Depletion of key protein components of the RISC pathway impairs pre-ribosomal RNA processing

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Supplementary Data

SUPPLEMENTARY MATERIALS AND METHODS

2'-MOE RNA/DNA chimeric antisense oligonucleotides used in this study

(2'-MOE substituted ribonucleotides are in bold, deoxyribonucleotides are in plain letters):

Drosha: **ISIS25690: 5'-ATCCC**TTTCTTCCGC**ATGTG**-3', which was used throughout the entire study. ISIS25691: 5'-**GCCAA**GGCGTGACAT**GATAT**-3'

Dicer: **ISIS138648:** 5'-**GCTGA**CCTTTTTGCT**TCTCA**-3', which was used throughout the entire study. ISIS138651: 5'-**AGGAG**GAAGCCAATT**CACAG**-3'. ISIS138678: 5'-**CATAA**ACATTTCCAT**CAGTG**-3'.

Ago2:

ISIS136764: 5'-**CTGCT**GGAATGTTTC**CACTT**-3', which was used throughout the entire study. ISIS136755: 5'-**GGCCT**TCAGAGACAC**CGTCG**-3'.

Ago1: **ISIS144239**: 5'-**TGAGT**CACATGAGAC**ACCTG**-3'.

Ago3: **ISIS241286**: 5'-**CTTGA**AAACAGTTAG**CCAGC**-3'.

Ago4: **ISIS309418**: 5'-**CATAG**TGATACACAT**CTATT**-3'

PTEN: **ISIS116847**: 5'-**CTGCT**AGCCTCTGGA**TTTGA**-3'

Control ASO: **ISIS141923**: 5'-**CCTTC**CCTGAAGGTT**CCTCC**-3'

Primer sets used in real-time PCR:

Drosha: RTS13816

Forward: 5'- CAAGCTCTGTCCGTATCGATCA-3'

Reverse: 5'-TGGACGATAATCGGAAAAGTAATCA-3'

Probe: 5'-CTGGATCGTGAACAGTTCAACCCCGAT-3'

Dicer: RTS519

Forward: 5'-ATTAACCTTTTGGTGTTTGATGAGTGT-3'

Reverse: 5'-GCGAGGACATGATGGACAAATT-3'

Probe: 5'-ATCTTGCAATCCTAGACCACCCCTATCGAGAA-3'

Ago2: RTS1302

Forward: 5'-CCAGCTACACTCAGACCAACAGA-3'

Reverse: 5'-GAAAACGGAGAATCTAATAAAATCAATGAC-3'

Probe: 5'-CGTGACAGCCAGCATCGAACATGAGA-3'

PTEN: RTS96

Forward: 5'- AATGGCTAAGTGAAGATGACAATCAT-3'

Reverse: 5'- TGCACATATCATTACACCAGTTCGT-3'

Probe: 5'- TTGCAGCAATTCACTGTAAAGCTGGAAAGG-3'

Ago1: RTS541

Forward: 5'-GAGCCTATGTTCCGGCATCTC-3'

Reverse: 5'-AGAGTGTATCTCCGACACGTTTCAC-3'

Probe: 5'-AGCATACACCGGCGTCTTCCCTGG-3'

Ago3: RTS2746

Forward: 5'-GGTGGTTGACTCAATGGTTCAG-3'

Reverse: 5'-GTGGATTGGCGGTGTAAAGAC-3'

Probe: 5'-ATTTGGAGACCGTAGACCAGTTTATGA-3'

Ago4: RTS1447

Forward: 5'- GGCCTGGAGCCACACTCTATAT-3'

Reverse: 5'- AAACACCCTTCACCATCATTTCC-3'

Probe: 5'- CTGAGGAAGGAGCTAGGGAAAGAGATAGACA-3'

Conventional oligonucleotides used in this study:

XL002, 5'-tcgccctccgggctccgttaatg-3', antisense, to the boundary of 18S/ITS1, used for RT-PCR. XL003, 5'- gcgattgatcggcaagcga-3', antisense, to the boundary of 5.8S/ITS2, used in northern hybridization. XL004, 5'- gaagacgaacggaaggacgg-3', antisense, to 3' ETS of pre-rRNA, used for RT-PCR. XL011, 5'-ttgctcagtaagaattttcg-3', antisense, to U16 snoRNA, used for

northern hybridization. XL016, 5'-accactcagaccgcgttctctcc-3', anti, to U3 snoRNA, used in northern hybridization. XL021, 5'-tggaacgcttcacgaatttgcg-3', antisense, to U6 snRNA, used in northern hybridization. XL022, 5'-gtgtcgatgatcaatgtgtc-3', antisense, to 5.8S rRNA, used in northern hybridization and primer extension sequencing. XL024, 5'-gccgccgggtctgcgcttag-3', anti, to ITS2, ~90 nt downstream to the 5' end of ITS2, used in RT-PCR. XL030, 5'-tccttcgagccggaattga-3', antisense, to $tRNA^{Tyr}$, used in northern hybridization. XL023, 5'-ccggggctac gcctgtctga-3', sense, specific to 5.8S rRNA, used for RT-PCR. XL056, 5'-tgaaagtcagccctcgacacaa-3', sense, specific to 28S rRNA, used for RT-PCR. XL062, 5'-cctggcggagcgctgagaag-3', sense, to 18S rRNA, used for RT-PCR. XL082, 5'-aagagtcgtacgaggtcgat-3', antisense, to ITS1/5.8S boundary, used in northern hybridization and primer extension (probe 2). XL083, 5'-gccgccgggtctgcgcttag-3', antisense, to ITS2, ~90 nt downstream from the 3' end of 5.8S rRNA, used in primer extension (probe 4) . XL038, 5'-cccgacgcgtgaccacgcag-3', antisense, to ITS2, ~150 nt downstream to the 5' end of ITS2, used in primer extension (probe 5). XL039, 5'-gcggcggccacgggaactcg-3', antisense, to ITS2, ~280 nt downstream to the 3' end of 5.8S rRNA, used in primer extension (probe 6). XL072, 5'-ggtcggaaggtttcacacca-3', antisense, to ITS1, ~175 nt upstream to the 5' end of 5.8S rRNA, used in primer extension (probe 1). XL097, 5'-cagccgcgcaccccgaggag-3', antisense, to ITS2, 31 nt downstream to 3' end of 5.8S rRNA, used in primer extension (probe 3). XL136, 5'-gggacgtgcc gcgccaggaa-3', sense, to ITS1, ~80 nt upstream to 5' end of 5.8S rRNA, used in RT-PCR.

RT-PCR. Co-immunoprecipitated RNA was treated with RNase-free DNase (RQ1, Promega), phenol extracted, and precipitated with ethanol in the presence of 10 μg glycogen. The RNA pellet was dissolved in 20 μl water. Synthesis of cDNA and PCR were performed as described in

(1), using primers specific to different regions of pre-rRNA, as indicated in Figure 5. PCR products were resolved and analyzed on 2% agarose gels.

Quantitative RT-PCR (qRT-PCR). About 150 ng total RNA in 15 μl water was mixed with 0.1 μl primer sets containing forward, reverse primers (10 μM of each) and fluorescently labeled probe (3 μM), 0.1 μl RT mix (Qiagen), and 15 μl of 2× reaction buffer. Reverse transcription was performed at 48 °C for 30 mins, and 40 cycles of PCR reaction were carried out at 94 °C for 15 seconds and 52 °C for 15 seconds within each cycle, using Stepone Plus RT-PCR system (Applied Biosystems). The mRNA levels were normalized to the total RNA present in each reaction as determined for duplicate RNA samples by Ribogreen assay (Invitrogen).

Western analysis: Whole cell extracts or sub-cellular fractions were separated in 4-12% gradient SDS-PAGE gels. Proteins were transferred to PVDF membrane using a semi-dry transfer apparatus. The membranes were blocked for 1 hr with block buffer (5% dry milk in 1×TBS), and incubated with primary antibodies against Drosha (anti-SR, in rabbit, 1:500), Dicer (ab14601, 1:1000), or Ago2 (ab32381, 1:1000) at 4 °C for overnight. After 3 washes with wash buffer (1×TBS, 0.1% Tween-20), membranes were incubated with anti-mouse or anti-rabbit secondary antibody in block buffer at room temperature for 1 hr. After 3 washes, proteins were detected using ECL (Abcam).

In vitro Dicer cleavage: 12 μg total RNA prepared from Hela cells labeled with [Methyl-^{3}H] methionine was heated at 94 ^{o}C for 3 mins in a 100 μl reaction containing 20 mM Tris.Cl (H8.0), 250 mM NaCl, and slowly cooled to 37 ^{o}C at room temperature. The sample was cooled on ice for 2 mins, and MgCl2 was added to 10 mM final concentration, followed by addition of 1 μl of 100XBSA and 0.25 μg Dicer protein, which was purified as described previously (2). The reaction was performed at 30 ^{o}C. 30 μl of the reaction was collected at time points 0, 2, and 4 hours, and precipitated with ethanol. As controls, 12 μg RNA was treated in the same way, without addition of Dicer protein. The precipitated samples were analyzed in an 8% polyacrylamide, 7M Urea gel, and results were visualized by autoradiography with X-ray film.

REFERENCES

1. Liang, X.H. and Fournier, M.J. (2006) The helicase Has1p is required for snoRNA release from pre-rRNA. *Mol Cell Biol*, **26**, 7437-7450.
2. Lima, W.F., Murray, H., Nichols, J.G., Wu, H., Sun, H., Prakash, T.P., Berdeja, A.R., Gaus, H.J. and Crooke, S.T. (2009) Human Dicer binds short single-strand and double-strand RNA with high affinity and interacts with different regions of the nucleic acids. *J Biol Chem*, **284**, 2535-2548.

FIGURE LEGENDS

Figure S1. Transfection of control ASOs does not cause similar defects on rRNA processing. Hela cells were treated with a control ASO (ISIS141923) or an ASO targeting PTEN (ISIS116847). Total RNA was prepared from cells 48 hours after transfection, and subjected to qRT-PCR analysis for PTEN mRNA (panel **A**). The error bars indicate standard deviation. Pre-

5.8S rRNA level was determined by northern hybridization (panel **B**), as in Figure 1E. The arrows indicate the species of pre-5.8S rRNA. U3 snoRNA was used as a control for loading.

Figure S2. **The pre-rRNA processing defects do not stem from off-target effects of the ASOs.** Hela cells were treated with 50 nM ASOs targeting Drosha (panel **A**), Ago2 (Panel **B**), or Dicer (Panel **C**). Cells were harvested 48 hrs after transfection, and total RNA was prepared. Reduction in the level of targeted mRNA was determined by qRT-PCR (upper panels), and the level of pre-5.8S rRNA was detected by northern hybridization, as in Fig. S1. U16 snoRNA was used as a loading control. In each case, the ASOs used throughout the study are boxed. The relative positions of the ASOs in mRNA are depicted.

Figure S3. Accumulation of pre-5.8S rRNA strongly correlates with the kinetics of Drosha reduction

Hela cells were transfected with 50 nM ASO targeting Drosha, and harvested at different times after transfection. Total RNA and whole cell lysate were prepared. (**A**) The level of Drosha mRNA was dramatically reduced, as determined by qRT-PCR. The error bars indicate standard deviation from three parallel experiments. (**B**) The level of Drosha protein was significantly reduced 12 hours after transfection, as analyzed by western analysis. Alpha-tubulin was used as a loading control. (**C**) Pre-5.8S rRNA starts to accumulate along the time course, as detected by northern hybridization. U3 snoRNA was used as a loading control. (**D**) Quantification of the level of pre-5.8S rRNA relative to untreated cells (time point 0) in panel C, as normalized to U3 snoRNA.

Figure S4. **The level of pre-5.8S rRNA negatively correlates with the level of Ago2 expression**. Hela cells were treated with 50 nM of Ago2 ASO, and harvested at different times after transfection. The levels of Ago2 mRNA (Panel **A**) and protein (Panel **B**) were detected as described in Fig S3, using primers specific to Ago2 mRNA, or anti-Ago2 antibody (ab57113). Alpha-tubulin was used as a loading control (Panel **B**). The level of pre-5.8S rRNA was determined by northern analysis (panel **C**) and quantified based on the level of U3 snoRNA (Panel **D**). The error bars in panel A indicate standard deviation from three independent experiments.

Figure S5. Strong correlation between the accumulated level of pre-5.8S rRNA and loss of Dicer protein. Cells were transfected with 50 nM ASO targeting Dicer, and collected at different time points after transfection. Subsequent analysis for the levels of Dicer mRNA (Panel **A**), protein (Panel **B**), and pre-5.8S rRNA (Panel **C** and **D**) were performed as described in Fig S3, using Dicer specific probes and antibody (ab14601). The sample for time point 6 was lost in western analysis (panel **B**), as shown with the level of control protein, alpha-tubulin. The error bars in panel A indicate standard deviation from three independent experiments.

Figure S6. Accumulation of pre-5.8S rRNA correlates with the extent of reduction in the level of Drosha mRNA. Cells were treated with different concentrations of ASO targeting Drosha, and harvested 48 hours after transfection. (**A**) The level of Drosha mRNA was reduced in cells treated with different dose of ASO, as determined by qRT-PCR assay. The error bars

indicate standard deviation from three independent experiments. (**B**) Northern hybridization for pre-5.8S rRNA, as in Fig S1. (**C**) Quantification of the level of pre-5.8S rRNA relative to untreated cells (time point 0) in panel B, as normalized to U3 snoRNA.

Figure S7. Reduction of Ago2, but not other Ago proteins significantly affects pre-5.8S rRNA accumulation. (A) Hela cells were treated with ASOs (60 mM) targeting different Ago proteins individually, or in combination with Ago2 depletion. Total RNA was prepared 48 hours after transfection, and mRNA levels were detected using qRT-PCR. The error bars indicate standard deviations of three parallel experiments. (**B**) Total RNA as used in panel A was subjected to northern hybridization, to detect the level of pre-5.8S rRNAs. U16 snoRNA was detected and served as a loading control.

Figure S8. Pre-rRNA can be cleaved by Dicer in vitro. (**A**) Pre-rRNA was labeled in vivo using [Methyl-^{3}H]methionine, and analyzed by gel electrophoresis in a 1.2% agarose gel. Different products are indicated. (**B**) In vitro cleavage of pre-rRNA by Dicer. In vivo labeled RNA was denatured, annealed, and incubated at 30 ^{o}C without [(-)Dicer] or with [(+)Dicer] purified Dicer protein. The incubation times are indicated above lanes. The distinct cleavage products are marked by arrows.

Fig S1

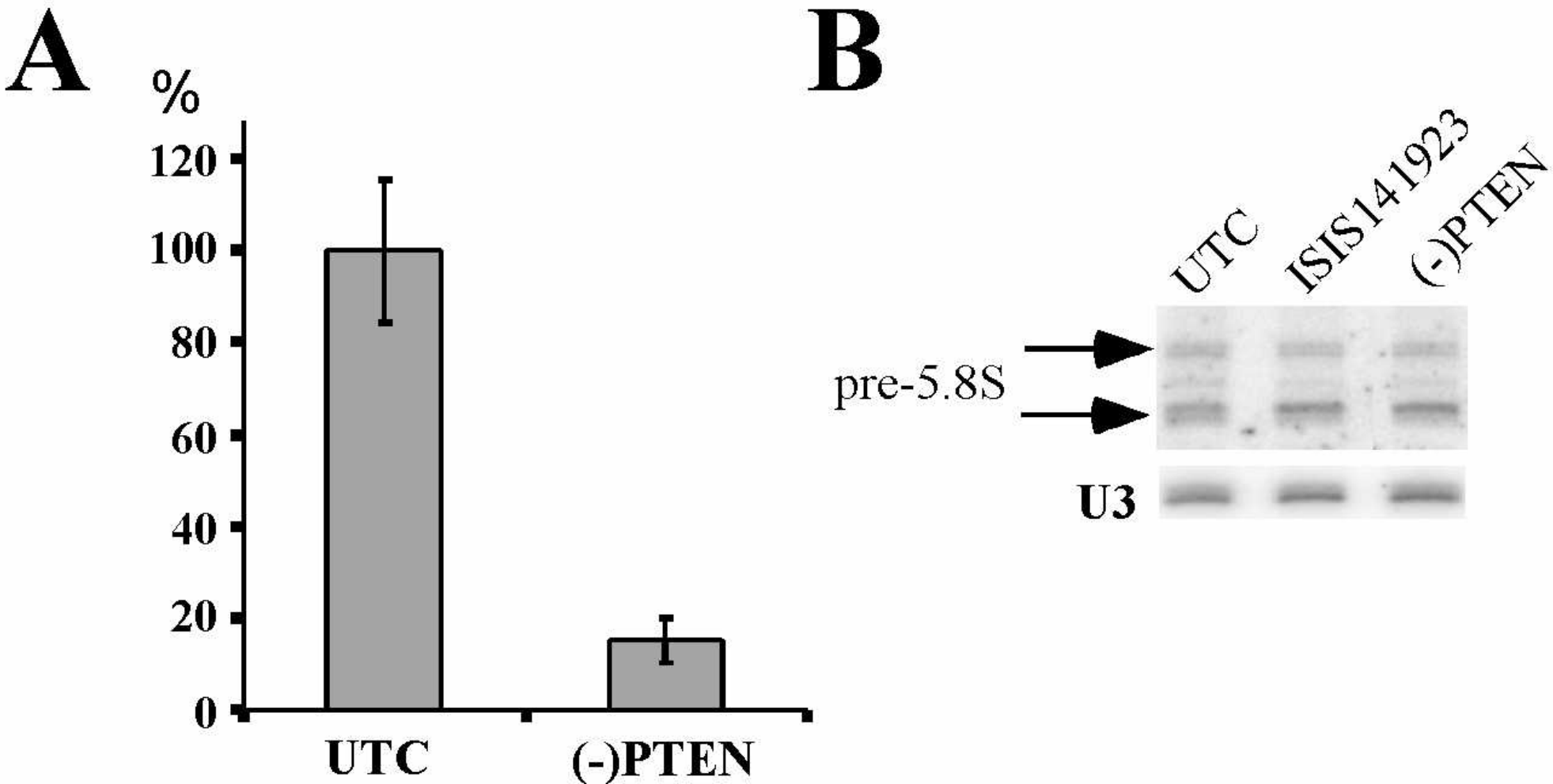
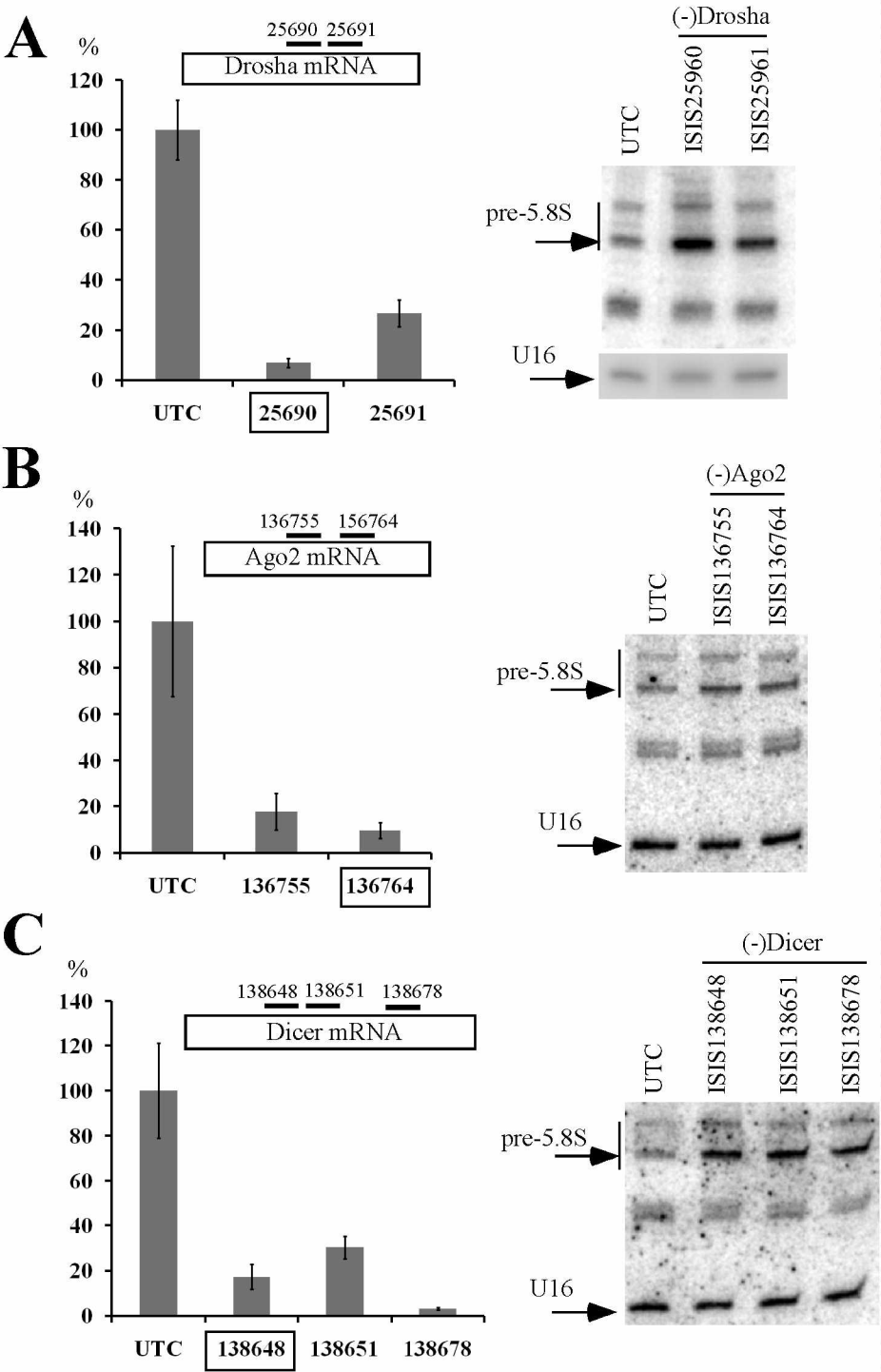


Fig. S2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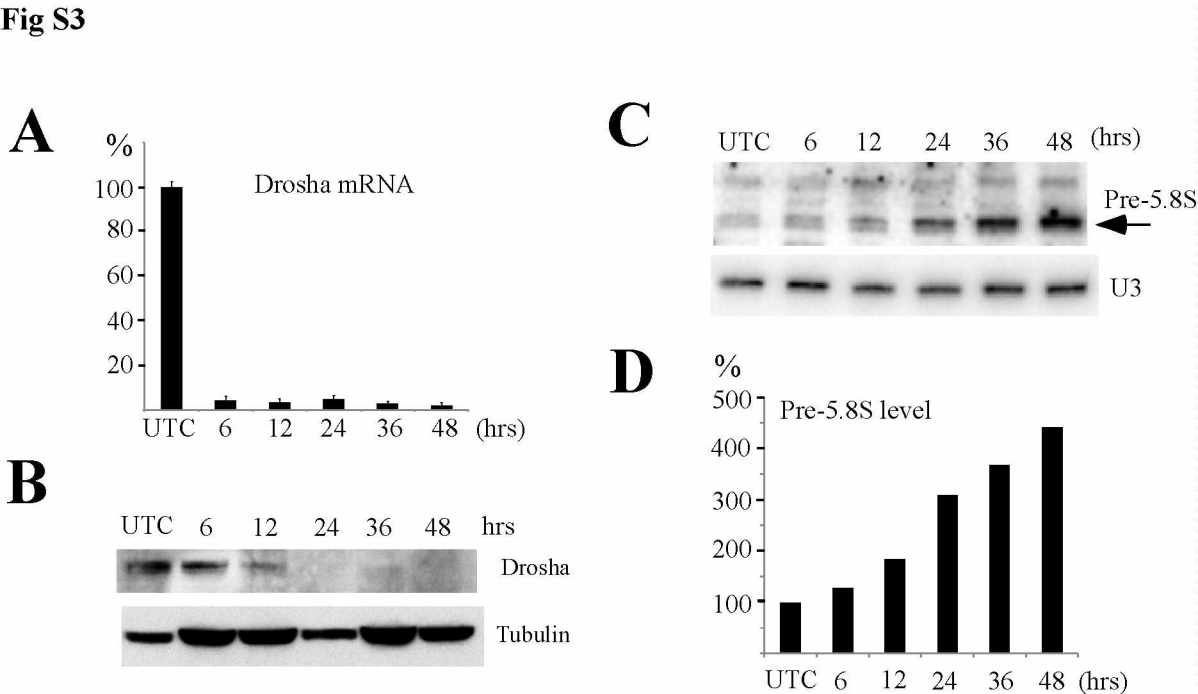
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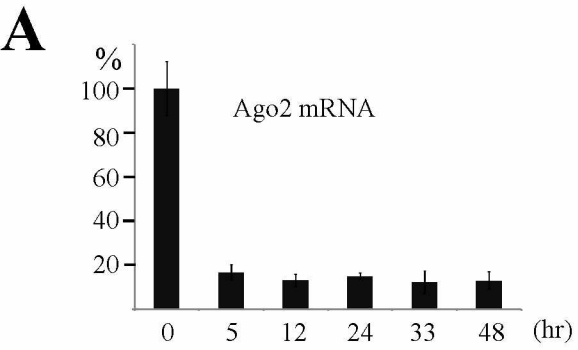
Fig S4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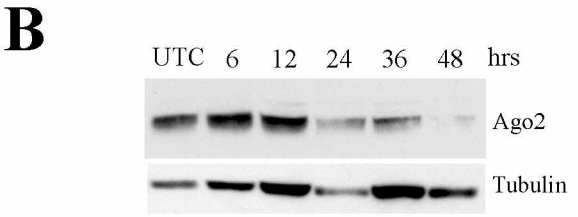
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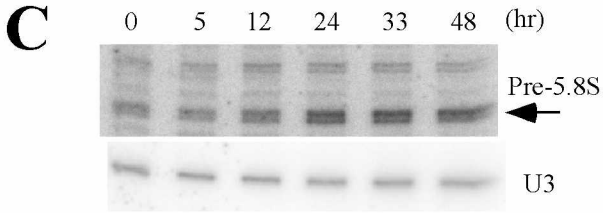
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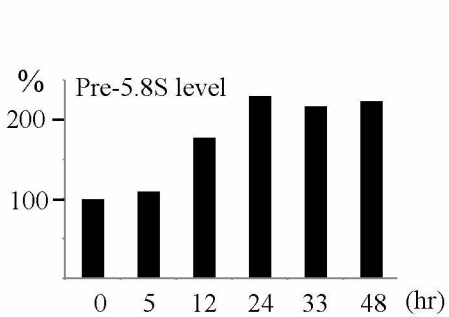
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Fig S5

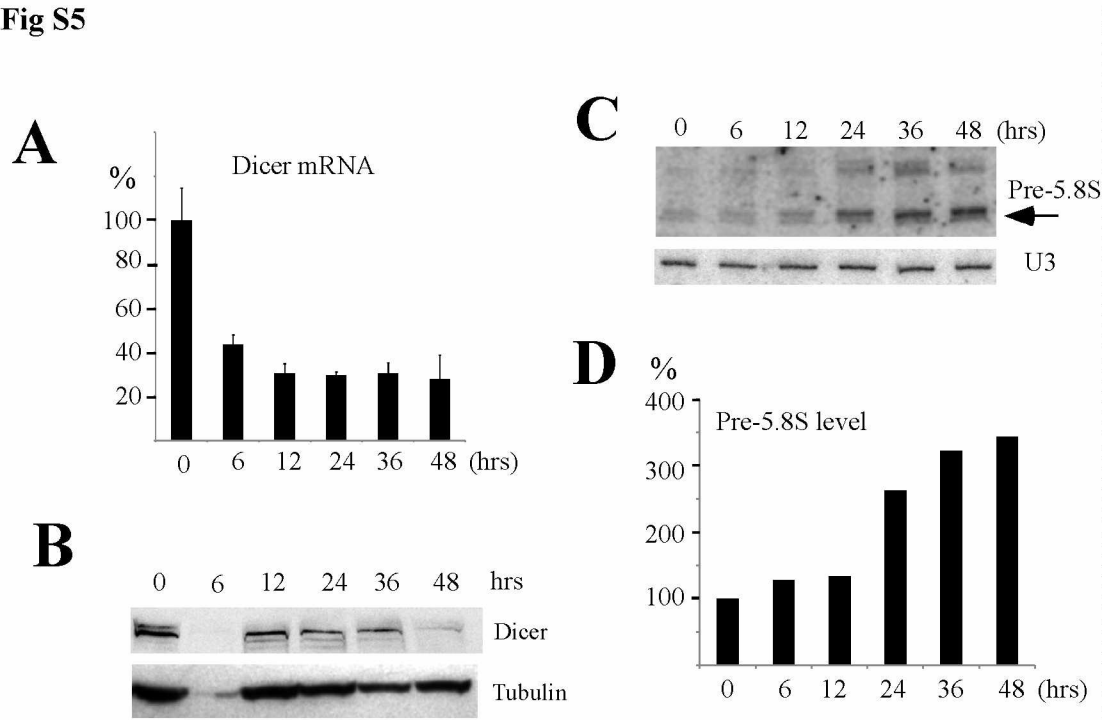
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Fig S6

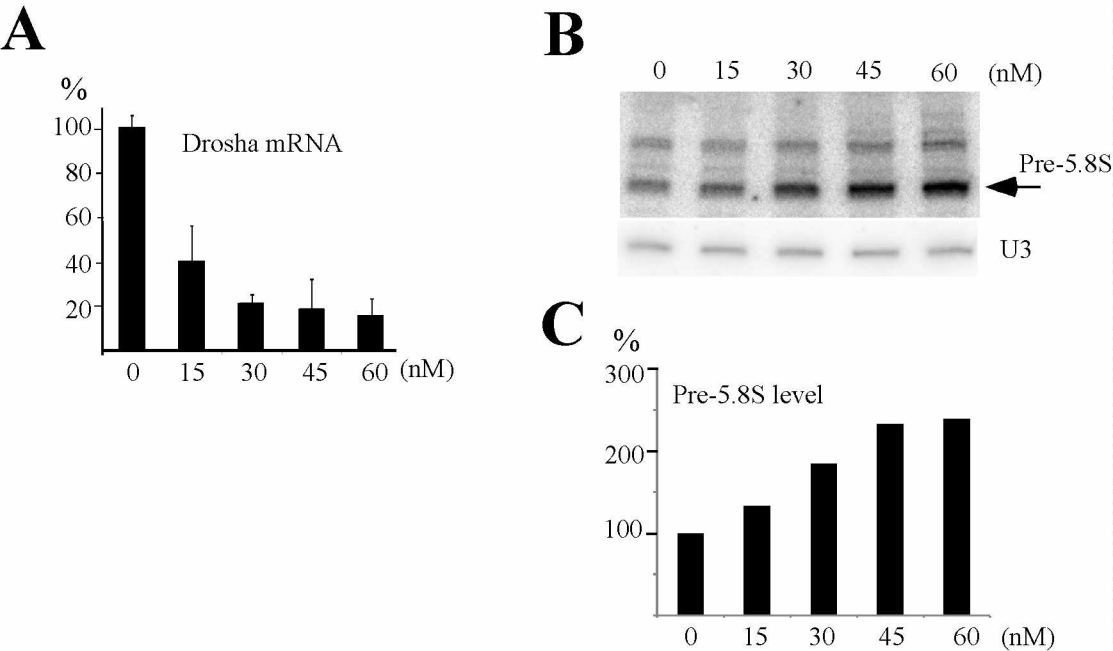
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Fig. S7

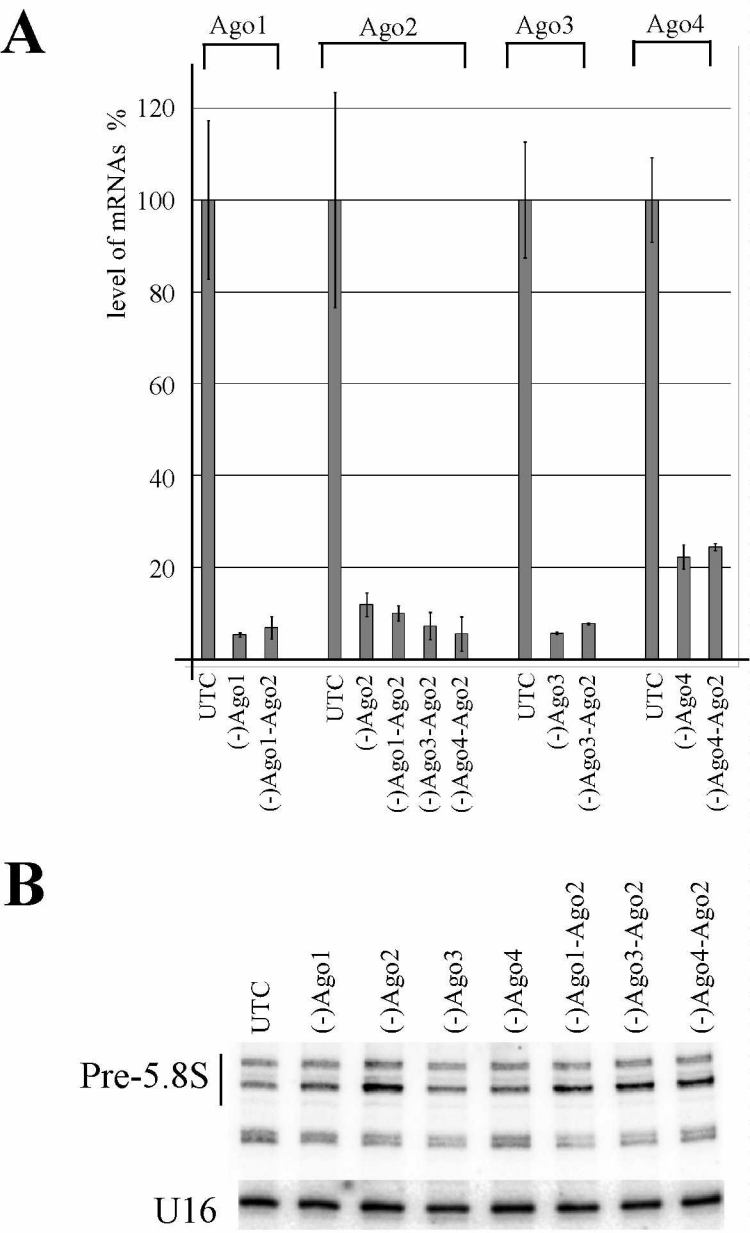
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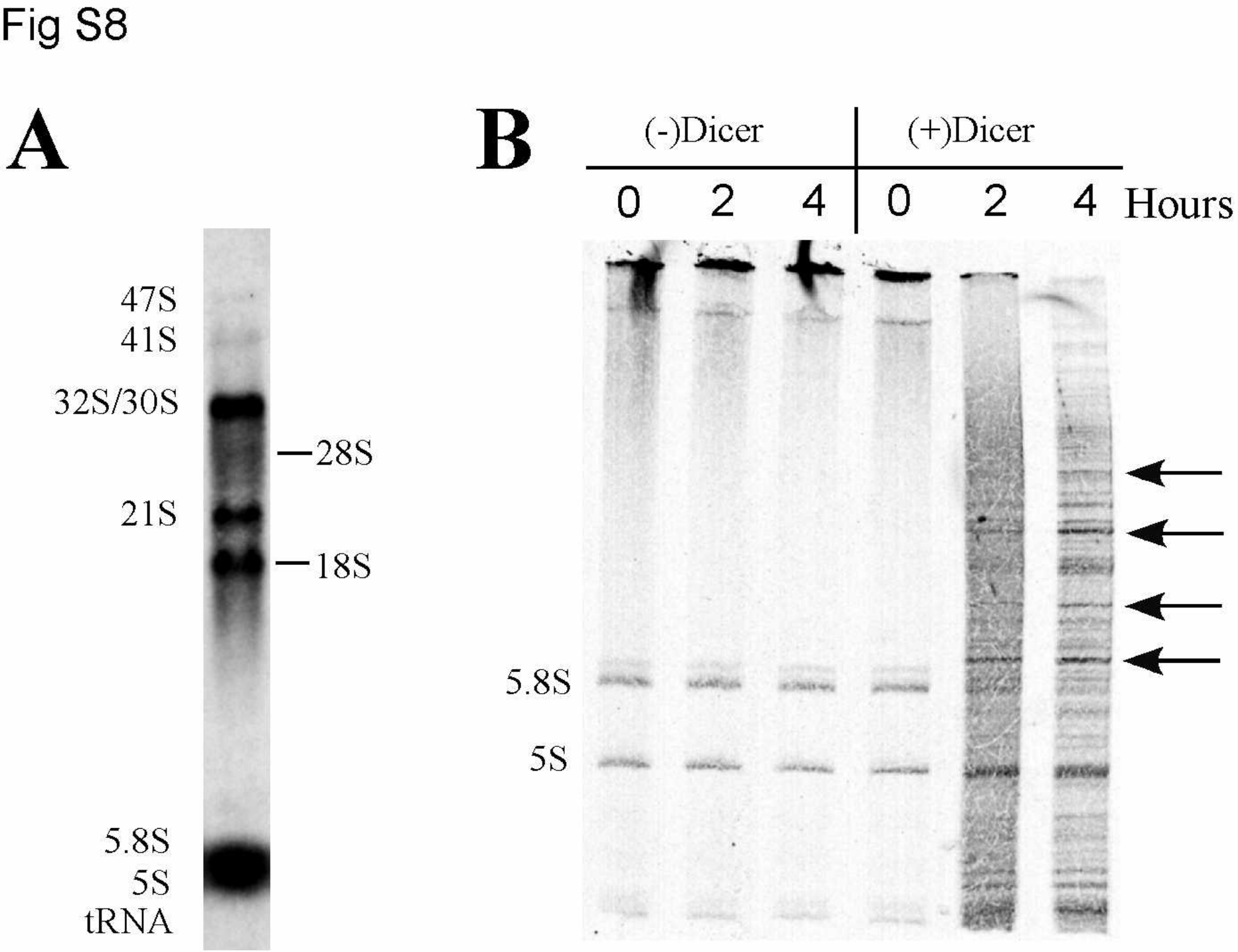
Fig S8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