Supporting Information

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SI Materials and Methods

Lentiviral Infection and Total RNA Preparation. For Lentivirus production, 293T cells were plated in six-well dishes at 6×10^5 cells/well. Forty-eight hours after cotransfection of viral and shRNA plasmids into 293T cells, lentivirus was harvested and used directly to infect mESCs in a six-well plate at 2×10^5 cells/well. The infection media was 1:2, viral media: mESC media with 2 mM polybrene. Infected mESCs were then selected for 24 hours with 2 μM puromycin. Total RNA was collected using the RiboPure Kit (Ambion/Applied Biosystems) according to the manufacturer's protocol.

RT-qPCR of RNA Transcripts. All other qPCR experiments were performed with *Power* SYBR Green PCR Master Mix (Applied Biosystems). Primers to detect uaRNAs, promoter RNAs, spliced mRNA (exon 1-2 junction, probe), and nascent mRNA (exon1-intron1, probe) are shown in Table S3. Analysis of relative transcript levels was calculated using the delta-delta Ct method. Once internal controls of β-Actin, GAPDH, and 28S rRNA were shown to be comparable standards, β-Actin was chosen as the internal control for all experiments. Error between biological replicates was calculated using SEM. Statistical significance was determined using a two-tailed, paired *t* test. *P*-values of <0.05 were reported for all RT-qPCR analysis.

Standard curves (for absolute quantification of uaRNAs) were generated with qPCR using uaRNA-specific primers to amplify ssDNA Ultramer Oligonucleotides. mESCs were collected, counted using a Coulter Counter (Millipore), and total RNA was prepared to determine average RNA concentration per mESC. A quantified number of cells were subjected to qPCR using the uaRNA primer pairs and resulting qPCR signal was converted to copy number based on the ssDNA molar equivalents.

Rapid Amplification of 5′ Complementary DNA Ends (5′-RACE). The following modifications were made to the FirstChoice RLM-RACE Kit (Ambion). First, T4 RNA Ligase 1 (ssRNA Ligase) was heat-inactivated for 15 minutes at 65 °C. Second, SuperScript III (SSIII, Invitrogen) was used for cDNA synthesis according to the manufacturer's protocol. Reverse transcription was performed with a target-specific primer, gsp1, at a 0.25 μM final concentration. In addition, two subsequent nested PCR reactions using HotStarTaq (Qiagen) was performed with two forward primers, P_o and P_i, 5′-RACE adaptor-specific primers (Ambion), and two reverse target-specific primers, gsp-2 and gsp-3, at a 0.4 μM final concentration (Table S4). PCR 1 and PCR 2 were amplified for 20 and 25 to 30 cycles, respectively. PCR reaction products were run on a 2% agarose gel, extracted using a QIAquick Gel Extraction Kit (Qiagen), and sequenced using Sanger methods.

Rapid Amplification of 3′ Complementary DNA Ends (3′-RACE). Large fractionated RNA (5 μg) was DNase-treated with the Turbo DNA-Free Kit (Ambion). The ligation reaction was performed with a 3′-RACE adaptor, synthesized with a 5′-phosphate and a 3′-dideoxy-C (IDT) (Table S5) and used at a final 50 μM concentration for 3′ end ligation. All primers for 3′-RACE are listed in Table S5. Reverse transcription was modified in the following manner: the SSIII protocol for GC-Rich templates was used in place of the standard and reverse transcription was performed with an adaptor-specific primer, 3′-RACE P_o, at 0.25 μM final concentration. Two subsequent PCR reactions were performed similarly as 5′-RACE, but instead with two forward target-specific primers, gsp-1 and gsp-2, and two reverse adaptor-specific primers 3′-RACE P_o and P_i.

Flavopiridol and Doxorubicin Treatment of mECSs. Flavopiridol was added directly to the mESC culture media to a final concentration of 1 μM and allowed to incubate for 1, 5, 10, 15, 30, and 60 min in a tissue culture incubator at 37 °C. For the wash-off experiments, flavopiridol treated mESCs were washed once with 37 °C 1× Phosphate Buffered Saline. Fresh mESC media was added to the flavopiridol treated mESCs and they were placed at 37 °C for 30, 60, or 90 min before total RNA was isolated as described above. Doxorubicin was added directly to the mESC culture media to a final concentration of 1 μM and incubated for 1.5, 4, or 6 h at 37 °C. After the specified time, RNA was isolated as previously described and RT-qPCR was preformed to assay transcript levels.

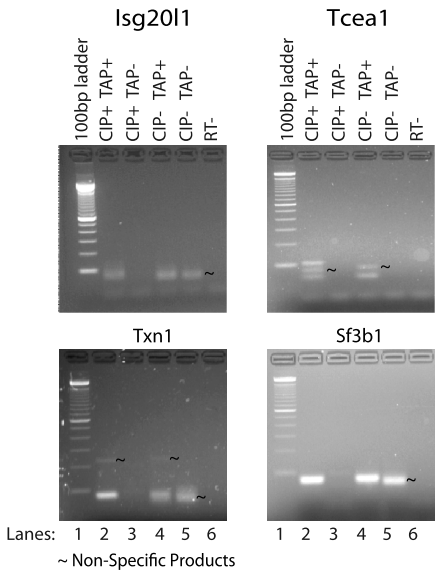

Fig. S1. uaRNAs contain a cap structure at the 5′ termini. All PCR products were collected from the gel, cloned into a TOPO vector, and transformed into competent cells. Five colonies for each band show above were selected for sequencing. From these sequencing results, specific products from the upstream antisense regions were only detected in the TAP+ lanes. For Tcea1, the addition of CIP prior to TAP treatment allowed for the detection of an additional antisense 5′ end cap site likely explained by the increase in PCR efficiency upon removal of uncapped background RNA (lanes 2 and 4). Size markers are shown to the left of each gel.

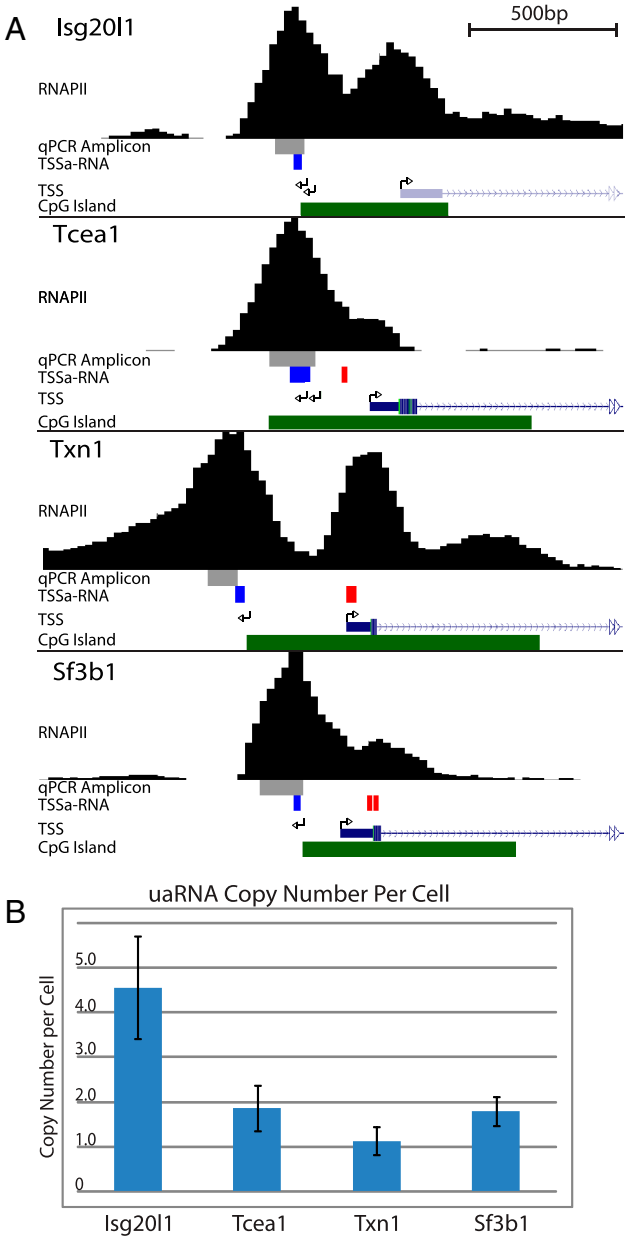

Fig. S2. Capped antisense RNA from divergent transcription initiate upstream of antisense TSSa-RNAs. (*A*) UCSC Genome browser view showing the location of detected antisense TSSs using rapid amplification of 5′ cDNA ends (5′-RACE) at four selected CpG island promoter genes: Isg20l1, Tcea1, Txn1, and Sf3b1. Arrows depicting antisense transcription are pointing to the left while sense TSSs are marked with arrows pointing to the right. Tracks: RNAPII ChIP-seq profiles in mESCs (black), qPCR amplicon (gray), antisense (blue) and sense (red) TSSa-RNAs, and CpG island (green). Each genomic region displayed spans 2 kb and the scale bar represents 500 bp. (*B*) Absolute quantification of the upstream antisense RNAs (uaRNAs) determined by qPCR using a ssDNA oligonucleotide standard. The values represent biological triplicates and error bars are SEM.

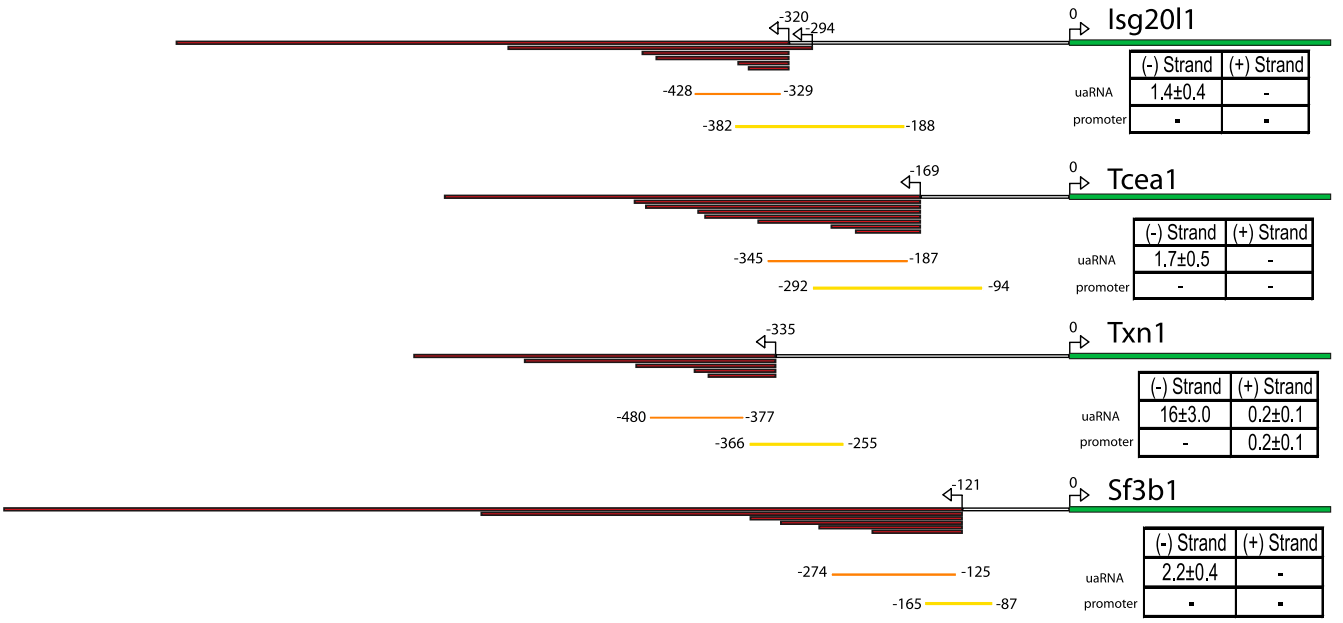

Fig. S3. Strand-specific RT-qPCR on RNAs that originate in the upstream antisense or promoter regions. Promoter regions are diagrammed for Isg20l1, Tcea1, Txn1, and Sf3b1, respectively. Sense TSSs are noted with a right facing arrow and set to position zero. Upstream antisense TSSs are noted with a left facing arrow. All uaRNA identified are represented by red bars and the first 300 nts of each sense transcript is shown as green bars. Amplicons for the "upstream" and "promoter" qPCR primers (Table S3) are shown for each gene as orange and yellow bars, respectively. Absolute quantitation was used to calculate copy number from strand-specific RT-qPCR reactions and copy numbers appear in the table below each sense TSS. The (–) and (+) strand columns represent transcripts amplified from either the "–" or "+" strand using a strand-specific RT primer, while the rows "upstream" and "promoter" correspond to the primer pair used in the experiment.

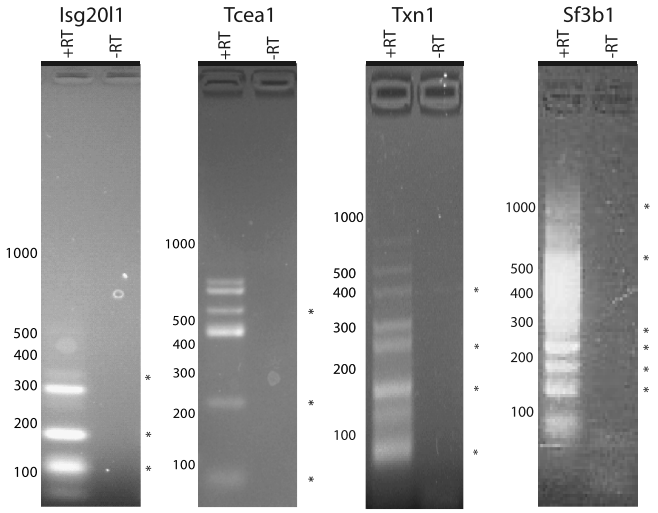

Fig. S4. 3′-RACE products are reverse-transcriptase dependent. 3′-RACE products were separated on a 2% agarose gel and the specific bands that were cloned and sequenced are marked with an asterisk (*) and shown in the UCSC Genome Browser in Fig. 1. The above gels are examples that yielded uaRNA sequences shown in Fig. 1. Samples displaying -RT did not receive reverse-transcriptase during cDNA synthesis. Size markers are shown to the left of each gel.

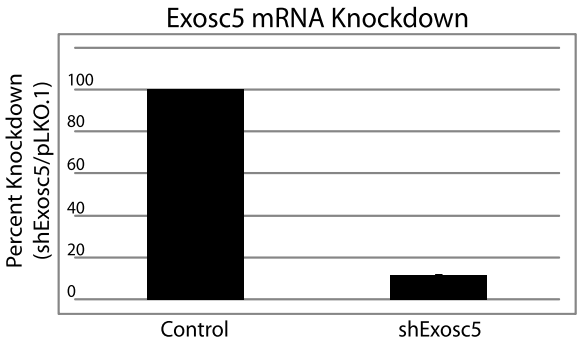

Fig. S5. shRNA-mediated Exosc5 knockdown in V6.5 ES cells. Relative gene expression of Exosc5, component of the exosome, as determined by RT-qPCR using Taqman probes (Table S1). Percent mRNA levels are shown compared to mock knockdown RNA samples. Values represent biological triplicates and error bars represent SEM of the biological replicates.

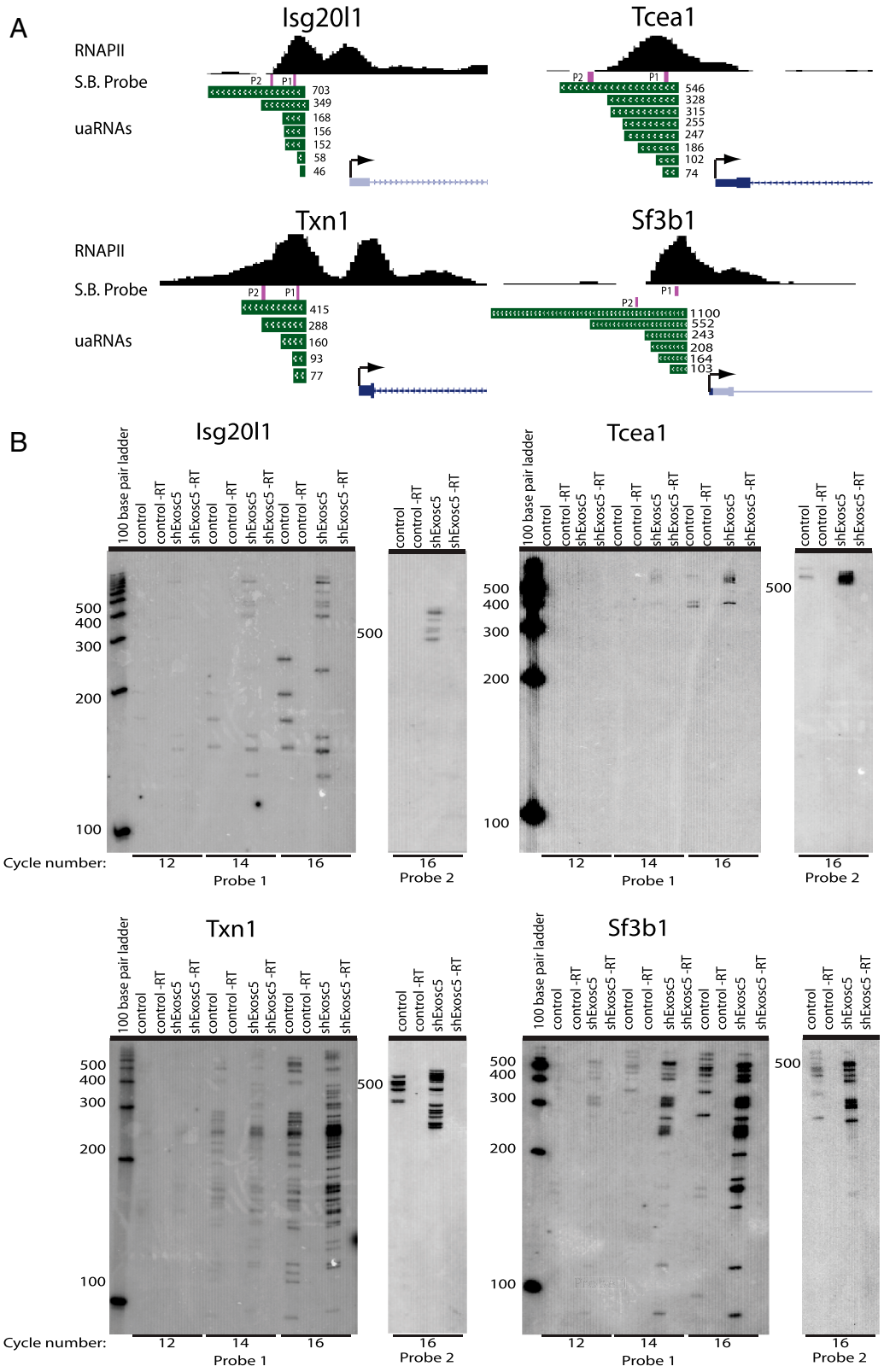

Fig. S6. uaRNAs have 3′ heterogeneity and their lengths are altered upon exosome depletion. (*A*) Genome browser views of the four divergent promoter regions displaying ChIP-Seq signal for RNAPII, the mapped uaRNA transcripts, and the southern blot probes used in *B* and in Fig. 2*B*. For each region, two probes were designed to be either proximal (probe 1) or distal (probe 2) to the antisense TSS. (*B*) 3′-RACE followed by Southern blot analysis from either control (pLKO.1) or knockdown (shExosc5) mESCs. For each uaRNA region, both probes described above were used to visualize the RNA species. A range of PCR cycles was used during the 3′-RACE protocol to assay the most abundant transcripts as determined by the detection of the initial products. The number of cycles used for control and knockdown samples are indicated below each blot.

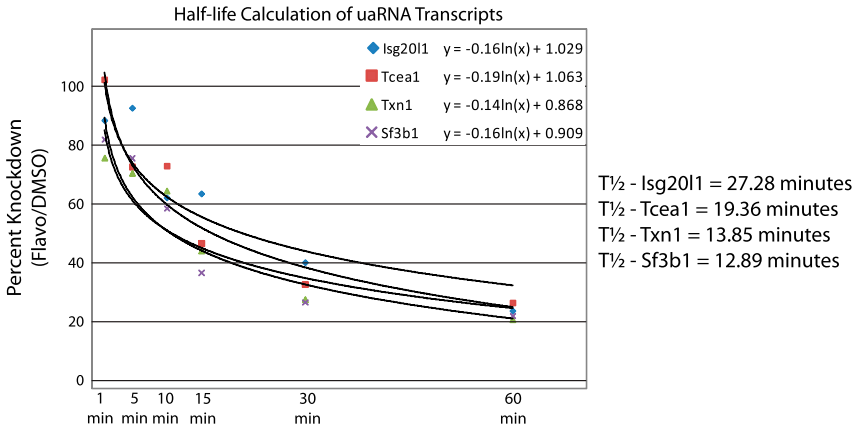

Fig. S7. uaRNA half-life calculations. Scatter plot of the relative uaRNA transcript abundances over a one hour flavopiridol treatment. For each gene, a best fit logarithmic curve was determined and equations corresponding to each gene are shown in the upper right-hand corner. Using the equations above, half-lives were calculated and shown for each gene to the right.

Table S1. Taqman primers and shRNAs used for mRNA knockdown

Gsym, Complex	Accession ID	Open Biosystems shRNA ID	Applied Biosystems Expression Assay
Supt4h, DSIF	NM_009296	RMM4534-NM_009296	Mm02527263_g1
Supt5h, DSIF	NM_013676	RMM4534-NM_013676	Mm01170629_m1
NELF-E, NELF	NM_138580	RMM4534-NM_138580	Mm01134804_m1
NELF-A, NELF	NM_011914	RMM4534-NM_011914	Mm01217228_m1
Exosc5, Exosome	NM_138586	RMM4534-NM_138586	Mm00506830_m1
GAPDH	NM_008084.2	N/A	4352932E

Table S2. ssDNA ultramer oligonucleotides for absolute copy number determination

	Sequence (5′-3′)
Isg20l1	TTATTTCACGGACTTCTCACAACCCCAAGCCTGGAGGGGCTGCAGTTCCCCGA
	GGCAGATCGGGATGTGCTCTTTGAGGCTTTAAGTCTTTGAAGGTTGCGGTTCACTAGGCGTCGGGTC
Tcea1	TGCTCATGCGCTTTAAGCCCTCGGCAATGCCTGTCCTGCGTCCCAGAGAACGCT
	CTGCCGGAGGGGTTTCGATGGAACTCGTAGCAACCTACCGCCTACTGCCTGAT
	CCCTCTGGCGTGAAAGCCGGACTCCGTCCAACTCCAGCTCGCCAGCAACGCGAG
	TCCGGATAGGGCCGGAAGT
Txn1	ATCTGACTTAGGTCTAGTTTGGGGCATGGGCAGTGTGATTACAGAAGGACTCTA
	CGGTGTGAGAGAGGACCGTGATCTACCCCGGCGCTGTTCGCTGTTAAAGTGCC
	CTTGAGGCAGCTGGAAGT
Sf3b1	GACAGGCTTTGTCTGTACAGCCCTGGCTTCGGGAACTCTCTTTGTAGACCAGGC
	TGGCCTCGAACTGCCTCTTCTCTTCCGAGTGCTTGAATTAACGGCACGTTACCC
	ACCACTGGCCGGACAGGCTACAGCCCTCTTGGGAAGTAGCCATCCTCTTCCGCGTTTT

Table S3. Primers used in RT-qPCR for relative transcript level analysis

Gene—Location	Forward Primer (5′ → 3′)	Reverse Primer (5′ → 3′)
Isg20l1—Upstream Antisense	CCTAGTGAACCGCAACCTTC	GACTTCTCACAACCCCAAGC
Isg20l1—Exon1-Exon2	GGGTTGGTTTGCAACTAGGC	GCTCACAGGTTGGGGTAAGA
Isg20l1—Exon1-Intron1	GGGTTGGTTTGCAACTAGGC	CCCAAAAGCTTACAGACCA
Isg20l1—Promoter	TGATCCTGCTCCTCCTCAGT	AGATCGGGATGTGCTCTTTG
Tcea1—Upstream Antisense	CTATCCGGACTCGCGTTG	CTTTAAGCCCTCGGCAATG
Tcea1—Exon1-Exon2	GATGGACAAAATGGTGCAGA	TTCATCTGTGCTCTGCTTGC
Tcea1—Exon1-Intron1	GTTCGCATTGCCAAGAAGAT	GCAGCACGGACCTGAAAG
Tcea1—Promoter	GATCGCAGGAGACTGGAAAG	GGGTTTCGATGGAACTCGTA
Txn1—Upstream Antisense	GCCTCAAGGGCACTTTAACA	GGTCTAGTTTGGGGCATGG
Txn1—Exon1-Exon2	GCCAAAATGGTGAAGCTGAT	TGATCATTTTGCAAGGTCCA
Txn1—Exon1-Intron1	TGGATCCATTTCCATCTGG	CCGAGAGTGTCCTCTTCAGC
Txn1—Promoter	GCTGCCGAACAAGAACCTTA	TTGGCTCTTAGGGGTAGCTG
Sf3b1—Upstream Antisense	GCGGAAGAGGATGGCTACT	GTCTGTACAGCCCTGGCTTC
Sf3b1—Exon1-Exon2	GTGGACAAAATGGCGAAGAT	TGCCTTCTTGCCTTGAATTT
Sf3b1—Exon1-Intron1	GTGGACAAAATGGCGAAGAT	CTCGGTCGAGACCAGAGATG
Sf3b1—Promoter	TCCTTAAAAAGCCAGCGAAA	GACAGGCTACAGCCCTCTTG
β-Actin mRNA	GACGAGGCCCAGAGCAAGAGAGG	GGTGTTGAAGGTCTCAAACATG
28S rRNA	AGCAGCCGACTTAGAACTGG	TAGGGACAGTGGGAATCTCG
GAPDH	GTGTTCCTACCCCCAATGTGT	AATGTGATACCAGGAAATGAGCTT

Table S4. 5′-RACE primers

	Gsp-1 (5′-3′)	Gsp-2 (5′-3′)
Isg20l1	GCTCTTTGAGGCTTTAAGTCTTTGAAGG	CTTTGAGGCTTTAAGTCTTTGAAGGTTGC
Tcea1	TAGCAACCTACCGCCTACTGCC	CTACCGCCTACTGCCTGATCC
Txn1	GTGCCCTTGAGGCAGCTGGAAGTTGG	CCTTGAGGCAGCTGGAAGTTGGCTC
Sf3b1	GGACAGGCTACAGCCCTCTTGG	CTACAGCCCTCTTGGGAAGTAGC
	Gsp-3 (5′-3′)	
Isg20l1	GAGGCTTTAAGTCTTTGAAGGTTGCGG	
Tcea1	CTCTGGCGTGAAAGCCGGACTCC	
Txn1	GAGGCAGCTGGAAGTTGGCTCTTAGG	
Sf3b1	CAGCCCTCTTGGGAAGTAGCCATCC	

Table S5. 3′-RACE primers and adaptor sequence

	Gsp-1 (5′-3′)	Gsp-2 (5′-3′)
Isg20l1	CGACGCCTAGTGAACCGCAACC	ACCGCAACCTTCAAAGACTTAAAGCC
Tcea1	ACGGAGTCCGGCTTTCACGCCAGAG	GGAGTCCGGCTTTCACGCCAGAG
Txn1	GGCAGCTACCCCTAAGAGCC	CCTAAGAGCCAACTTCCAGCTGCC
Sf3b1	CGCGGAAGAGGATGGCTACTTCC	CCAAGAGGGCTGTAGCCTGTCC
Po	CGACTACCGCTACTTACTTGTGAC	
Pi	CTTGTGACGCAAACGACCGAAACTAC	
Adaptor Sequence	5′-/5Phos/rArArArGrUrArGrUrUrUrCrGrGrUrCrGrUrUrUrGrCrGr UrCrArCrArArGrUrArArGrUrArGrCrGrGr UrArGrUrCrG/3ddC/-3′	