Supporting Information

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SI Materials and Methods

Plasmid Construction for PCR Standards. To create the 1–2-3–4-5 plasmids for UBA52 and RPL23, cDNA from HeLa cells was used as a template to amplify each mRNA by PCR using primers UBA52 Fwd 5′GGATCCTCTAGAGGCCGAGCTGACG-CAAACATG3′ and UBA52 Rev 5′CAGCTGAAGCTTGG-GACACTTTATTGAGGCTCCAGG3′, or RPL23Fwd 5′GT-TCAAGATGTCGAAGCGAGGAC3′ and RPL23Rev 5′GATGCAATCCGGGGCCACAAGTC3′. The resulting fragment from the latter reaction was used to seed a subsequent PCR for cloning (primers RPL23ExoFwd 5′ACCGGCCTCGAGGT-TCAAGATGTCGAAGCGAGGAC3′ and RPL23ExoRev 5′TCGGTACCCGGGGATGCAATCCGGGGC-CACAAGTC3′). The PCR products were inserted into SP73 vector digested with *Hin*dIII and *Xba*I. To create the 1–3-4–5, 1–4-5, 1–5, 1–2-4–5, 1–2-5, and 1–2-3–5 plasmids for UBA52 and RPL23, the 1–2-3–4-5 plasmids were used with appropriate forward and reverse primers (primer information is available upon request). Cloning was performed using a procedure first used by Li and Evans (1).

RT-PCR Primer Specificity Calculations. To define the specificity of each primer set, two reactions were set up for each primer set. The first reaction included primers specific for the skipping event and matching plasmid. In a second reaction, the same primer set was added along with plasmid containing the constitutively spliced (1–2-3–4-5) plasmid. For example, to determine the specificity of the primer that amplifies the product that splices exon 1 to exon 3, we set up two reactions. The first reaction included the 1–3 primer set and the 1–3 plasmid. The second reaction included the 1–3 primer set and the 1–2-3–4-5 plasmid. The cycle threshold (CT) values were recorded for each reaction, and the difference in CT values was used to calculate the difference in amplification rounds. From the CT difference, the difference in copy number between the two reactions was calculated (Specificity = $2^{\Delta CT}$). Every reaction was carried out in triplicate. The specificity calculation is a low estimate of how specific the primers are because in these reactions, only plasmid is included. In the context of a cDNA sample with many different DNA molecules, the potential for the primer to interact with a similar target sequence is much less than within a PCR sample that only contains the similar target sequence. Therefore, there should be much more mis-priming in the sample that only contains the plasmid compared with a cDNA sample with many different potential target sequences. To more accurately report the fidelity of splice-site pairing in our cDNA sample, we subtracted the potential number of molecules due to mis-priming events as calculated in our specificity measurements.

RNAi Conditions and Sequences. HeLa cells were grown and plated at 1.25×10^5 cells/mL in MEM medium (Cellgrow) containing 10%FBS, and siRNA was transfected at a concentration of 0.1 pmol/μL using Lipofectamine 2000 (Invitrogen). hUpf1, SMN, or control siRNA were down-regulated using, respectively, 5′-GAUGCAGUUCCGCUCCAUUdTT-3′ (2), anti-SMN: 5′-GAAGAAUACUGCAGCUUCCdTT-3′ (3), and control sequence (non-targeting siRNA #1), all from Dharmacon. Media was changed after 5 h and replaced with fresh media. Cells were harvested 3 days later and assayed for knockdown. Primers to check for hUpf1 knockdown were first described by (4), Fwd 5′-CCTGCTGCAGGGCGAGGCAC-3′ and Rev 5′-CTGCAT-TCTAGTTGTGGTTTG-3′. Splicing of gene #3 and #263 were shown to be differentially spliced upon hUpf1knockdown (5). These genes were analyzed by RT-PCR. Primers to check for SMN knockdown; Fwd 5′-ATAATTCCCCCACCACCTC-3′ and Rev 5′-GCCTCACCACCGTGCTGG-3′.

1. Li C, Evans RM (1997) Ligation independent cloning irrespective of restriction site compatibility. *Nucleic Acids Res* 25:4165–4166.
2. 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* 120:195–208.
3. Carissimi C, Saieva L, Gabanella F, Pellizzoni L (2006) Gemin8 is required for the architecture and function of the survival motor neuron complex. *J Biol Chem* 281:37009–37016.
4. Sun X, Perlick HA, Dietz HC, Maquat LE (1998) A mutated human homologue to yeast Upf1 protein has a dominant-negative effect on the decay of nonsense-containing mRNAs in mammalian cells. *Proc Natl Acad Sci USA* 95:10009–10014.
5. Pan Q, *et al.* (2006) Quantitative microarray profiling provides evidence against widespread coupling of alternative splicing with nonsense-mediated mRNA decay to control gene expression. *Genes Dev* 20:153–158.

Fig. S1. Specificity of real-time PCR primer sets. Specificity of the UBA52 and RPL23 junction primer sets. Specificity is defined as the number of correct priming events per 1 mis-priming event.

A

UBA52

Splice Site Strength

25
20
15
10
5
0

0 0.0005 0.001 0.0015 0.002

Error Rate

B

RPL23

Splice Site Strength

19
18.5
18
17.5
17
16.5
16

0 0.0002 0.0004 0.0006 0.0008 0.001 0.020 0.025

Error Rate

Fig. S2. Error rate correlated with splice-site strength. Using maxentscan score from Chris Burge's website (http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html) the combined splice-site strength was computed for each skipping event. For example, for the 1–3 splicing event, the 5′ splice site of exon 1 was combined with the 3′ splice site of exon 3. The combined splice-site strength was plotted against the error rate for each splicing event.

Fig. S3. Error rates observed using cDNAs generated from oligo(dT) priming. Graph displaying the number of copies detected for each splicing event in cDNA made by reverse transcribing with oligo(dT).

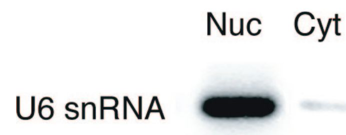
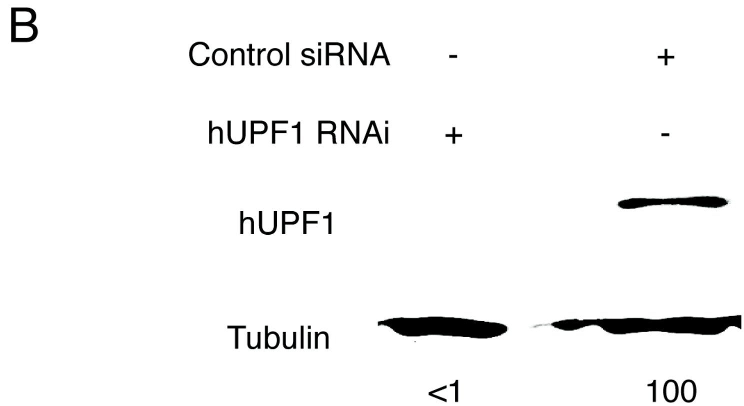


Fig. S4. U6 snRNA PCR. RT-PCR of cytoplamic and nuclear RNA fractions using primers for U6 snRNA. U6 is predominately found in the nucleus and is used as a marker to control for nuclear and cytoplasmic fractionation efficiency.



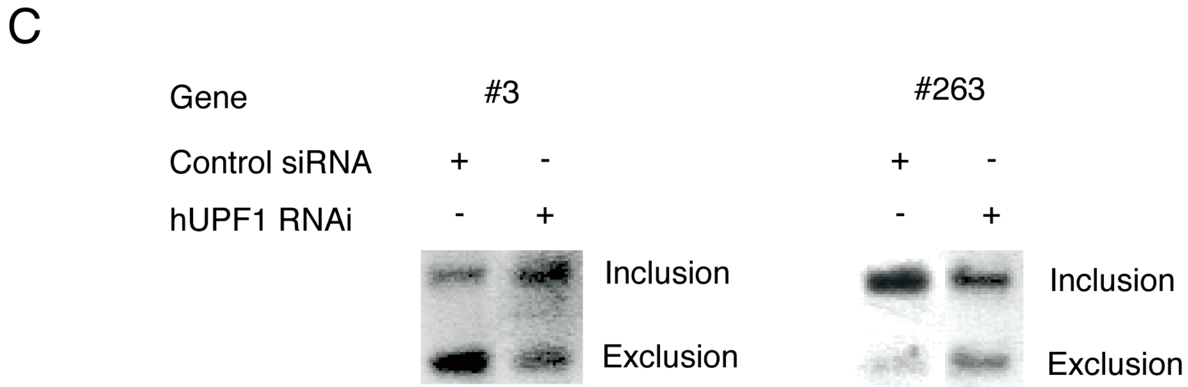


Fig. S5. Levels of hUpf1 after hUpf1 knockdown in HeLa cells. (*A*) RT-PCR showing levels hUpf1 mRNA after control or hUpf1 RNAi knockdown. Levels of UBA52 were used as a loading control. (*B*) Western blot showing protein hUpf1 mRNA after control or hUpf1 RNAi knockdown. Tubulin was used as a loading control. (*C*) Alternative splicing of two NMD target genes was used as a control for functional hUpf1 knockdown. Gene #3 contains an exon that is alternatively included upon hUpf1 knockdown, and gene #263 contains an exon that is alternatively excluded upon hUpf1 knockdown (5).

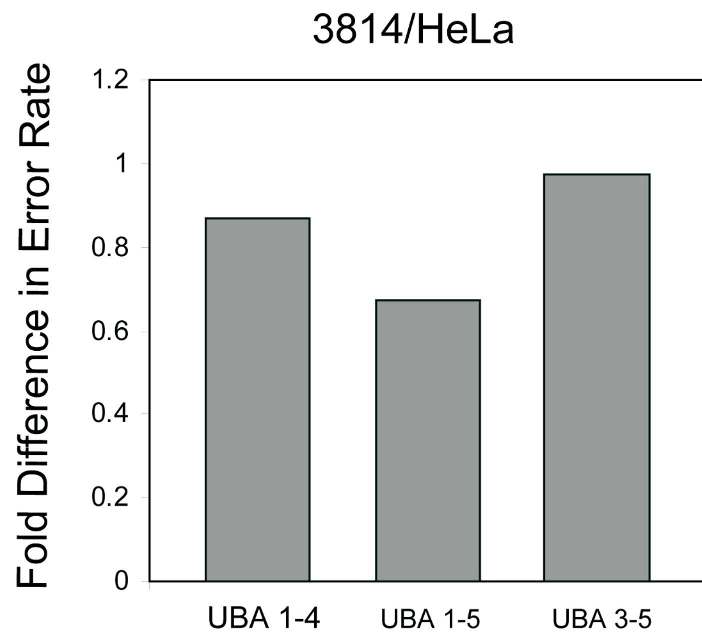


Fig. S6. Error rate comparison between HeLa and fibroblast cell lines. The relative difference in splice-site pairing error levels is plotted. The RNA samples compared were obtained from HeLa cells or from the SMA control cell line 3814.

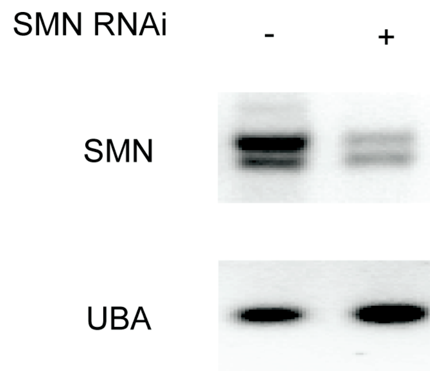


Fig. S7. Levels of SMN mRNA after SMN knockdown in HeLa cells. RT-PCR showing SMN mRNA levels after control or SMN RNAi knockdown. UBA52 levels were used as a loading control.

Table S1. UBA52 exon junction primers used

Primer name	Sequence	Annealing temp., °C	Concentration, nM
1–3 Fwd	5′GGCCGAGCTGGTATCC3′	56	300
1–3 Rev	5′GCAGGGTGGACTCTTTC3	56	300
1–4 Fwd	5′GCCGAGCTGAGTCAA3′	56	300
1–4 Rev	5′GCGAGCATAGCACTTG3′	56	300
1–5 Fwd	5′CCGAGCTGGTGCTATG3′	56	300
1–5 Rev	5′AACCACCTTATTTGACCTTC3′	56	300
2–4 Fwd	5′GACAGGGAGGAGTCCAC3′	53	300
2–4 Rev	5′TCTTGCGGCAGTTGAC3′	53	300
2–5 Fwd	5′GACAAGGAGGGTGCTATG3′	53	300
2–5 Rev	5′AACCACCTTATTTGACCTTC3′	53	300
3–5 Fwd	5′TCCAGAAAGGTGCTATGC3′	56	300
3–5 Rev	5′GAGGCTGCCCTTCAAG3′	56	300
1–2 Fwd	5′CGAGCTGACGCAAAC3′	53	300
1–2 Rev	5′GATACCCTCCTTGTCTTG3′	53	300
2–3 Fwd	5′AGACAAGGAGGGTATCC3′	56	300
2–3 Rev	5′TGAAGGCGAGCATAGC3′	56	300
3–4 Fwd	5′CATCCAGAAAGAGTCCAC3′	53	300
3–4 Rev	5′TGAAGGCGAGCATAGC3′	53	300
4–5 Fwd	5′CTGCCGCAAGTGCTATG3′	53	300
4–5 Rev	5′AACCACCTTATTTGACCTTC3′	53	300

Table S2. RPL23 exon junction primers used

Primer name	Sequence	Annealing temp, , ° C	Concentration
1–3 Fwd	5′GTCGAAGCGAGGAGCCAAAAACC3′	68	100
1–3 Rev	5′GCTCTGGTTTGCCTTTCTTGACTG3′	68	100
1–4 Fwd	5′GTCGAAGCGAGTACATCCAGC3′	66	200
1–4 Rev	5′GTCGAAGCGAGTACATCCAGC3′	66	200
1–5 Fwd	5′ GTCGAAGCGAGGTTCTGCCAT3′	63	200
1–5 Rev	5′GATGCAATCCGGGGCCACAAGTC3′	63	200
2–4 Fwd	5′TGACAACACAGACATCCAGCAG3′	66	200
2–4 Rev	5′AGAACCTTTCATCTCGCCTTTATTG3′	66	200
2–5 Fwd	5′TGGGTCCTCTGGTGCGAAATTC3′	61	100
2–5 Rev	5′GTAATGGCAGAACCTGTGTTGTCA3′	61	100
3–5 Fwd	5′CGCTGCTGGTGTGGGTGAC3′	63	100
3–5 Rev	5′GGCAGAACCCTTTTTTCTGA3′	63	100
1–2 Fwd	5′CGAAGCGAGGACGTGGTG3′	62	200
1–2 Rev	5′AGTCTGTTCAGCCGTCCCTTG3′	62	200
2–3 Fwd	5′GACAACACAGGAGCCAAAAACC3′	62	200
2–3 Rev	5′GCTCTGGTTTGCCTTTCTTGACTG3′	62	200
3–4 Fwd	5′GCTCAGAAAAAAGGTACATCCAGC3′	62	200
3–4 Rev	5′ACCTTTCATCTCGCCTTTATTGTTC3′	62	200
4–5 Fwd	5′ACATCCAGCAGTGGTCATTCG3′	62	200
4–5 Rev	5′TAATGGCAGAACCTTTCATCTCG3′	62	200

Table S3. Oligo-dT and Random Hexamer Comparison

	1–3	1–4	1–5	2–4	2–5	3–5
			UBA52 Skipping Event			
Oligo-dT	9.8×10^{-5}	2.7×10^{-4}	1.8×10^{-3}	4.3×10^{-4}	1.0×10^{-4}	3.3×10^{-4}
Random	1.6×10^{-4}	2.5×10^{-4}	7.4×10^{-4}	2.7×10^{-4}	2.9×10^{-4}	1.9×10^{-3}
Oligo/Random	0.6	1.1	2.4	1.6	0.35	0.2
			RPL23 Skipping Event			
Oligo-dT	9.0×10^{-5}	6.7×10^{-5}	2.1×10^{-4}	1.5×10^{-3}	2.1×10^{-2}	2.6×10^{-3}
Random	4.7×10^{-5}	5.7×10^{-6}	5.4×10^{-5}	4.5×10^{-4}	2.3×10^{-2}	9.4×10^{-4}
Oligo/Random	1.9	11	3.8	3.3	0.9	2.8

The numbers represent the splicing error rate for each mis-splicing event. The standard error calculated from at least 6 repetitions is less than 10%.