Supporting Information

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SI Materials and Methods

Bioinformatics Analysis. Novoindex (www.novocraft.com) was used to create a mouse genome index (M_musculus_Jul_2007 version) with extended splice junctions. Novoalign (Novocraft) was used to align raw reads in FASTAQ format against the indexed mouse genome and to strip adapter sequences from each end before alignment. Default parameters were used except ≤100 alignments per read were allowed if the read could be aligned to more than one genomic location (-r All100 was used). Dataset S4 shows the number of total, aligned, and unique reads for each sample.

Aligned reads were analyzed with USeq (www.sourceforge.net/projects/USeq) (1). The SamTranscriptome Parser application was used with parameters to convert coordinates to genomic space and to sort and save alignments in BAM format. Default parameters were used except we allowed up to about five mismatches (-a 150), one alignment/read (-n 1, -d), and merged proper paired alignments (-p).

To identify enriched annotated genes, we used DRSS and DRDS applications. We used DRSS and DRDS with default parameters. DRSS uses a binomial distribution to calculate *P* values and Storey's *q*-value method to convert to FDRs; DRDS uses DESeq's variance-corrected count to calculate fold change and FDR (2, 3). Mm9 Ensembl transcriptome file downloaded from University of California at Santa Cruz was used as an input gene table. The ScanSeqs application was used to identify enriched annotated and unannotated regions using a 50-bp sliding window across the genome. The binomial *P* value was converted to FDR using Storey's *q*-value method.

UV–Cross-Linking RNA Immunoprecipitation and RT-qPCR. Cells were irradiated with 254 nm UV light at 200 mJ/cm^2. Twenty percent of each cell lysate was saved to serve as an input sample for RT-qPCR. Immunoprecipitation was performed as described in *Materials and Methods* except beads were washed eight times with lysis buffer containing 750 mM NaCl. Beads and input samples were treated with 0.8 mg proteinase K (Invitrogen) for 20 min at 37 °C in buffer A (100 mM Tris·Cl, pH 7.5, 50 mM NaCl, 10 mM EDTA) and for another 20 min in buffer B (buffer A supplemented with 7 M urea). RNA was extracted from cell lysates (Input) and immunoprecipitated fractions using TRIzol. RNA samples were treated with Turbo DNase and ethanol-precipitated.

cDNAs were generated using random hexamer primers and SuperScript Reverse transcriptase II (Invitrogen). qPCR was performed using SYBR green dye (Roche) on a real-time PCR machine (LightCycler 2.0, Roche); qPCR primers are in Dataset S3.

Protein Expression and Purification. pPET-PKR/PPase plasmid (Addgene) was used to express human wild-type PKR. The expression vector was transformed into BL21(DE3) Rosetta cells (New England Biolabs). PKR was expressed and purified as described (4, 5).

In Vitro Transcription. cDNAs for snoRNAs were PCR-amplified from mouse genomic DNA and cloned into a TOPO vector (Invitrogen). Unlabeled and uniformly ^{32}P-labeled RNA was synthesized using T7 RNA polymerase. To generate RNA with a 5′-monophosphate, RNA was treated with Antarctic phosphatase followed by T4 PNK (NEB). Some mouse snoRNAs were cloned into pss419 (gift of Sandra Searles, Medical Research Council Laboratory of Molecular Biology, Cambridge, United Kingdom) and transcribed as described (6).

In Vitro Autophosphorylation Assays. Purified PKR (100 nM) was incubated with various concentrations of RNA in PKR activation buffer as described (7). Gels were exposed to PhosphorImage screens. Activation was determined using ImageQuant software.

Gel Mobility Shift. A total of 50 pM of ^{32}P-RNA was incubated alone or with various amounts of dephosphorylated PKR in binding buffer [25 mM Hepes, pH 7.5, 100 mM NaCl, 5% (vol/vol) glycerol, 5 mM DTT, 0.1 mM EDTA] for 7 min at 25 °C. The complexes were electrophoresed on a 6% native polyacrylamide gel (37.5:1) and visualized using a PhosphorImager (Typhoon Trio, GE Healthcare).

Cell Fractionation, Western Blots, and RT-qPCR. Cells were scraped off plates in hypotonic buffer [10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 1× protease inhibitor mixture (Roche)] and centrifuged (1,000 × *g*, 5 min). Cell pellets were incubated in hypotonic buffer supplemented with 0.1% Nonidet P-40 for 30 min on ice and centrifuged (6,000 × *g*, 1 min). Supernatants (cytoplasmic fractions) were transferred to new tubes, and salt was adjusted to 150 mM. Remaining pellets were incubated with nuclear buffer (20 mM Hepes, pH 7.9, 400 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1× protease inhibitor mixture) for 30 min on ice and centrifuged (10,000 × *g*, 15 min); supernatants were transferred to new tubes, and salt was adjusted to 150 mM.

Equivalent amounts of protein were analyzed on a 4–15% gradient gel (Bio-Rad) and transferred to PVDF by electroblotting. PKR was detected with the anti-flag M2 antibody (Sigma). As a control for fractionation, cytoplasmic and nuclear proteins were analyzed by Western using an antibody against α/β-tubulin and PARP (Cell Signaling), respectively.

Total RNA was extracted from cytoplasmic and nuclear fractions using TRIzol. qPCR was performed as above.

Indirect Immunofluorescence. Cells were fixed with 3.7% (vol/vol) paraformaldehyde in PBS for 20 min on ice and then permeabilized for 3 min in 0.2% Triton X-100 at room temperature (RT). Cells were blocked overnight at 4 °C in blocking buffer [10% (vol/vol) goat serum, 0.2% gelatin, 0.1% Triton X-100]. Cells were incubated with PKR antibody (K17, SCBT) overnight at 4 °C, washed six times with PBS, and incubated with Alexafluor 488 antibody (Life Technologies) for 2 h at RT. Cells were mounted in ProLong gold antifade reagent with DAPI (Life technologies) for 5 min at RT. Images were obtained using an Olympus FV1000 confocal microscope (University of Utah, Cores Research Facility).

RNA Transfection and Western Blot. MEFs were transfected with 3 μg of 5′-triphosphorylated or 5′-monophosphorylated RNA using Lipofectamine 3000 (Life Technologies) for 4 h at 37 °C. Cells were washed two times with PBS and lysed in SDS protein-loading buffer. Western analyses were performed as above using p-PKR antibody (Thr-451, SCBT). PKR phosphorylation activity was normalized to the number of molecules of transfected snoRNA. RNA copy number was calculated using the formula [copy number = (amount (ng) × 6.022 × 10^{23})/(length (nt) × 10^9 × 330)].

eIF2α Phosphorylation Activity in Cell Lysate. MEFs or CHO cells were lysed in lysis buffer. Cell lysate was incubated with or without snoRNA for 1 h at 30 °C. Western analysis was performed as described above using phospho-eIF2α antibody (Ser51, Cell Signaling).

Nuclease Structure Mapping of snoRNAs. A total of 200 nM of 5′-end–labeled RNA was incubated with 0.01 or 0.1 U RNase T1 (Life Technologies) or 0.01 or 0.1 ng RNase A (Sigma) for 15 min at 30 °C or 37 °C in PKR activation buffer. RNA alkaline hydrolysis ladders were generated by incubating RNA with 50 mM sodium carbonate at pH 9.5 and 1 mM EDTA for 1 min at 95 °C. RNase T1 ladders (ΔT1) were generated by incubating the RNA in 20 mM sodium citrate at pH 4.5, 1 mM EDTA, and 7 M urea for 10 min at 50 °C. The cleavage products were separated on a 12% 8 M urea polyacrylamide gel.

1. Nix DA, Courdy SJ, Boucher KM (2008) Empirical methods for controlling false positives and estimating confidence in ChIP-Seq peaks. *BMC Bioinformatics* 9:523.
2. Anders S, Huber W (2010) Differential expression analysis for sequence count data. *Genome Biol* 11(10):R106.
3. Storey JD, Tibshirani R (2003) Statistical significance for genomewide studies. *Proc Natl Acad Sci USA* 100(16):9440–9445.
4. Anderson E, Pierre-Louis WS, Wong CJ, Lary JW, Cole JL (2011) Heparin activates PKR by inducing dimerization. *J Mol Biol* 413(5):973–984.
5. Lemaire PA, Lary J, Cole JL (2005) Mechanism of PKR activation: Dimerization and kinase activation in the absence of double-stranded RNA. *J Mol Biol* 345(1):81–90.
6. Welker NC, et al. (2011) Dicer's helicase domain discriminates dsRNA termini to promote an altered reaction mode. *Mol Cell* 41(5):589–599.
7. Nallagatla SR, et al. (2007) 5′-Triphosphate-dependent activation of PKR by RNAs with short stem-loops. *Science* 318(5855):1455–1458.

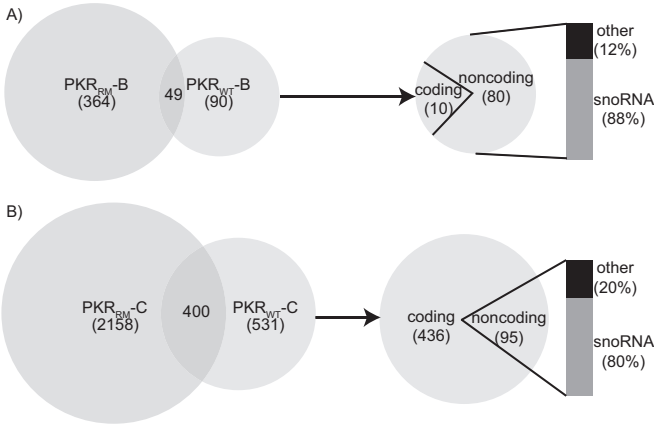

Fig. S1. High-throughput sequencing of additional samples confirmed that snoRNAs are enriched in PKR$_{WT}$ after PA treatment. Venn diagrams show annotated genes enriched in PKR$_{WT}$ and PKR$_{RM}$ samples after PA treatment, for dataset B (fold increase ≥ 2, FDR ≤ 5%) (*A*), or dataset C (fold increase ≥ 2, Storey's *q*-value FDR ≤ 5%) (*B*). (*Right*) Pie charts display number of coding and noncoding RNAs enriched in PKR$_{WT}$ in dataset B and dataset C, and stacked columns show percentage of snoRNAs in the enriched noncoding RNAs. More enriched genes were observed in dataset C because the binomial test used for this single replicate analysis does not control for overdispersion in the data.

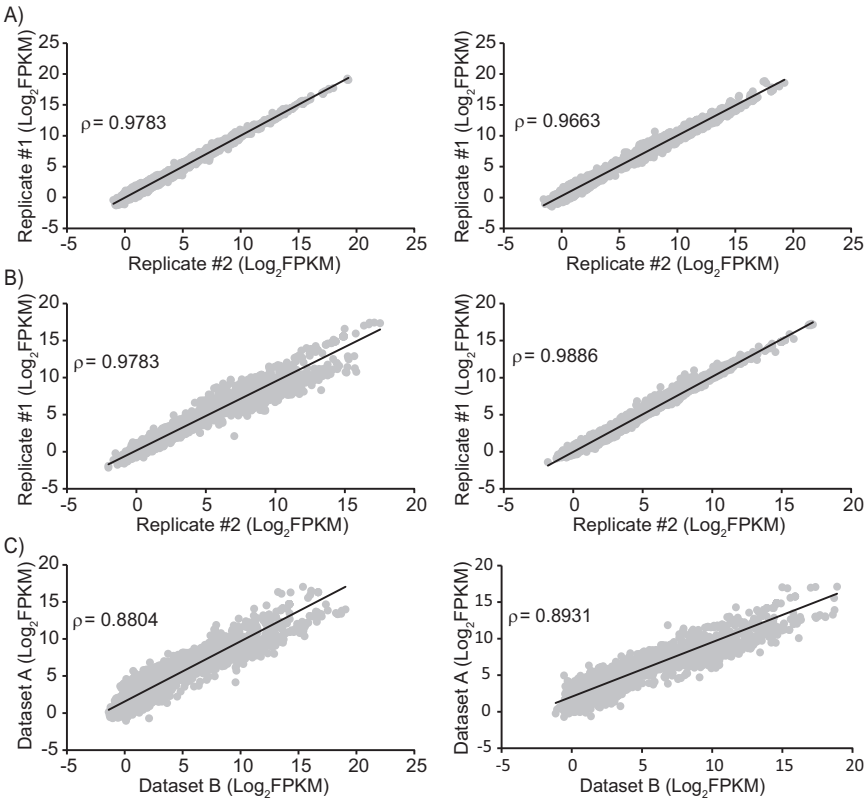

Fig. S2. Scatter plots with Spearman's correlation show good agreement between biological replicates. Correlation scatter plots between biological replicates 1 and 2 in dataset A (*A*) and dataset B (*B*) and between biological replicates in dataset A and B (*C*). Mock-treated samples (*Left*) and PA-treated samples (*Right*). Fragments per kilobase of exon per million fragments mapped (FPKM) of dataset A and dataset B in *C* is calculated as an average FPKM of the three biological replicates in each dataset. "ρ" indicates the Spearman correlation coefficient.

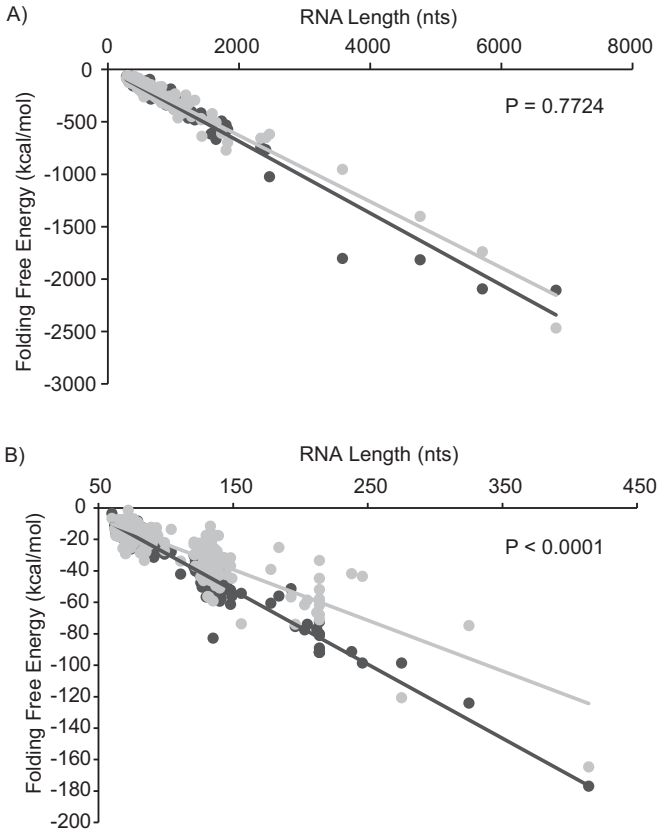

Fig. S3. Structural stability of non-snoRNAs and snoRNAs from RIP-Seq data. Scatter plots of predicted folding free energy (*y* axis) against RNA length (*x* axis) of enriched non-snoRNA regions using Scan-Seqs data (*A*) and snoRNAs using Scan-Seqs and DRDS data (*B*). Enriched RNA, dark gray. Length-matched randomly generated transcribed regions, light gray. RNA secondary structure free energies were calculated using UNAFold 3.8. *P* value is from the Wilcoxon matched-pairs signed rank test.

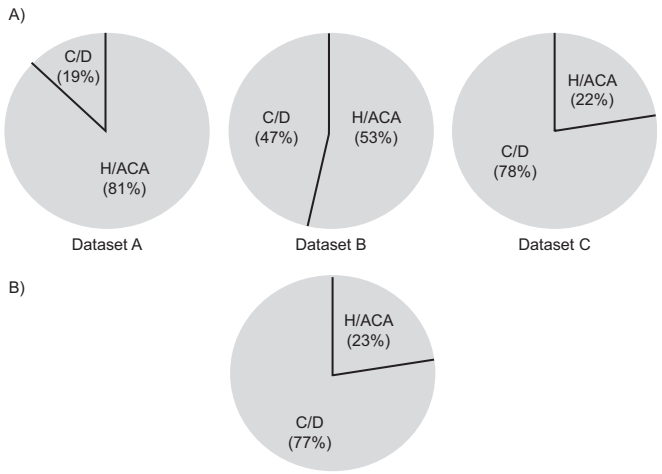

Fig. S4. Classification of snoRNAs enriched only in PKR_{WT} in the three datasets. Pie charts display the snoRNA classes in the annotated regions enriched only in PKR_{WT} in each of the three datasets identified by DRDS (datasets A and B) and by DRSS (dataset C) (*A*) and identified by the ScanSeqs approach using all three datasets (*B*).

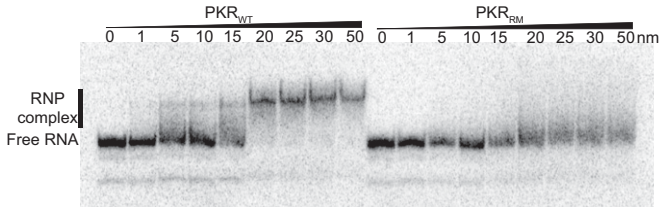

Fig. S5. snoRNAs bind to PKR_{WT} but not PKR_{RM} in vitro. In vitro-transcribed ^{32}P-uniformly labeled SNORD113 (50 pM, triphosphorylated) was incubated with increasing amounts of PKR_{WT} or PKR_{RM} (nM as indicated) followed by electrophoresis on a native gel to separate PKR-bound species from free RNA (*SI Materials and Methods*). The RNA signal was quantified from a PhosphorImage using ImageQuant software (GE Healthcare).

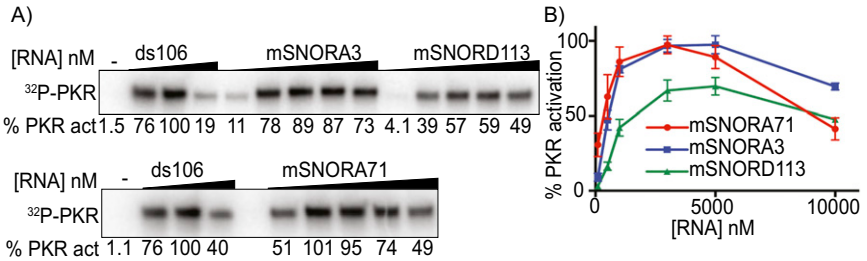

Fig. S6. PKR activation by snoRNAs follows a bell-shaped curve. (*A*) PKR autophosphorylation by snoRNAs. snoRNA concentrations were 100, 1,000, 3,000, 5,000, and 10,000 nM; ds106 concentrations were 1, 5, and 50 nM. Phosphorylation activities were normalized with reactions of 5 nM ds106. (*B*) Averaged activation data for SNORA3, SNORA71, and SNORD113. Error bars represent mean ± SE, $n \geq 2$. snoRNA (2000 nM) was tested and is shown on the graph but not on the gel.

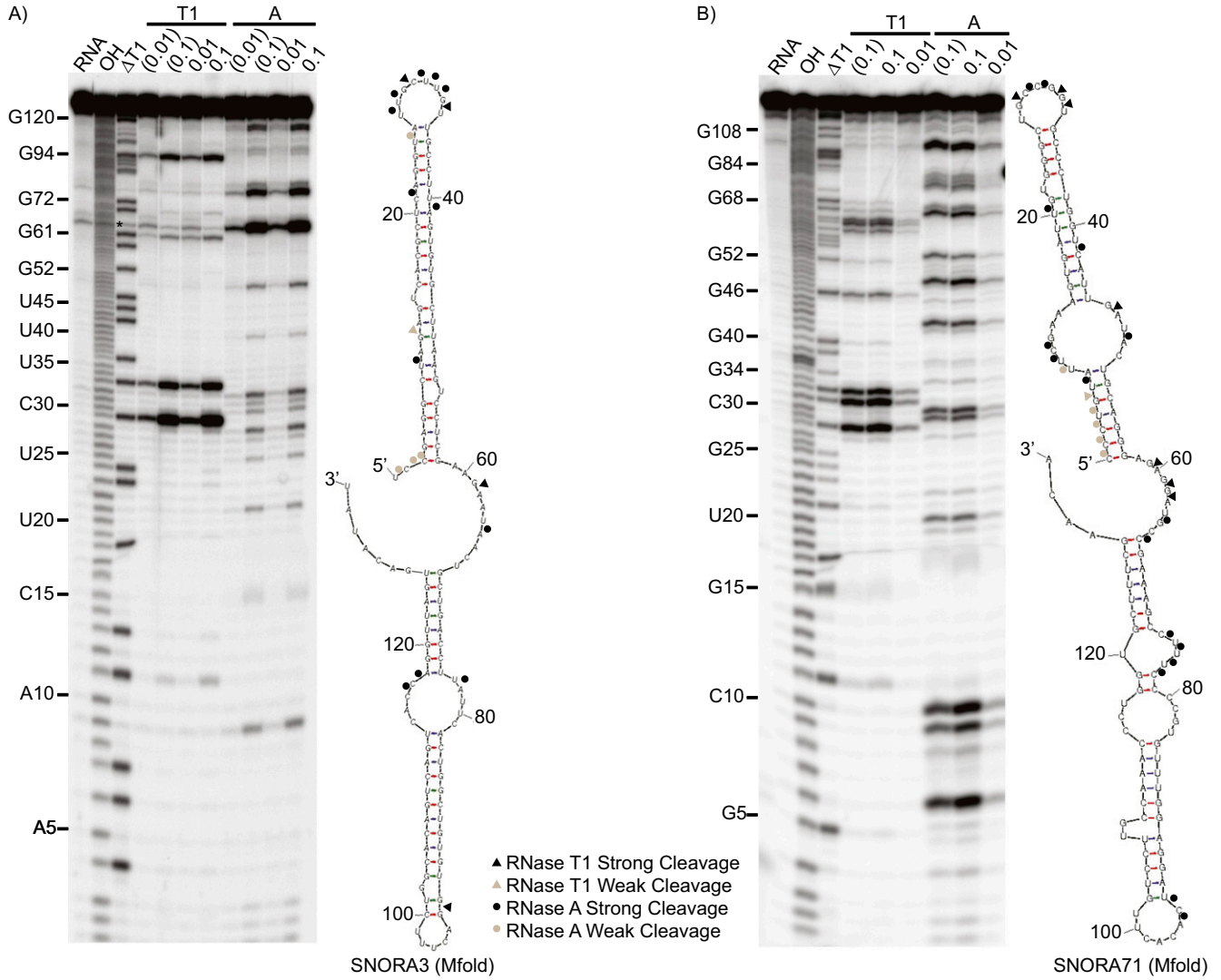

Fig. S7. Secondary structure mapping of SNORA3 and SNORA71. 5′-End–labeled SNORA3 (*A*) and SNORA71 (*B*) was digested with indicated concentrations of RNase T1 (unit) and RNase A (ng). RNA indicates no treatment, OH indicates alkaline hydrolysis, ΔT1 indicates RNase T1 cleavage under denaturing conditions, T1 and A indicate RNase T1 and A cleavage under native conditions, respectively. Secondary structure of each snoRNA (predicated using Mfold) is shown to the right side of the corresponding sequencing gel. RNase T1 and A cleavages were performed at 30 °C (parentheses) or 37 °C under native conditions. U121–U132 in SNORA3 and C109–A131 in SNORA71 were not mapped. The asterisk indicates nonspecific cleavage.

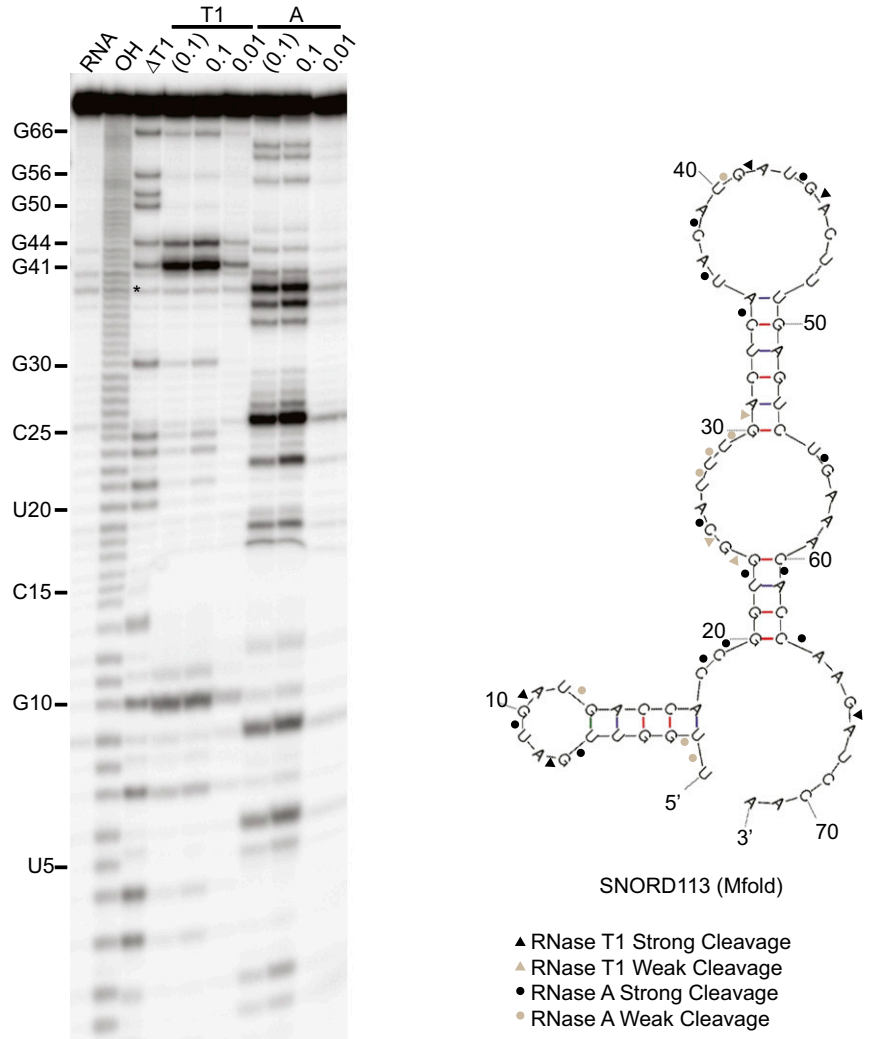

Fig. S8. Secondary structure mapping of SNORD113. Nuclease cleavage and secondary structure of SNORD113. Labels are as in Fig. S7. A67–A72 in SNORD113 was not unmapped. An asterisk indicates nonspecific cleavage.

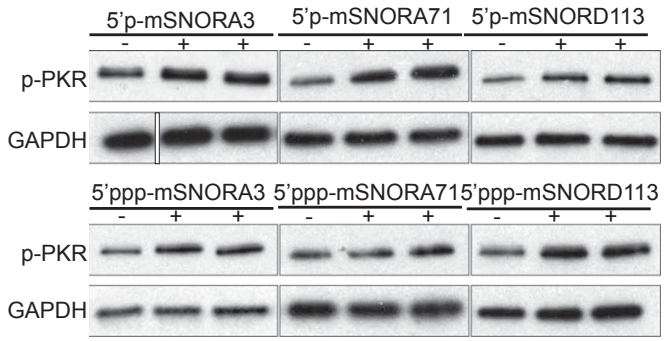

Fig. S9. snoRNAs activate PKR in cells. Western analysis of p-PKR in mock-treated (–) and snoRNA-treated (+) cells. GAPDH, loading control. White space indicates where intervening lanes were removed from the figures.

Dataset S1. Enriched annotated genes in PKR_{WT} and PKR_{RM} after PA treatment in the three datasets identified using DRDS/DRSS

Dataset S1

Dataset S2. Enriched annotated and unannotated regions in PKR_{WT} and PKR_{RM} after PA treatment common to the three datasets identified using ScanSeqs

Dataset S2

Dataset S3. Primers used for qPCR and PCR (to generate templates for in vitro transcription)

Dataset S3

Dataset S4. Number of total, aligned, and unique reads in the three datasets (extracted from Novoalign outputs)

Dataset S4