Supporting Information

Kurosaki and Maquat 10.1073/pnas.1219908110

SI Materials and Methods

Plasmids. pCMV-MYC-UPF1 (1), pRLUC-Gl Norm, and pRLUC-Gl 39Ter (where 0 specifies the AUG initiation codon and CMV refers to the cytomegalovirus promoter) have been described (2). To remove cryptic splice sites from pRLUC-Gl Norm and pRLUC-Gl 39Ter, pdRLUC-Gl derivatives were generated in which the RLUC open translational reading frame was trimmed from 936 bp to 69 bp. To this end, KOD DNA polymerase (Novagen) was used to PCR amplify the entire plasmid except for 867 bp of the RLUC open translational reading frame by using the primer pair 5′-CTCGAGCTCAGTACATCAAGAGCTTCGTGGAG-3′(sense) and 5′-CCTTGGAAGCCATGGCGC-3′ (antisense). The resulting blunt-ended PCR products were ligated together by using T4 DNA ligase (TAKARA).

To construct pdRLUC-Gl 7Ter, pdRLUC-Gl 26Ter, pdRLUC-Gl 65Ter, pdRLUC-Gl 82Ter, pdRLUC-Gl 120Ter, or pdRLUC-Gl 131Ter, all of pdRLUC-Gl Norm was PCR amplified by using, respectively, the mutagenic primer 5′-CCTGACTCCTGAGTAGAAGTCTGCCG-3′(sense; 7Ter), 5′-GAAGTTGGTGGTTAGGCCCTGGGCAG-3′(sense; 26Ter), 5′-GTGAAGGCTCATGGCTAGAAAGTGCTCGGTG-3′(sense; 65Ter), 5′-CACCTGGACAACCTCTAGGGCACCTTTG-3′(sense; 82Ter), 5′-GGCCACCACCTGGGCTAGGATTTCACCCCCG-3′(sense; 120Ter), or 5′- GCACAGGCTGCCTTCTAGAAGGTGGTGGCTG-3′ (sense; 131Ter), where underlined nucleotides generate the denoted Ter codon, and a completely complementary antisense primers. Each resulting blunt-ended PCR fragment was then circularized by ligation.

To construct pdRLUC-Gl 155Ter, pdRLUC-Gl 163Ter, or pdRLUC-Gl 171Ter, the normal termination codon of pdRLUC-Gl Norm was converted to CAA by using the mutagenic primer 5′-CACAAGTACCACCAAACCCCCTTTC -3′(sense), where underlined nucleotides specify the CAA codon, and a completely complementary antisense primer. The resulting plasmid was PCR amplified by using, respectively, the mutagenic primer 5′-CCTGCTCTTGCCTGAGAACAATGGTTA -3′ (sense; 155Ter), 5′-GTTAATTGTTCCCTAGAGAGCATCTGTCAG -3′ (sense; 163Ter), or 5′-CTGTCAGTTGTTGGTAAAATGATAGAC -3′ (sense; 171Ter), where underlined nucleotides generate the denoted Ter codon, and a completely complementary antisense primer. Each resulting blunt-ended plasmid PCR fragment was then circularized by ligation.

To generate pdRLUC-Gl 101Ter, pmCMV-Gl 101Ter (3) was digested by using XbaI and DraIII, and the resulting 1.6-kbp fragment was introduced into the corresponding sites of pdRLUC-Gl Norm.

To construct pcDNA3.1-HIS-eRF3(KAKA), pEQ863-His-eRF3 (4) [which we refer to here as pcDNA3.1-HIS-eRF3(WT)] was PCR amplified by using the mutagenic primer pair 5′-AAAAAGCCCGCCTGCCAACGTCCACGCC-3′ (sense) and 5′- GGCGACGTTTTTTTGCCGGCTGAAGGCCGCGCT-3′ (antisense). The resulting blunt-ended plasmid PCR fragment was then circularized by ligation. pcDNA3.1-HIS-LACZ was purchased (Invitrogen).

To generate pcDNA3-Gl(Δ intron 2)-MS2bs, 5′-GTGGATCCTGAGAACTTCAGGCTCCTGGGCAACGTGCTGGTCTGTGTGCTGGCCCATCACTTTGGCAAAGAATTCA-3′, where underlined nucleotides denote a BamHI or EcoRI site, was annealed to a cDNA oligonucleotide. The annealed product was cleaved with BamHI and EcoRI and inserted into the BamHI and EcoRI sites of pcDNA3-Gl-MS2bs (5). To generate pcDNA3-Gl and pcDNA3-Gl(Δ intron 2), pcDNA3-Gl-MS2bs and pcDNA3-Gl(Δ intron 2)-MS2bs were digested by using NotI and XbaI to remove the MS2bs sequence. The ends of the resulting 6.8-kbp fragments were made blunt by using Klenow fragment (New England Biolabs) and circularized by ligation. The integrity of all constructs was validated by using DNA sequencing.

Immunoprecipitations. For MYC-UPF1 transfected cells, immunoprecipitation (IP) was performed by using anti-c-MYC agarose-conjugated beads (Sigma). For UPF1 IP, samples were generated before and after IP in the presence and/or absence of RNase A (Sigma) as reported (6) or before and after oligonucleotide-directed RNase H-mediated cleavage (7, 8) by using specific DNA oligonucleotides (Oligo 1 is 5′-AAGGGTAGACCAC-3′; Oligo 2 is 5′-CATAACAGCATCA-3′; Oligo 3 is 5′-TTGAGGTTGTCCAG-3′; and Oligo 4 is5′-TGCCAAAGTGATG-3′). IPs used anti-UPF1 (6) or antibodies that are described under Western blotting (see below).

siRNA-mediated Down-Regulation of XRN1. HeLa cells stably expressing pIRE-Gl Norm (7) were transiently transfected with 40 nM XRN1 siRNA (9) or a nonspecific control siRNA (Ambion) by using Lipofectamine RNAi Max (Invitrogen). After 24 h, cells were cultured in serum-supplemented DMEM containing 100 μM Df for 24 h and subsequently in serum-supplemented DMEM containing 100 μM of either Df or hemin. Cells were harvested 24 h thereafter.

Western Blotting. Proteins were electrophoresed in 6–14% polyacrylamide and transferred to either a nitrocellulose (GE Healthcare) or polyvinylidene difluoride (Millipore) membrane. Blots were probed by using anti-UPF1 (6), anti-MYC (Calbiochem), anti-calnexin (Enzo Life Sciences), anti–β-actin (Sigma), anti-ferritin (Abcam), anti-eRF3 (Sigma), anti-CBP80 (10), anti-HIS (Qiagen), anti-GAPDH (Santa Cruz Biotechnology), anti–α-tubulin (Santa Cruz Biotechnology), anti-XRN1 (Bethyl Laboratories), and anti–c-JUN (Santa Cruz Biotechnology).

RT-PCR. RT-PCR was performed essentially as described (8). Briefly, total-cell cDNA or cDNA after oligo-directed RNase H-mediated RNP cleavage was synthesized by using SuperScript III reverse transcriptase (Invitrogen) and either random hexamers or an mRNA-specific primer (Table S1). RT-PCR products were electrophoresed in 6% polyacrylamide and quantitated by using Typhoon PhosphorImaging (Molecular Dynamics).

Quantitative RT-PCR. RT coupled to quantitative real-time PCR (qRT-PCR) was undertaken essentially as specified (6–8) (Table S1) by using the 7500 Fast Real-Time PCR System (Applied Biosystems) and Fast SYBR Green Master Mix (Applied Biosystems).

1. Kim YK, Furic L, Desgroseillers L, Maquat LE (2005) Mammalian Staufen1 recruits Upf1 to specific mRNA 3′UTRs so as to elicit mRNA decay. *Cell* 120(2):195–208.
2. Woeller CF, Gaspari M, Isken O, Maquat LE (2008) NMD resulting from encephalomyocarditis virus IRES-directed translation initiation seems to be restricted to CBP80/20-bound mRNA. *EMBO Rep* 9(5):446–451.
3. Matsuda D, Hosoda N, Kim YK, Maquat LE (2007) Failsafe nonsense-mediated mRNA decay does not detectably target eIF4E-bound mRNA. *Nat Struct Mol Biol* 14(10):974–979.
4. Janzen DM, Geballe AP (2004) The effect of eukaryotic release factor depletion on translation termination in human cell lines. *Nucleic Acids Res* 32(15):4491–4502.

5. Lykke-Andersen J, Shu MD, Steitz JA (2000) Human Upf proteins target an mRNA for nonsense-mediated decay when bound downstream of a termination codon. *Cell* 103(7):1121–1131.
6. Gong C, Kim YK, Woeller CF, Tang Y, Maquat LE (2009) SMD and NMD are competitive pathways that contribute to myogenesis: Effects on PAX3 and myogenin mRNAs. *Genes Dev* 23(1):54–66.
7. Sato H, Maquat LE (2009) Remodeling of the pioneer translation initiation complex involves translation and the karyopherin importin beta. *Genes Dev* 23(21):2537–2550.
8. Hwang J, Sato H, Tang Y, Matsuda D, Maquat LE (2010) UPF1 association with the cap-binding protein, CBP80, promotes nonsense-mediated mRNA decay at two distinct steps. *Mol Cell* 39(3):396–409.
9. Franks TM, Singh G, Lykke-Andersen J (2010) Upf1 ATPase-dependent mRNP disassembly is required for completion of nonsense- mediated mRNA decay. *Cell* 143(6):938–950.
10. Izaurralde E, et al. (1994) A nuclear cap binding protein complex involved in pre-mRNA splicing. *Cell* 78(4):657–668.

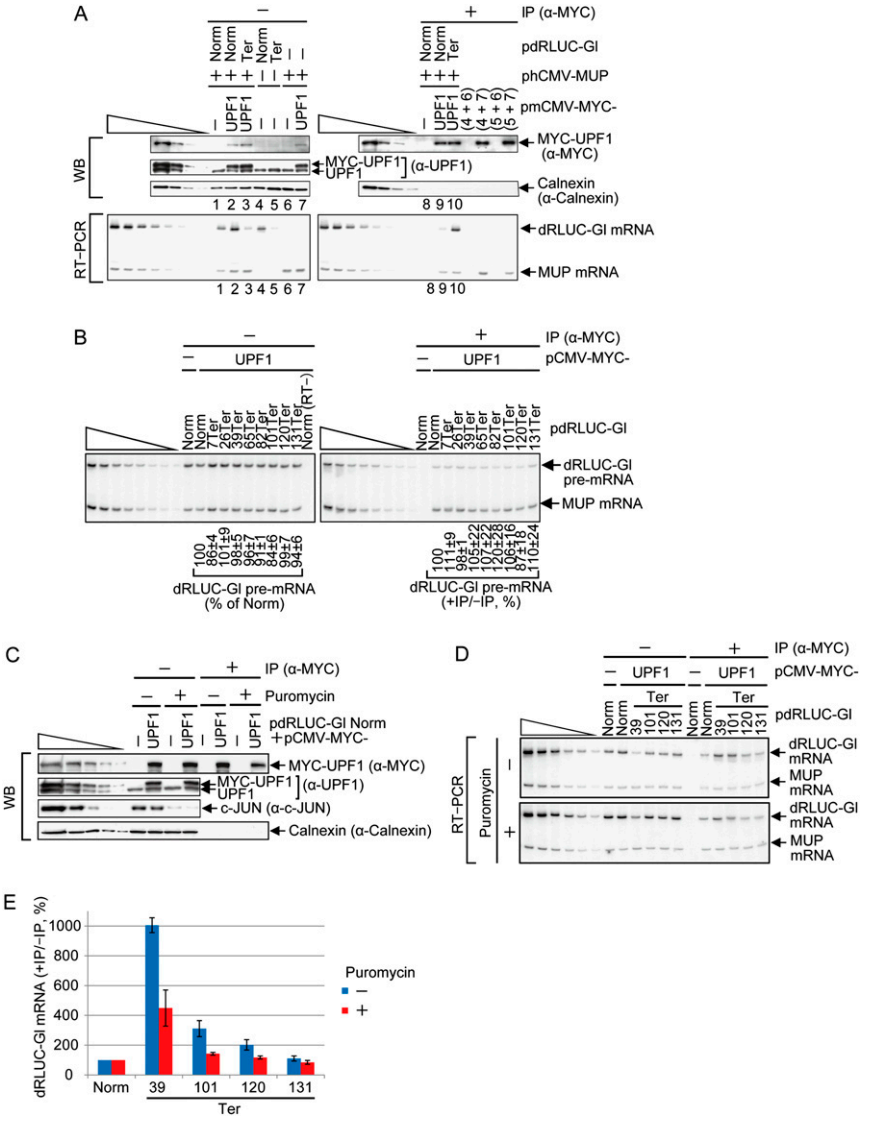

Fig. S1. Lysate mixing experiments, demonstration that UPF1 binding to pre-mRNA is nonspecific, and demonstration that UPF1 binding to mRNAs that are and are not NMD targets depends on translation. (*A*) HEK293T cells (8×10^7) were transiently transfected with the following plasmids so the amount of introduced DNA was constant: lanes 1 and 8, pdRLUC-Gl Norm (1 μg), phCMV-MUP (0.5 μg), and pmCMV-MYC (1 μg); lanes 2 and 9, pdRLUC-Gl Norm (1 μg), phCMV-MUP (0.5 μg), and pmCMV-MYC-UPF1 (1 μg); lanes 3 and 10, pdRLUC-Gl Ter (1 μg), phCMV-MUP (0.5 μg), and pmCMV-MYC-UPF1 (1 μg); lane 4, pdRLUC-Gl Norm (1 μg), pmCMV-MYC (1 μg), and pCMV-HA (0.5 μg); lane 5, pdRLUC-Gl Ter (1 μg), pmCMV-MYC (1 μg), and pCMV-HA (0.5 μg); lane 6, pCMV-HA (1 μg), phCMV-MUP (0.5 μg), and pmCMV-MYC (1 μg); lane 7, pCMV-HA (1 μg), phCMV-MUP (0.5 μg), and pmCMV-MYC-UPF1 (1 μg). Lysates were analyzed before (–) or after (+) IP by using anti-MYC before (lanes 8–10) or after (lanes 4+6, 4+7, 5+6, 5+7) the specified lysates were mixed. Shown are Western blotting (*Upper*) and RT-PCR (*Lower*). (*B*) RT-PCR of most RNA samples analyzed in Fig. 1*C*, except dRLUC-Gl pre-mRNAs rather than dRLUC-Gl mRNAs were analyzed. The level of UPF1 binding to Norm pre-mRNA was ∼40-fold lower than the level of UPF1 binding to Norm mRNA. (*C* and *D*) HEK 293T cell were transfected as in Fig. 1 *B* and *C*. However, the specified plasmids were used, and cells were incubated in the presence (+) or absence (–) of puromycin (100 μg/mL) for 6 h before lysis. (*C*) Western blotting before (–) or after (+) IP by using anti-MYC. The level of MYC-UPF1 was comparable (<1.5-fold) to the level of cellular UPF1. Measurements of c-JUN protein, which has a half-life of only ∼90 min (1, 2), was used to demonstrate that translation was indeed inhibited by puromycin. (*D*) RT-PCR of dRLUC-Gl mRNA before (–) or after (+) IP. (*E*) Histograms representation of MYC-UPF1 binding to each dRLUC-Gl mRNA using data shown in *D*, where the level of dRLUC-Gl mRNA was normalized to the level of MUP mRNA, and the normalized level of dRLUC-Gl Norm mRNA was defined as 100%. See Table S1 for primer pairs used in PCR. All quantitations derive from two to three independently performed experiments and represent the mean ± SDs.

1. Lamph WW, Wamsley P, Sassone-Corsi P, Verma IM (1988) Induction of proto-oncogene JUN/AP-1 by serum and TPA. *Nature* 334(6183):629–631.
2. Treier M, Staszewski LM, Bohmann D (1994) Ubiquitin-dependent c-Jun degradation in vivo is mediated by the δ domain. *Cell* 78(5):787–798.

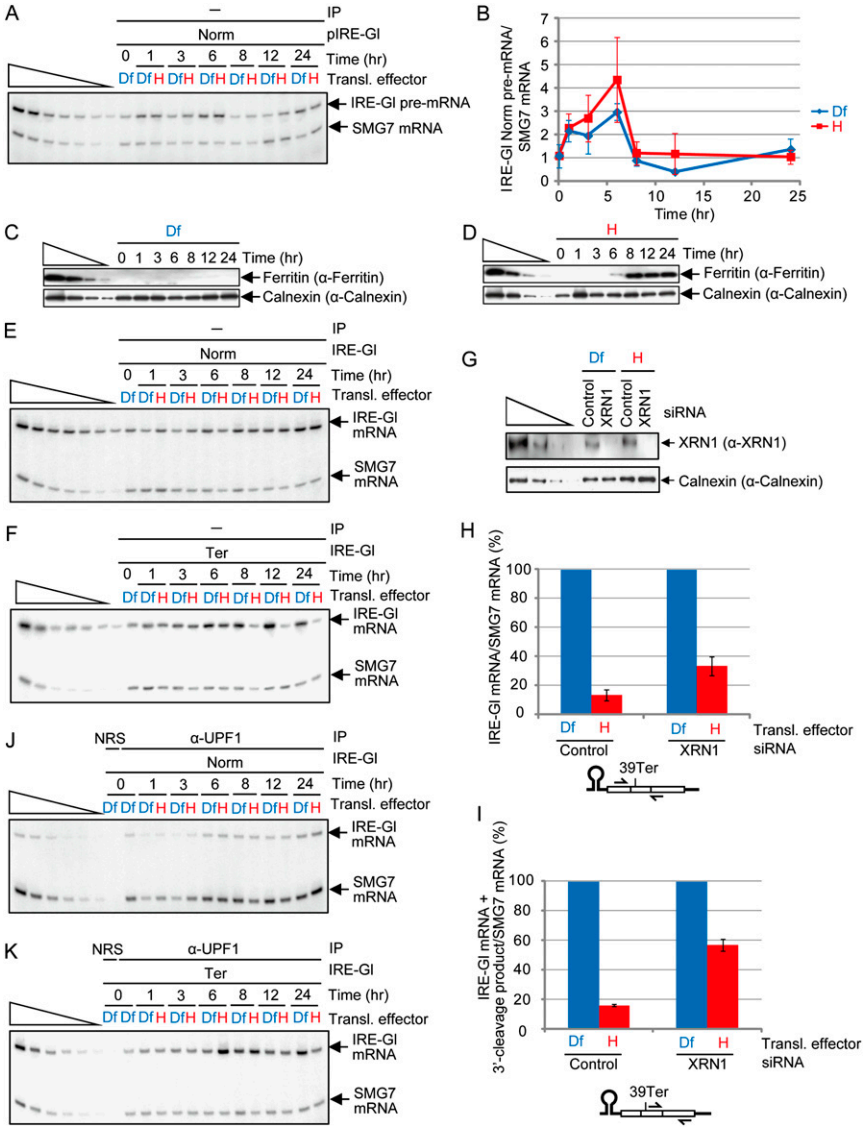

Fig. S2. Evidence that hemin, unlike Df, promotes formation of a SMG6-generated IRE-Gl Ter mRNA 3′-cleavage product. (*A*) RT-PCR of IRE-Gl Norm pre-mRNA and SMG7 mRNA using RNA from samples analyzed in Fig. 2*C*, demonstrating that the burst in IRE-Gl Norm gene transcription returned to steady-state by 8 h after the addition of fresh medium at 0 h. (*B*) Plot of data in *A*, where the level of IRE-Gl Norm pre-mRNA was normalized to the level of SMG7 mRNA. Results show that IRE-Gl Norm pre-mRNA is induced upon the addition of fresh DMEM supplemented with 10% FBS and either Df or hemin. (*C*) Cells were cultured as in *A* except only Df was used. Western blotting using anti(α)-ferritin reveals that Df-mediates translational inhibition. (*D*) As in *C* only Df was replaced by hemin. Western blotting using anti(α)-ferritin reveals that hemin-mediates translational induction. (*E*) As in *A*, except IRE-Gl Norm mRNA was analyzed. (*F*) As in *E*, except cells expressing IRE-Gl Ter mRNA were analyzed. (*G–I*) HeLa cells (1×10^7) stably expressing IRE-Gl Ter mRNA were cultured DMEM supplemented with 10% FBS and 100 μM Df and transiently transfected with 40 nM Control siRNA (Ambion) or XRN1 siRNA. After 24 h, cells were cultured for an additional 24 h in DMEM supplemented with 10% FBS and 100 μM Df, and subsequently incubated with 100 μM of either Df or hemin for another 24 h. (*G*) Western blotting shows that XRN1 siRNA reduced the cellular level of XRN1, which is a 5′-to-3′ exonuclease, to an undetectable level. (*H*) RT-qPCR of IRE-Gl Ter mRNA using PCR primers that amplify the full length of the mRNA, i.e., primers that span exon 1–exon 3, reveals that XRN1 siRNA inhibits the decay of mRNA. (*I*) RT-qPCR of IRE-Gl Ter mRNA using PCR primers that amplify downstream of the PTC, i.e., primers that span exon 2–exon 3 so as to measure not only full-length mRNA but also the SMG6-generated 3′-cleavage product of mRNA. The finding that XRN1 siRNA up-regulates the abundance of IRE-Gl Ter mRNA sequences downstream of the PTC more than it up-regulates the abundance of uncleaved IRE-Gl Ter mRNA indicates that NMD indeed occurs during the drop in the level of full-length mRNA. (*J* and *K*) As in *E* and *F* except cells were analyzed after anti-UPF1 IP. See Table S1 for primer pairs used in PCR. Quantitations derive from three independently performed experiments and represent the mean ± SDs.

Table S1. List of RT, PCR, or qPCR primers used in the specified figures

Figure	RNA	RT primer(s)	Sense (S) and antisense (AS) PCR primers
Fig. 1*C* and Fig. S1 *A*, *B*, and *D*	dRLUC-Gl mRNA	Random hexamers	S: 5′-TGGAGCGCGTGCTGAAGAAC-3′ AS: 5′-CACGATCATATTGCCCAGGAG-3′
	MUP mRNA	Random hexamers	MUP-S: 5′-CTGATGGGGCTCTATG-3′ MUP-AS: 5′-TCCTGGTGAGAAGTCTCC-3′
Fig. 1*F*	Gl mRNA	Random hexamers	S: 5′-CAAGGTGAACGTGGATGAAG-3′ AS: 5′-CCACTTTCTGATAGGCAGCC-3′
	MUP mRNA	Random hexamers	MUP-S and MUP-AS
Fig. S1*B*	dRLUC-Gl pre-mRNA	Random hexamers	S: 5′-GGTTACAAGACAGGTTTAAGGAGAC-3′ AS: 5′-CCCAGGAGGTGCCCATCAGAC-3′
	MUP mRNA	Random hexamers	MUP-S and MUP-AS
Fig. 2 *B*–*F* and Fig. S2 *E*, *F*, *J*, and *K*	IRE-Gl mRNA	Random hexamers	S: 5′-CCTGCTTCAACAGTGCTTGGACGGAACCC-3′ AS: 5′-CCGAGCACTTTCTTGCC-3′
	SMG7 mRNA	Random hexamers	SMG7-S: 5′-CCAAAGGAGACCATCTGA-3′ SMG7-AS: 5′-CCTCATCTCGGCTTTCC-3′
Fig. S2 *A* and *B*	IRE-Gl pre-mRNA	Random hexamers	S: 5′-CCTGCTTCAACAGTGCTTGGACGGAACCC-3′ AS: 5′-TAAACCTGTCTTGTAACC-3′
	SMG7 mRNA	Random hexamers	SMG7-S and SMG7-AS
Fig. S2*H*	IRE-Gl mRNA	Random hexamers	S: 5′-CAAGGTGAACGTGGATGAAG-3′ AS: 5′-CACACAGACCAGCACGTTG-3′
	SMG7 mRNA	Random hexamers	SMG7-S and SMG7-AS
Fig. S2*I*	IRE-Gl mRNA	Random hexamers	S: 5′-CCTTTGCCACACTGAGTGAG-3′ AS: 5′-ACACAGACCAGCACGTTGCC-3′
	SMG7 mRNA	Random hexamers	SMG7-S and SMG7-AS
Fig. 3 *C* and *D*	Gl mRNA	Random hexamers	S: 5′-ACCACCGTAGAACGCAGATCG-3′ AS: 5′-CACGATCATATTGCCCAGGAG-3′
	GPx1 mRNA	Random hexamers	S: 5′-ACCACCGTAGAACGCAGATCG-3′ AS: 5′-CTTCTCACCATTCACCTCGCACTT-3′
	MUP mRNA	Random hexamers	MUP-S and MUP-AS
Fig. 4*D*	FL IRE-Gl mRNA	5′-GAATCCAGATGCTCAAGGCCC-3′	S: 5′-CAAGGTGAACGTGGATGAAG-3′ AS: 5′-CCACTTTCTGATAGGCAGCC-3′
	5′-CP IRE-Gl mRNA	5′-CACAGGGCAGTAACGGCAG-3′	S: 5′-ACTGTGTTCACTAGCAACCC-3′ AS: 5′-CACAGGGCAGTAACGGCAG-3′
	3′-CP IRE-Gl mRNA	5′-GAATCCAGATGCTCAAGGCCC-3′	S: 5′-GGTTCCTTTGTTCCCTAAGTCC-3′ AS: 5′-GAATCCAGATGCTCAAGGCCC-3′
	SMG7 mRNA	5′-CCTCATCTCGGCTTTCC-3′	S: 5′-ACTATAGGCATGCAGCTCAG-3′ AS: 5′-GTGCTTTAGAAAGTGCTTTTTGC-3′