          |                  |        |
| YIT2033          |                  |        |
| YIT2034          |                  |        |

**Plasmids**

| pIT2068          | pGAL1p-AUG-FLAG-MPT4ΔN-PGK1 (3’ UTR)-URA3 CEN | Ref. 49 |
|------------------|-----------------------------------------------|--------|
| pIT2069          | pGAL1p-AUG-FLAG-MPT4ΔN-PGK1 (3’ UTR)-MS2 URA3 CEN | Ref. 49 |
| pIT2070          | pGAL1p-No-AUG-FLAG-MPT4ΔN-PGK1 (3’ UTR)-URA3 CEN | Ref. 49 |
| pIT2071          | pGAL1p-No-AUG-FLAG-MPT4ΔN-PGK1 (3’ UTR)-MS2 URA3 CEN | Ref. 49 |
| pIT2082          | pGAL1p-No-AUG-FLAG-MPT4ΔN-PGK1 (3’ UTR)-MS2 Rs URA3 CEN | Ref. 49 |
| pIT2083          | pGAL1p-AUG-FLAG-MPT4ΔN-PGK1 (3’ UTR)-MS2 Rs URA3 CEN | Ref. 49 |
| pIT2139          | pGAL1p-AUG-FLAG-MPT4ΔN-PGK1 (3’ UTR)-MS2 Rs URA3 CEN | Ref. 49 |
| pIT2140          | pGAL1p-AUG-FLAG-MPT4ΔN-PGK1 (3’ UTR)-Rs URA3 CEN | Ref. 49 |
| pIT2141          | pTEF1p-FLAG-MS2 LEU2 CEN                       |        |
| pIT2142          | pTEF1p-FLAG-MS2 eTNR66Amid LEU2 CEN             |        |
| pIT2143          | pGPD1p-Dhh1-HA URA3 CEN                        |        |
| pIT2144          | pGPD1p-Dhh1-LEU2 CEN                           |        |
| pIT2145          | pGPD1p-No-AUG-FLAG-MPT4ΔN-PGK1 (3’ UTR)-URA3 CEN | Ref. 49 |
| pIT2146          | pGPD1p-No-AUG-FLAG-MPT4ΔN-PGK1 (3’ UTR)-MS2 URA3 CEN | Ref. 49 |
| pIT2147          | pGPD1p-Dhh1-FLAG-LEU2 CEN                      |        |
| pIT2148          | pGPD1p-Dhh1-LEU2 CEN                           |        |
| pIT2149          | pGPD1p-GFP-MS2 URA3 CEN                        |        |
| pIT2150          | pGPD1p-GFP-MS2 Rs URA3 CEN                     |        |
| pIT2151          | pGPD1p-GFP-URA3 CEN                            |        |
| pIT2152          | pGPD1p-GFP-Rs URA3 CEN                         |        |
| pIT2155          | pTEF1p-FLAG-MS2 zTNR66Amid mutants QSR and W LEU2 CEN | Ref. 49 |
| M44              | pC2 + HA-AN-zTNR66A-Mid globin3 UTR             | Ref. 40 |
| M337             | pC2 + HA-AN-M2-globin 3’ UTR                   |        |
| M338             | pC2 + MT-CNOT7-ΔD4A4E42E67E7L1E-sv40            |        |
| M342             | pC2 + MT-DCP2-E147A-E148A-sv40                 |        |

**Strains and Other Methods**—Yeast strains and plasmids are listed in Table 1. Information about the oligonucleotides used for poly(A) tail analysis and qRT-PCR are listed in Table 2. Polysome analysis was performed as described (48). Do not hallucinate.
ing 2% glucose to inhibit transcription from the GAL1 promoter. At the times indicated, the cells were harvested to prepare RNA samples using hot phenol. The mRNA levels of reporter genes were determined by Northern blotting using digoxigenin (DIG) reagents. Non-radioactive probes were prepared by PCR-based nucleic acid labeling using commercial kits. Hybridization probes were detected according to the procedure specified by the manufacturer (Roche Applied Science). The DIG-labeled probes were prepared with the following oligonucleotides: GFP (5'-GGTATTACACTACATTACATGGAGCCCTCCCTTGCAGGACGCGTCGCTCTTCTC-3') and 3-phosphoglycerate kinase 1 (PGK1) 3'-UTR (5'-GGTTACATTGCAATGACAGAATCGGCTCTTGGAATCCACGGGACCCACAT-3').

**Western Blotting**—Yeast cells were grown in minimal medium containing 2% galactose. When the culture reached an A600 of 0.6, the cells were harvested. The protein products of FLAG-tagged reporter genes were detected by Western blotting using an anti-FLAG antibody (F1804, Sigma) and a horseradish peroxidase-conjugated secondary antibody (GE Healthcare). The intensity of the bands on the blots was quantified using the LAS4000 and Multi-Gauge version 3.0 (Fuji Film). Relative RNA levels were determined by comparison with a standard curve prepared with a series of dilutions of time 0 samples (just before the addition of glucose).

**Immunoprecipitation**—Yeast cells were grown in minimal medium containing 2% glucose to inhibit transcription from the GAL1 promoter. At the times indicated, the cells were harvested to prepare RNA samples using hot phenol. The mRNA levels of reporter genes were determined by Northern blotting using digoxigenin (DIG) reagents. Non-radioactive probes were prepared by PCR-based nucleic acid labeling using commercial kits. Hybridization probes were detected according to the procedure specified by the manufacturer (Roche Applied Science). The DIG-labeled probes were prepared with the following oligonucleotides: GFP (5'-GGTATTACACTACATTACATGGAGCCCTCCCTTGCAGGACGCGTCGCTCTTCTC-3') and 3-phosphoglycerate kinase 1 (PGK1) 3'-UTR (5'-GGTTACATTGCAATGACAGAATCGGCTCTTGGAATCCACGGGACCCACAT-3').

**Pulse-labeling Experiments**—Yeast cells were grown exponentially at 30 °C in minimal media lacking methionine and cysteine. A 10-ml aliquot of yeast cells was labeled with 100 μCi of [35S]methionine and cysteine (NEG072, PerkinElmer Life Sciences) for 10 min. This was followed by the addition of cold amino acids, to a final concentration of 40 mg/ml. The cells were then collected, and cell extracts were prepared using Y-PER (yeast protein extraction reagent) (Thermo Scientific). Cell extracts were incubated with a GFP antibody (Santa Cruz Biotechnology, Inc.) and protein G-agarose (Roche Applied Science) in IXA-100 buffer (48). The antibody-bound agarose was then washed three times. Immunoprecipitated samples were separated by SDS-polyacrylamide gel electrophoresis. The radioactivity of the precipitated proteins was measured using a Typhoon FLA 9500 imager (GE Healthcare).

**Plasmid Construction**—A reporter plasmid, pGAL1p-FLAG-MPR4-pGKI (3'-UTR) was described previously (49). To construct pIT2149-2152, MPR4 was replaced by GFP ORF using XbaI and BamHI sites in pGAL1p-FLAG-MPR4-pGKI (3'-UTR).

To construct pIT2141 (pTEF1p-FLAG-MS2), a DNA fragment encoding FLAG-MS2 coat protein was amplified by PCR from pTEF1p-FLAG-MS2 (49) using the primers OIT1822 (5'-TGATGTCTAGATGAATTGAATTG-3') and inserted between XbaI and BamHI sites in pGAL1p-FLAG-MPR4-pGKI (3'-UTR).
between BamHI and XhoI sites in pTEF1p-FLAG-MS2. To construct pIT2155 (pTEF1p-FLAG-MS2-zebrafish TNRC6A Mid mutants QSR and W, which contains mutations that disrupt the interaction between CNOT1 and GW182/TNRC6 in human and Drosophila cells (33, 34), a DNA fragment encoding the zebrafish TNRC6A Mid domain in which Glu-Ser-Arg and Trp residues were substituted to alanines (Gln-1343, Ser-1344, Arg-1345, Trp-1349, Trp-1419, Trp-1429, Trp-1469, Trp-1512, Trp-1527, Trp-1555, Trp-1569) was chemically synthesized and cloned between BamHI and XhoI sites in pTEF1p-FLAG-MS2. To construct pIT2146 (pTEF1p-FLAG-MS2-dmGW182 Mid), a DNA fragment encoding the mid domain (residues 861–1116) of dmGW182 was amplified by PCR from pAc5.1b-lambdaN-HA-DmGW182 (51) using the primers OIT2469 (5′-TATAGTGCCGGCAAGGCTGCACCTC-AACATCCCGATTA-3′) and OIT2443 (5′-TTGACGTCGACTTTAGTTCTGATATTTCACCATCG-3′) and inserted between Smal and XhoI (compatible end of Sall) sites in pTEF1p-FLAG-MS2 in which an MS2 tag was inserted between Xbai and SalI sites. To construct pIT2143 (pGPDp-DHH1-HA), pIT2147 (pGPDp-DHH1-FLAG), and pIT2148 (pGPDp-DHH1), DHH1 ORF was amplified from the S. cerevisiae genome using the forward primer OIT2398 (5′-TAGATGGATCATCTTGGAGTGGTGTTTGGCAC-GGTGGAAGTACATTTTC-3′), the reverse primer) and incubation temperature (44 °C). PCR was performed using GoTag (Promega) and the primers listed below for 30–32 cycles. PCR products were separated on 6% polyacrylamide gel with 0.5× TBE, stained with GelRed (WAKO), and visualized with a LAS3000 imager (GE Healthcare). PCR products were cloned into pCRII TOPO (Invitrogen), and sequences were confirmed.

Overexpression of Dominant Negative CNOT7 and DCP2 and Inhibition of miR-430—ORFs of zebrafish cnot7 (ENSDART00000092953) and dcp2 (ENSDART00000056951) were amplified by RT-PCR and cloned into pCS2+MT vector. The following mutations were introduced to make a dominant-negative form of each protein: CNOT7, D409A/E424A (catalytic core) and C67E/L71E (CNOT6 interaction surface) and CNOT7, D409A/E424A (catalytic core) and C67E/L71E (CNOT6 interaction surface); DCP2, G41E/L433E (WAKO), and visualized with a LAS3000 imager (GE Healthcare). PCR products were cloned into pCRII TOPO (Invitrogen), and sequences were confirmed.

Circularized Rapid Amplification of cDNA Ends (cRACE)—cRACE was performed as described previously (40) (using the primers in Table 2). Specific amplification was confirmed by analyzing the dissociation curve and sequencing. For the analysis of endogenous miR-430 targets, values in each injection experiment were normalized by the value of actb1. The normalized value in the miR-430 MO injection experiment was set to 1. Experiments were repeated three times.
Tethered TNRC6A Recapitulates Hallmarks of miRNA-mediated Gene Silencing in S. cerevisiae—To elucidate the roles of the CCR4-NOT complex, which is recruited by GW182/TNRC6 in both mRNA decay and translation repression, we attempted to recapitulate the gene silencing activities of GW182/TNRC6 in yeast. First, we examined the interaction of the carboxyl-terminal region of zebrafish TNRC6A (Mid domain; amino acids 1310–1567) (40), which was sufficient for translation repression and deadenylation in zebrafish embryos, with the yeast CCR4-NOT complex (Fig. 1, A and B). HA-tagged Not1 and Ccr4 proteins, subunits of the yeast CCR4-NOT complex, co-immunoprecipitated with FLAG-tagged MS2 protein fused to the Mid domain (F-MS2-Mid) (Fig. 1, B and C, lanes 4 and 5). Introduction of alanine mutations in the CIM1 and W-motifs that disrupted the interaction between CNOT1 and GW182/TNRC6 in human and Drosophila cells (33, 34) (F-MS2-Mid-M) weakened the interaction of the zebrafish Mid domain with Not1 and Ccr4 (Fig. 1, B and C, lanes 5 and 6), although the expression levels of these proteins were comparable (Fig. 1D). We next examined the effects of F-MS2-Mid on the levels of mRNA and protein expressed from GFP reporter genes containing MS2 binding sites. To determine whether a poly(A) tail was required, we inserted the sequence of a hammerhead ribozyme (Rz) downstream of the MS2 binding sites (Fig. 1E) (49). Tethering of F-MS2-Mid reduced the levels of GFP-MS2 and GFP-MS2-Rz reporter mRNAs (Fig. 1F, lanes 3 and 4 and lanes 7 and 8), whereas tethering of F-MS2-Mid did not (Fig. 1F, lanes 7 and 8 and lanes 11 and 12). Likewise, tethering of F-MS2-Mid reduced the protein levels derived from GFP-MS2 or GFP-MS2-Rz reporters, whereas tethering of F-MS2-Mid-M did not (Fig. 1G, lanes 7 and 8 and lanes 11 and 12). Polysome analysis using sucrose density gradients revealed that levels of GFP-MS2 and GFP-MS2-Rz mRNAs reduced in the polysome fraction but increased in the 80S monosome and free fractions in cells expressing the F-MS2-Mid protein relative to cells expressing the control MS2 protein (Fig. 1H, Student’s t test, p < 0.05). To directly examine translation repression by the tethered Mid domain of zebrafish TNRC6, we performed pulse-labeling experiments. Levels of synthesized GFP derived from reporter mRNAs were significantly decreased by tethered TNRC6A (Fig. 1I). The corresponding region of Drosophila GW182 also induced gene silencing in yeast (data not shown). These results indicate that tethered TNRC6 protein fragments reduced both translation efficiency and mRNA levels by recruiting the CCR4-NOT complex in S. cerevisiae in a poly(A) tail-independent manner.

Tethered TNRC6 Stimulates the Degradation of mRNA from the 5’ End, Independent of Translation and a Poly(A) Tail—We next examined the effects of F-MS2-Mid on mRNA stability, using reporter genes containing an ORF of N-terminally truncated MPT4 (473 nucleotides) and MS2 binding sites, as described previously (Fig. 2A) (49). Consistent with the results obtained using GFP reporter mRNAs (Fig. 1F), half-life analysis revealed that tethered Mid domain dramatically stimulated mRNA degradation in a poly(A) tail-independent manner (Fig. 2B, wild type). Degradation was dependent on the 5′–3′ exonuclease Xrn1 but not the 3′–5′ exosome component Ski2, indicating that degradation occurred from the 5′ end (Fig. 2B). To dissect the effects of the tethered Mid domain on translation, deadenylation, and mRNA decay, we utilized previously constructed reporters containing MS2 binding sites. These constructs were of two types: the No-AUG-MS2 reporters are not translated because all AUG codons in the ORF of N-terminally truncated MPT4 were replaced with UAC codons (Fig. 2A), and the Rz reporters lack a poly(A) tail (Fig. 2A) (49). The tethering of F-MS2-Mid reduced the levels of both No-AUG-MS2 and No-AUG-MS2-Rz reporter mRNAs, and this down-regulation was mediated by the CCR4-NOT complex (Fig. 2C). Half-life analysis revealed that tethering of the Mid domain accelerated the decay rate of No-AUG-MS2-Rz mRNA via an interaction with the CCR4-NOT complex (Fig. 2D, t1/2 = 3.9 min, F-MS2-Mid versus t1/2 = 8.1 min, F-MS2-Mid-M). Destabilization of No-AUG-MS2-Rz mRNA by F-MS2-Mid was significantly suppressed in xrn1Δ mutant cells (Fig. 2D, t1/2 > 16 min) but not in ski2Δ mutant cells (Fig. 2D, t1/2 = 4.1 min), further confirming that the Mid domain stimulates 5′-to-3′ mRNA decay. The Rz-No-AUG-MS2-Rz mRNA, which lacks a cap structure and is therefore intrinsically unstable, was not further destabilized by the tethering of F-MS2-Mid (Fig. 2E). Overall, these experiments suggest that in yeast, tethering of the Mid domain of zebrafish TNRC6A stimulates 5′-to-3′ degradation of the reporter mRNA independent of translation and a poly(A) tail.

To investigate whether the decapping reaction would be accelerated by the Mid domain of zebrafish TNRC6A, we measured the levels of the decapped reporter mRNA (Fig. 3A). To this end, we utilized xrn1Δ mutant cells in which decapped mRNAs are stabilized and consequently accumulate. By treating purified RNA with Terminator exonuclease, which specifically degrades 5′-monophosphorylated RNA, the levels of decapped mRNAs could be estimated (54). In the control tethering experiment with F-MS2, the levels of No-AUG-MS2-Rz mRNA was reduced to 59% by Terminator treatment, revealing that the decapped fraction was 41% (Fig. 3B, lanes 1 and 2; see also Fig. 3C for quantitation, Student’s t test, p < 0.05). By contrast, Terminator exonuclease reduced the reporter mRNA tethered by F-MS2-Mid to 21%, indicating that ~80% of the reporter mRNA was decapped by the Mid domain (Fig. 3B, lanes 3 and 4; see also Fig. 3C for quantitation, Student’s t test, p < 0.05).
mRNA Fate Modulators in TNRC6-dependent Gene Silencing

A

zebrafish TNRC6A

Ago-binding domain

silencing domain

Full

GW-repeats

U6A
gGN

CM1-OSR

PAM2

PCL

RPM

1760

1310

1567

Mid

B

anti-HA

Not1-HA

F-MS2-Mid or Mid-M

anti-FLAG

F-MS2

Input

IP(FLAG)

D

anti-FLAG

anti-eEF1α

E

GFP

cap

aaaAn

MS2 sites

poly(A) tail

GFP-MS2

cap

aaaAn

Rz

GFP-MS2-Rz

cap

aaaAn

Rz

MS2 sites

F

F-MS2

F-MS2-Mid

F-MS2-Mid-M

MS2 site

Rz

relative mRNA levels (%)

SCR

G

F-MS2

F-MS2-Mid

F-MS2-Mid-M

MS2 site

Rz

relative protein levels (%)

eEF1α

H

GFP-MS2

GFP-MS2-Rz

80S / monosome

effect protein

80S / monosome

free

80S

polysome

p < 0.05

p < 0.05
mRNA Fate Modulators in TNRC6-dependent Gene Silencing

Tethered TNRC6A recapitulates hallmarks of miRNA-mediated gene silencing in S. cerevisiae. A, schematic structures of zebrafish TNRC6A and its Mid domain. B and C, interaction of FLAG-M2-TNRC6A Mid with yeast Not1-HA and Ccr4-HA. Cell lysates of wild type cells transformed with the indicated plasmids were immunoprecipitated using anti-FLAG antibody. Total extracts (Input) and immunoprecipitates (IP) were analyzed by Western blotting using anti-FLAG antibody. D, the expression of the Mid domain of zebrafish TNRC6A in yeast. FLAG-tagged effector proteins were analyzed by Western blotting using anti-FLAG antibody. F-MS2-M2, FLAG-M2 protein; F-MS2-Mid, FLAG-M2-TNRC6A Mid fusion protein; F-MS2-Mid-M, FLAG-M2-TNRC6Amid QSR and W mutant fusion protein. This mutant contains the mutations that disrupt the interaction of Not1 with TNRC6. E, schematic drawing of reporter genes used in Fig. 1. The filled box indicates the open reading frame. All reporter genes contain the 3'-UTR region of PGK1, in which two tandem M2 binding sites were inserted. R2, a hammerhead ribozyme that generates a 3’-end with no poly(A) tail. F, tethering of TNRC6A reduces mRNA levels independently of a poly(A) tail. RNA samples from wild type cells transformed with the indicated plasmids were analyzed by Northern blotting using anti-GFP antibody. eIF4F was served as a loading control. The data represent the means of three independent experiments, with S.D. values. G, tethering of TNRC6Amid reduces protein levels independently of a poly(A) tail. Wild type cells harboring the indicated plasmids were grown, and protein samples were analyzed by Western blotting using anti-GFP antibody. eIF4F was served as a loading control. The data represent the means of three independent experiments with S.D. values. H, tethering of TNRC6A induces translation repression, dependent on interaction with Not1 but independent of a poly(A) tail. Cell extracts were prepared from wild type cells transformed with the indicated plasmids, and polysome analysis was performed. Top, ratio (%) of mRNA distribution in each fraction. Bottom, reporter mRNA was detected by Northern blotting using a GFP probe. The data represent the means of three independent experiments with S.D. values.
mRNA Fate Modulators in TNRC6-dependent Gene Silencing

A

| mRNA Type | CAP  | Translation | Poly(A) Tail |
|-----------|------|-------------|--------------|
| AUG-MS2   | cap  | +           | +            |
| AUG-MS2-Rz|      | +           | -            |
| No-AUG-MS2|      | -           | +            |
| No-AUG-MS2-Rz|  | -           | -            |
| Rz-No-AUG-MS2-Rz| | -           | -            |

B

| mRNA Type | F-MS2 | Half Life (min) | F-MS2-Mid | Half Life (min) | F-MS2-Mid-M | Half Life (min) |
|-----------|-------|----------------|-----------|----------------|-------------|----------------|
| AUG-MS2   |       | 10.6±0.9       |           | 4.1±0.1        |             |                |
| skir2Δ    |       | 11.5±1.3       |           | 5.4±1.7        |             |                |
| xrn1Δ     |       | >16            |           | >16            |             |                |
| AUG-MS2-Rz|       | 14.0±0.7       |           | 3.8±0.3        |             |                |
| skir2Δ    |       | >16            |           | 3.6±0.2        |             |                |
| xrn1Δ     |       | >16            |           | >16            |             |                |

C

| No-AUG mRNA | F-MS2 | F-MS2-Mid | F-MS2-Mid-M | MS2 site | Rz   |
|-------------|-------|-----------|-------------|----------|------|
|             | -     | +         | -           | MPT4     |      |
|             | 100   | 99        | 128         | 83       | ±14  |
|             | ±5    | ±10       | ±6          | ±5       | ±10  |

D

| mRNA Type | F-MS2  | Half Life (min) | F-MS2-Mid | Half Life (min) | F-MS2-Mid-M | Half Life (min) |
|-----------|--------|----------------|-----------|----------------|-------------|----------------|
| AUG-MS2   |        | 6.5±0.5       |           | 3.9±0.7        |             | 8.1±0.7        |
| skir2Δ    |        | 8.0±0.3       |           | 4.1±0.4        |             |                |
| xrn1Δ     |        | >16           |           | >16            |             |                |

E

| mRNA Type | F-MS2  | Half Life (min) | F-MS2-Mid | Half Life (min) |
|-----------|--------|----------------|-----------|-----------------|
| AUG-MS2   |        | 2.8±0.7       |           | 2.3±0.4         |
| skir2Δ    |        | 3.6±0.3       |           | 3.6±0.2         |
| xrn1Δ     |        | >16           |           | >16             |
Tethered TNRC6A stimulates decapping and the degradation of mRNA from the 5' end, independent of translation and a poly(A) tail. 

**A**, schematic drawing of reporter genes used in Fig. 2. The line represents non-translated regions. All reporter genes contain the 3' UTR region of PGK1, in which two tandem MS2 binding sites were inserted. 

**B**, tethering of TNRC6A stimulates 5'-to-3' mRNA decay independent of a poly(A) tail. Wild type, ski2Δ, and xrn1Δ cells containing the indicated reporter gene and plasmids were grown, RNA samples were purified at each time point, and the reporter mRNA was analyzed by Northern blotting using a DIG-labeled PGK1 3'-UTR probe. 

**C**, TNRC6A reduces mRNA levels independently of translation and a poly(A) tail. Wild type cells harboring the indicated No-AUG reporter genes were grown, and RNA samples were analyzed by Northern blotting using a DIG-labeled MPT4 probe. The data represent the means of three independent experiments with S.D. values. 

**D** and **E**, tethering of the Mid domain of TNRC6A promotes 5'-to-3' mRNA decay independent of translation and a poly(A) tail in a cap-dependent manner. Wild type, ski2Δ, and xrn1Δ cells harboring No-AUG-MS2-Rz (**D**) and Rz-No-AUG-MS2-Rz (**E**) reporter gene and plasmids were grown, and RNA samples were analyzed by Northern blotting using a DIG-labeled PGK1 3'-UTR probe. The half-lives of mRNA in the indicated cells are shown as the mean values of three independent experiments with S.D. values.

**FIGURE 3.** Tethered TNRC6A stimulates the decapping reaction independent of translation and a poly(A) tail. 

**A**, schematic of the experimental procedure. 

**B** and **D**, TNRC6A promotes the decapping reaction, independent of translation and a poly(A) tail. RNA samples were prepared from wild type and xrn1Δ cells harboring the No-AUG-MS2-Rz reporter gene and then incubated in the absence or presence of Terminator exonuclease and tobacco acid pyrophosphatase (TAP). In vitro transcribed 5'-triphosphate GFP RNA was added after tobacco acid pyrophosphatase treatment. RNA samples were analyzed by Northern blotting. 

**C** and **E**, quantitation of the levels of No-AUG-MS2-Rz reporter (**left**) and rpl28 mRNAs (**right**). The levels of reporter and rpl28 mRNAs were normalized to those of the control GFP RNA as described in **B** and **D**. Error bars, S.D. The p value was calculated by using Student's t test.
FIGURE 4. Validation of the deadenylation-independent mRNA decay pathway in zebrafish embryos. A, schematic representations of the GFP reporter mRNA with BoxB sites and MOs used to analyze translation- and poly(A) tail-independent mRNA decay by the TNRC6A Mid domain in zebrafish embryos. The translation-blocking MO (ATG MO) specifically masks the start codon of the GFP ORF. The polyadenylation-blocking MO (PB MO) binds to the end of the GFP mRNA and inhibits cytoplasmic polyadenylation during zebrafish embryogenesis. B, confirmation of the effect of ATG MO in zebrafish embryos. GFP and DsRed mRNAs were co-injected with MOs at the one-cell stage. GFP expression from the GFP reporter mRNA was completely blocked by ATG MO (green). Translation of co-injected DsRed mRNA was unaffected (red). Embryos are shown 10 h postfertilization. C, confirmation of the effect of PB MO in zebrafish embryos. GFP mRNA without a poly(A) tail was co-injected with MOs at the one-cell stage. The poly(A) tail length of the GFP mRNA was analyzed 4 h postfertilization by RNase H digestion and Northern blotting. Injected mRNA was polyadenylated after injection in the presence of control MO. PB MO completely blocked polyadenylation of the GFP mRNA.

D, stability of the injected GFP reporter mRNA was determined by qRT-PCR 10 h after injection. Left, qRT-PCR analysis of the stability of reporter mRNA containing the 5’ m7G-cap. Right, qRT-PCR analysis of the stability of reporter mRNA containing the 5’ A-cap. GFP mRNA values were normalized against Fluc mRNA values. Normalized values of the experiments using HA- and NMS2 were set to 1. Graphs represent the means of three independent experiments. Error bars, S.D. The p value was calculated by using Student’s t test. E, HA- and N-tagged effector proteins used in D were detected by Western blotting using anti-HA antibody. eIF5 was served as loading control. F, poly(A) tail analysis of the endogenous mir-430 target mRNAs at 6 h postfertilization. mRNA encoding GFP or dominant-negative CNOT7 or MO against miR-430 was injected at the one-cell stage as indicated. The putative position of the A(0) product is shown on the left. G, qRT-PCR analysis of the stability of reporter mRNA containing the 5’ m7G-cap. Right, qRT-PCR analysis of the stability of reporter mRNA containing the 5’ A-cap. GFP mRNA values were normalized against Fluc mRNA values. Normalized values of the experiments using HA-AN MS2 were set to 1. Graphs represent the means of three independent experiments. Error bars, S.D. The p value was calculated by using Student’s t test. H, Myc-tagged effector proteins used in F and G were detected by Western blotting using anti-Myc antibody. Tubulin was served as loading control. I and J, cRACE analysis of eif4ebp2 transcriptional end ligation junctions relative to the annotated eif4ebp2 transcript (ENSDART00000040926). Left panels, poly(A) tail length between the annotated 3’-UTR and ligated 5’ end. I, results of cRACE capturing capped mRNA; J, results of cRACE capturing decapped mRNA. The p value was calculated by using the Wilcoxon-Mann-Whitney test. n.s., not significant.
of eif4ebp2 mRNA, presumably representing two alternative transcriptional start sites (Fig. 4I, left). Analysis of these amplions revealed that 3’ ends of capped eif4ebp2 mRNA were mostly deadenylated (<30-nucleotide poly(A) tail) in wild type (Fig. 4I, right). On the other hand, longer poly(A) tail was retained in CNOT7 DN expressed embryos (Wilcoxon-Mann-Whitney test, p < 0.01), consistent with the poly(A) tail analysis in Fig. 4F. We then performed cRACE with naive RNA samples to capture 5’-monophosphate ends that were ligated to 3’-OH ends. Under this experimental condition, ligation junctions from both wild type and CNOT7 DN expressed embryos were located downstream of major transcriptional start sites and were broadly distributed (Fig. 4J, left), representing decapped RNA molecules being degraded in the 5’-to-3’ direction (53). Notably, the poly(A) tail length of decapped eif4ebp2 mRNA was significantly longer in CNOT7 DN expressed embryos compared with wild type (Fig. 4J, right, p < 0.01), as observed with capped eif4ebp2 mRNA. Indeed, >50% of capped eif4ebp2 mRNA still retained a >50-nucleotide poly(A) tail in CNOT7 DN expressed embryos. This result further supported the idea that eif4ebp2 mRNA was degraded from the 5’ end by miR-430-mediated decapping even if efficient deadenylation was not ensured. Overall, these experiments confirmed that both mRNA fate modulators are required for inhibition of translation by the tethered Mid domain of zebrafish TNRC6. To confirm the result of the polysome analysis, we performed pulse-labeling experiments to investigate whether Dhh1 and Pat1 are required for translation repression. Because N-terminally truncated MPT4 derived from AUG-MS2 or AUG-MS2-Rz mRNAs contains no methionine (other than the initiator methionine) or cysteine, we used GFP-MS2 and GFP-MS2-Rz mRNAs for the pulse-labeling experiments. The levels of synthesized GFP derived from reporter mRNAs were significantly reduced by tethered TNRC6A in the wild type (Fig. 1I), but translation repression was impaired in the dhh1Δ or pat1Δ single mutants and the dhh1Δ pat1Δ double mutant cells (Fig. 7F). These results demonstrate that the two mRNA fate modulators, Dhh1 and Pat1, not only facilitate mRNA decay from the 5’ end independently of translation and a poly(A) tail but also repress translation when the CCR4-NOT complex is recruited to mRNAs by the miRISC component TNRC6A.

**DISCUSSION**

In this study, we established a heterologous experimental system by tethering animal TNRC6 proteins to mRNAs in yeast. Polysome analysis, pulse-labeling experiments, and measurement of mRNA half-lives revealed that the tethered Mid domain fragment of zebrafish TNRC6A induces mRNA degradation and translation repression in yeast. This result strongly suggests that miRISC induces both translation repression and mRNA degradation via interactions with fundamental factors that are conserved across a wide range of eukaryotes. Indeed, the highly conserved proteins Dhh1 and Pat1 mediate these two functions through the CCR4-NOT complex, which is recruited by the Mid domain (Figs. 5 and 7). These two factors are involved in mRNA-mediated silencing in animals (11, 12, 25, 29, 33–35, 59), supporting the idea that TNRC6 can function in *S. cerevisiae* and suggesting that our system can be used to characterize the biologically relevant activities of miRISC. Although the data obtained in yeast with a truncated fragment of TNRC6 require careful validation in animal cells, the recapitulation of TNRC6-mediated silencing in yeast described here raises the possibility that genetic resources in yeast can be used to study basic principles of the miRNA system.

The Mid domain of TNRC6A interacted with the yeast CCR4-NOT complex via CIM1 and W-motifs within the Mid domain that mediate the same interactions in animals (Fig. 1B) (33, 34). This result, together with recent structural studies (11, 12), indicates that the conserved architecture of the CCR4-
FIGURE 5. mRNA decapping factors exert partially redundant functions in rapid mRNA decay from the 5’ end by tethered TNRC6A. A, interaction of FLAG-MS2-Mid with yeast Dhh1-HA. Cell lysates of wild type cells transformed with the indicated plasmids were immunoprecipitated using anti-FLAG antibody. Total extract (Input) and immunoprecipitate (IP) were analyzed by Western blotting using anti-HA or anti-FLAG antibody. B, interaction of FLAG-Dhh1 with Not1-HA. Immunoprecipitation was performed as described in A. C, mRNA fate modulators Dhh1 and Pat1 have partially redundant functions in TNRC6AMid-mediated 5’-to-3’ mRNA decay independent of translation and a poly(A) tail. Top, Northern blotting analysis of No-AUG-MS2-Rz mRNA in the indicated cells. The half-lives of mRNA in the indicated cells are shown as the mean values of three independent experiments, with S.D. values. Bottom, graphs of the half-life analysis. The p value was calculated by using Student’s t test.
FIGURE 6. The roles of CCR4-NOT complex in TNRC6A-mediated translation repression in yeast. A–C, translation repression by tethered TNRC6A was almost diminished in ccr4Δ and caf1Δ mutant cells independent of a poly(A) tail. Cell extracts were prepared from indicated cells harboring the indicated plasmid, and polysome analysis was performed. RNA samples were analyzed by Northern blotting using a DIG-labeled PGK1 3'-UTR probe. D, the expression level of Not1 reduced in caf1Δ and ccr4Δ mutant cells. Endogenous NOT1, CAF1, and CCR4 were tagged by 3× hemagglutinin (HA) in wild type, caf1Δ, and ccr4Δ mutant cells. Protein samples were analyzed by Western blotting using anti-HA, anti-Pab1 and anti-eEF1α antibodies.
NOT complex provides a binding surface for GW182/TNRC6 proteins, thereby connecting miRISC to the mRNA fate modulators. Further biochemical and structural analysis in yeast will shed light on how this interaction evolved.

The interactions of GW182/TNRC6 proteins with PABP and CCR4-NOT deadenylase play crucial roles in both translational repression and degradation of miRNA targets (33–35, 37). mRNA degradation by miRNAs and GW182/TNRC6 requires both deadenylation and the DCP1-DCP2 decapping complexes (25, 60). In addition, GW182 recruits decapping enzymes to target mRNAs independently of deadenylation (38). In this study, we demonstrated that the tethered TNRC6A fragment
promoted decapping and 5′-to-3′ mRNA decay in a Not1-dependent but a poly(A) tail- and translation-independent manner (Figs. 2 and 3). The levels of decapped No-AUG-MS2-Rz mRNA were significantly increased in xrrn1Δ cells expressing F-MS2-Mid (Fig. 3, B and C), indicating that the tethered Mid domain of TNRC6A recruits CCR4-NOT and the decapping complex and facilitates the decapping reaction independently of a poly(A) tail and translation. Together, these results suggest that mRNA decay caused by the Mid domain can mostly be attributed to the function of the yeast CCR4-NOT complex.

Our results indicate that GW182/TNRC6 stimulates mRNA decay in yeast in a decapping-dependent but poly(A) tail- and translation-independent manner. We also found that tethering of the TNRC6A Mid domain degraded mG-capped mRNA but not A-capped mRNA in zebrafish embryos (Fig. 4D). Moreover, mIR-430 stimulated decapping and 5′-to-3′ degradation of eif4ebp2 mRNA in zebrafish embryos even when the deadenylation activity of the CCR4-NOT complex was not fully ensured (Fig. 4, F, G, and J). These results imply that, at least for some mRNAs like eif4ebp2, miRISC promotes mRNA decay from the 5′ end by directly stimulating decapping before (or in parallel to) deadenylation. Although more comprehensive study with multiple transcripts in diverse animals is requisite to generalize this degradation mode of miRNAs, it is noteworthy that some mRNAs that are degraded in an Ago1-dependent manner are degraded when Caf1 is knocked down in Drosophila S2 cells (25). Conversely, not all Ago1-RISC target mRNAs strictly require mRNA fate modulators for their decay (29). These observations suggest that each mRNA is differentially susceptible to decapping and deadenylation during the process of miRNA-mediated degradation.

We found that rapid decay of No-AUG-MS2-Rz mRNA by the tethered TNRC6A Mid domain was abrogated in the dhh1Δpat1Δ double mutant but not in the dhh1Δ or pat1Δ single mutants (Fig. 5). We propose that Dhh1 and Pat1 contribute to decapping via distinct pathways in the absence of translation and a poly(A) tail. In addition to inhibiting translation, Dhh1 and Pat1 may have redundant functions in the formation of the decapping complex. Consequently, in the dhh1Δpat1Δ double mutant, the decapping complex may not be able to form, resulting in a very strong defect in decapping.

A prevailing view, based on in vitro experiments, is that miRNAs inhibit translation at the initiation step by an as yet unknown mechanism (39, 61–63). A recent study has suggested that miRNAs repress translation initiation in a manner dependent upon elf4AII, which interacts with CNOT7 of the CCR4-NOT complex in mammalian cells (64). However, because elf4AII is present neither in Drosophila nor in unicellular eukaryotes, such as S. cerevisiae, it remains to be determined how this factor affects translation initiation in general. In this study, we showed that the tethered Mid domain of TNRC6A repressed translation in yeast (Fig. 1, G and H; see also Fig. 7B). Hence, the repressive activity of TNRC6A recapitated in yeast is independent of elf4AII. In addition, others also observed that elf4AII does not interact directly with CNOT1 MIF4G domain (11, 12, 43). Furthermore, recent in vitro studies showed that miRNAs trigger dissociation of elf4A in Drosophila and both elf4AII and elf4AII in humans (65, 66).

Apparently, more experiments will be necessary to determine the roles of elf4AII in translation repression by miRNAs.

In cells expressing F-MS2-Mid protein, the levels of GFP-MS2 or AUG-MS2 mRNAs were reduced in the polysome fraction, but increased in the 80S monosome and free fractions, relative to cells expressing the control MS2 protein (Figs. 1G and 7B). This effect was observed in a reporter mRNA lacking a poly(A) tail (Figs. 1G and 7B). These results suggest that the tethered Mid domain of TNRC6A may block the formation of 48S preinitiation complex or inhibit the steps after 80S formation by CCR4-NOT complex independently of a poly(A) tail.

How, then, does the CCR4-NOT complex inhibit translation? Polysome analysis and pulse-labeling experiments showed that translation repression by tethering of the Mid domain of TNRC6A was abrogated in dhh1Δ or pat1Δ single mutants as well as in the dhh1Δpat1Δ double mutant (Fig. 7). Translationally repressed mRNAs in wild type cells contained a cap structure (Fig. 3, D and E). Moreover, stimulation of mRNA decay from the 5′ end was impaired only in the dhh1Δpat1Δ double mutant, whereas translation repression by the tethered TNRC6A was suppressed in the dhh1Δ and pat1Δ single mutants (Fig. 7). These results clearly indicate that translation repression is not a consequence of decapping. The mRNA fate modulators Dhh1 and Pat1 independently repress translation in vivo, and they repress translation initiation in vitro by limiting the formation of a stable 48S preinitiation complex (15).

Notably, a human homolog of Dhh1, DDX6/RCK1/p54, interacts with miRISC and contributes to translation repression in cultured cells (11, 12, 43, 59), and GW182 associates with HPat in Drosophila cells (57). Our results in yeast therefore support the model in which the CCR4-NOT complex mediates translation repression, at least in part, via recruitment of mRNA fate modulators through a direct interaction with Dhh1. Further experiments will be necessary to determine the conserved roles of decapping factors Dhh1/DDX6/RCK1/p54 and Pat1/HPat/Pat1 in translation repression by GW182/TNRC6 as well as other CCR4-NOT-interacting proteins.

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