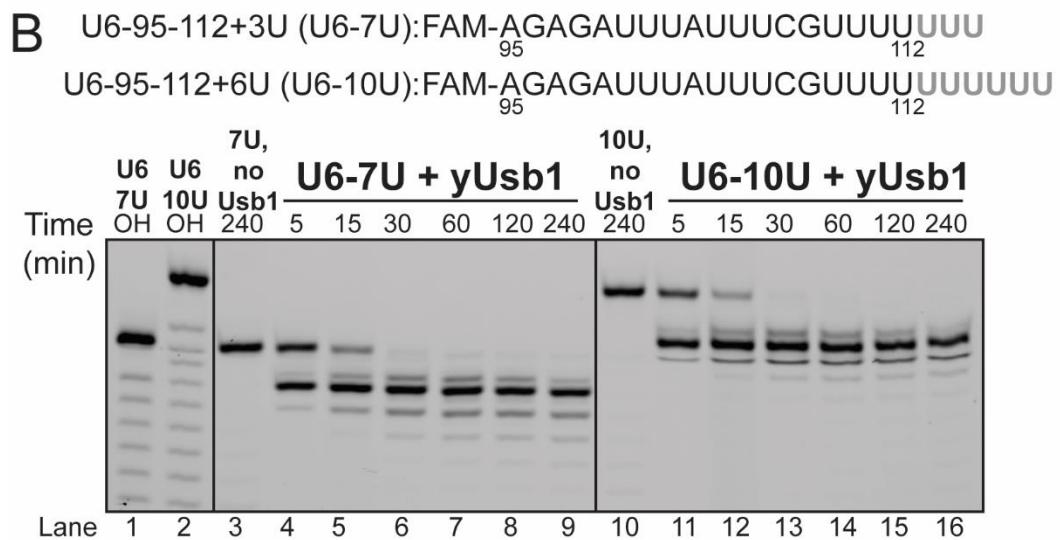
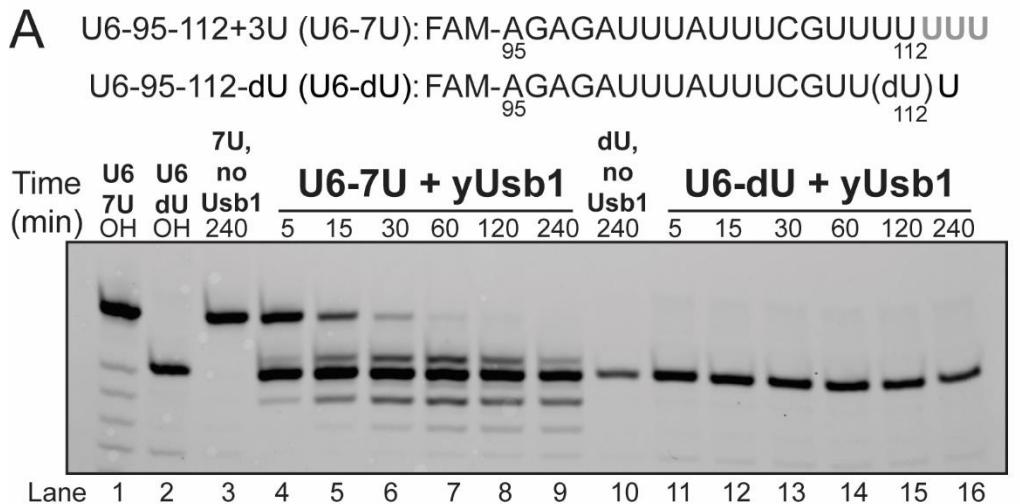


Description of Supplementary Files

File name: Supplementary Information

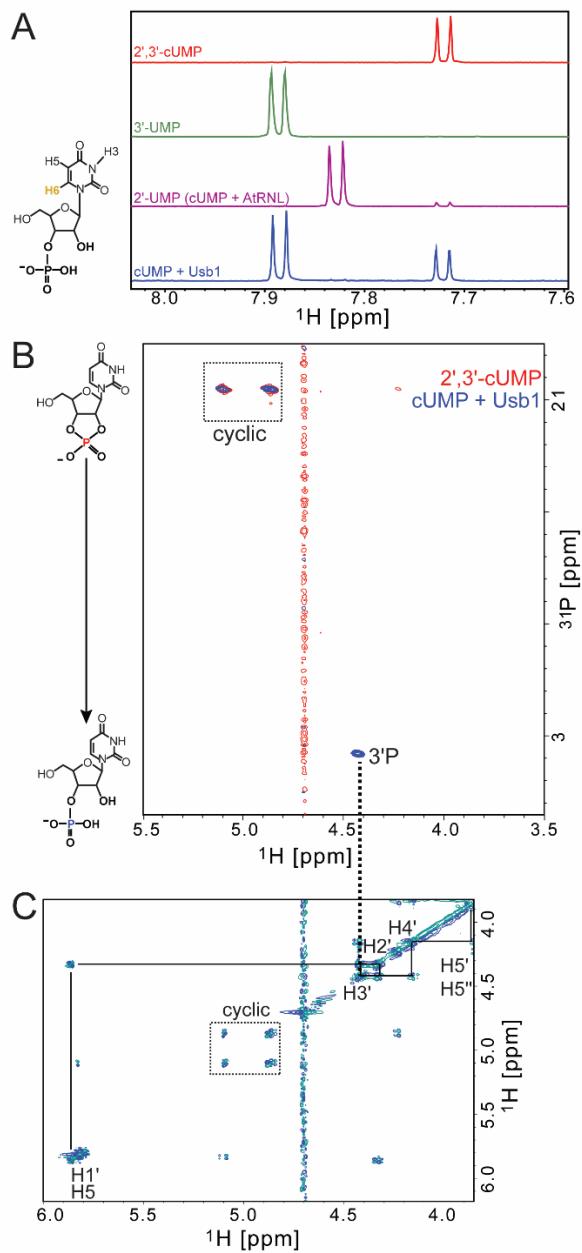
Description: Supplementary figures, supplementary tables and supplementary references.

File name: Peer review file



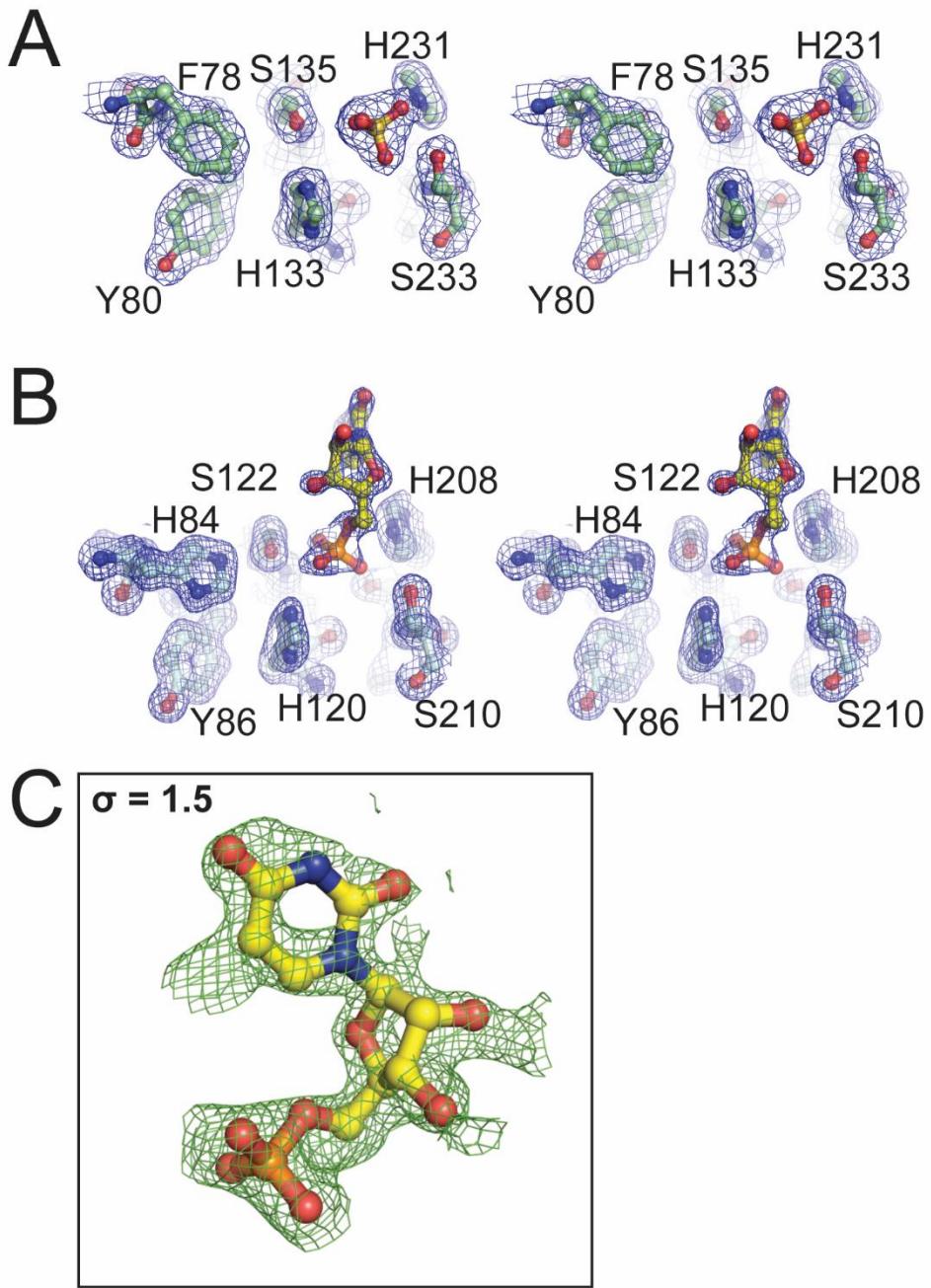
Supplementary Figure 1. Usb1 is inactive on substrates with a deoxyuridine adjacent to the scissile phosphate.

(a) Time course of Usb1 processing on substrates with three additional uridines (U6-7U, lanes 3-9) or where the *n*-1 nucleotide is a deoxyuridine (U6-dU, lanes 10-16) indicate that yUsb1 processing is exonucleolytic and depends on a 2' hydroxyl. Alkaline hydrolysis ladders of the RNAs (lanes 1-2) show the mobility of oligonucleotide products of different length. (b) Time course of Usb1 processing on substrates with three additional uridines (U6-7U, lanes 3-9) or six additional uridines (U6-10U, lanes 10-16) indicate that yUsb1 processing is independent of U-tail length. Alkaline hydrolysis ladders of the RNAs (lanes 1-2) show the mobility of oligonucleotide products of different length.



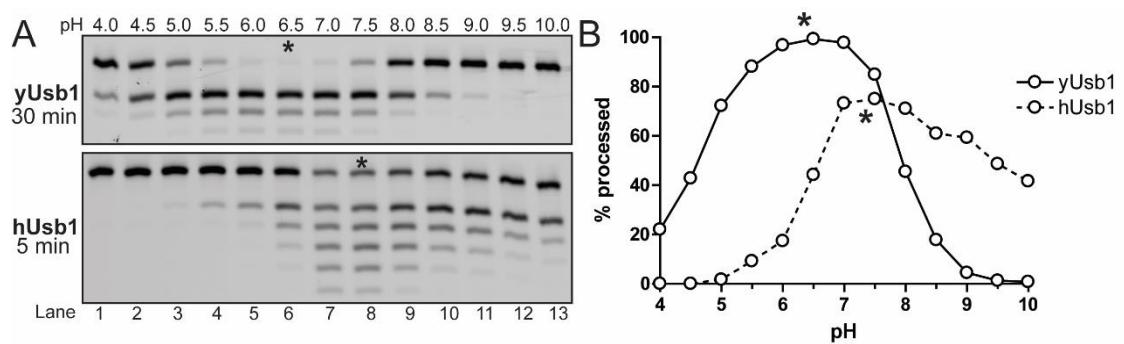
Supplementary Figure 2. NMR of Usb1-treated 2',3'-cUMP reveals Usb1 has CPDase activity and leaves a 3' phosphate.

(a) 1D-¹H spectra focusing on the ¹H chemical shift region for uracil H6 protons. 2',3'-cyclic UMP (red) was incubated with AtRNL (purple) or Usb1 (blue). The spectra of a 3' UMP standard is also shown. The H6 proton peaks are split by proton-proton coupling between H5 and H6. (b) 2D-¹H-³¹P HMBC overlay of 2',3'-cUMP before (red) and after incubation with AtRNL (purple) or Usb1 (blue). (c) 2D-¹H-¹H COSY of 2',3'-cUMP after incubation with Usb1.



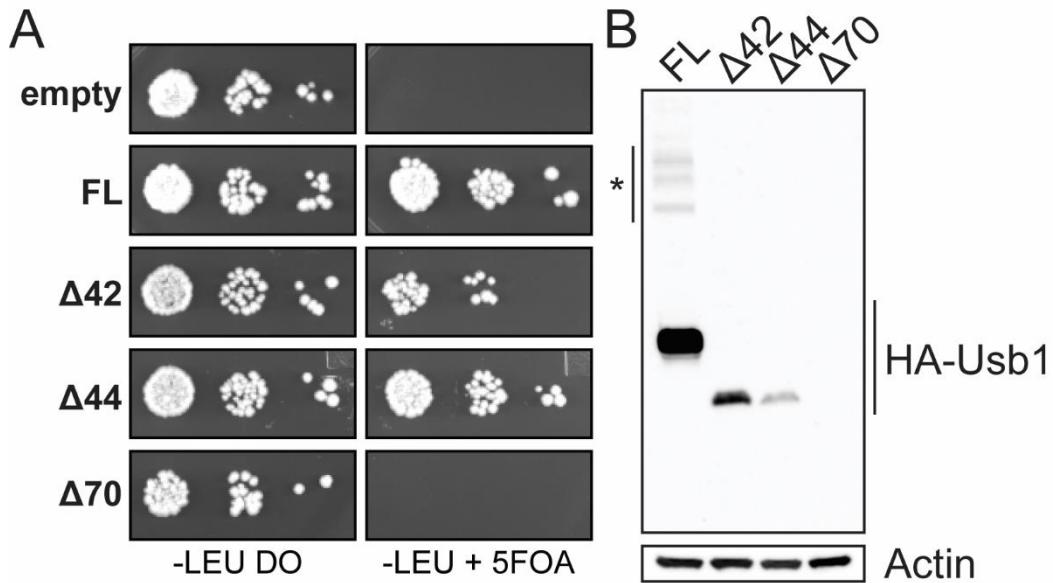
Supplementary Figure 3. Active site densities and annealed omit map.

- (a) Stereo view of the $2mF_o-DF_c$ electron density map of the yUsb1 active site, contoured at 1σ .
- (b) Stereo view of the $2mF_o-DF_c$ electron density map of the hUsb1/5'UMP active site, contoured at 1σ .
- (c) Annealed omit map of the hUsb1/5'UMP active site. The occupancy of the bound ligand was refined to a value of 0.72 in phenix.refine. The residual F_o-F_c density near to the C5 atom overlaps with a bound water molecule in the ligand-free structure reported previously¹ (PDB 4H7W).



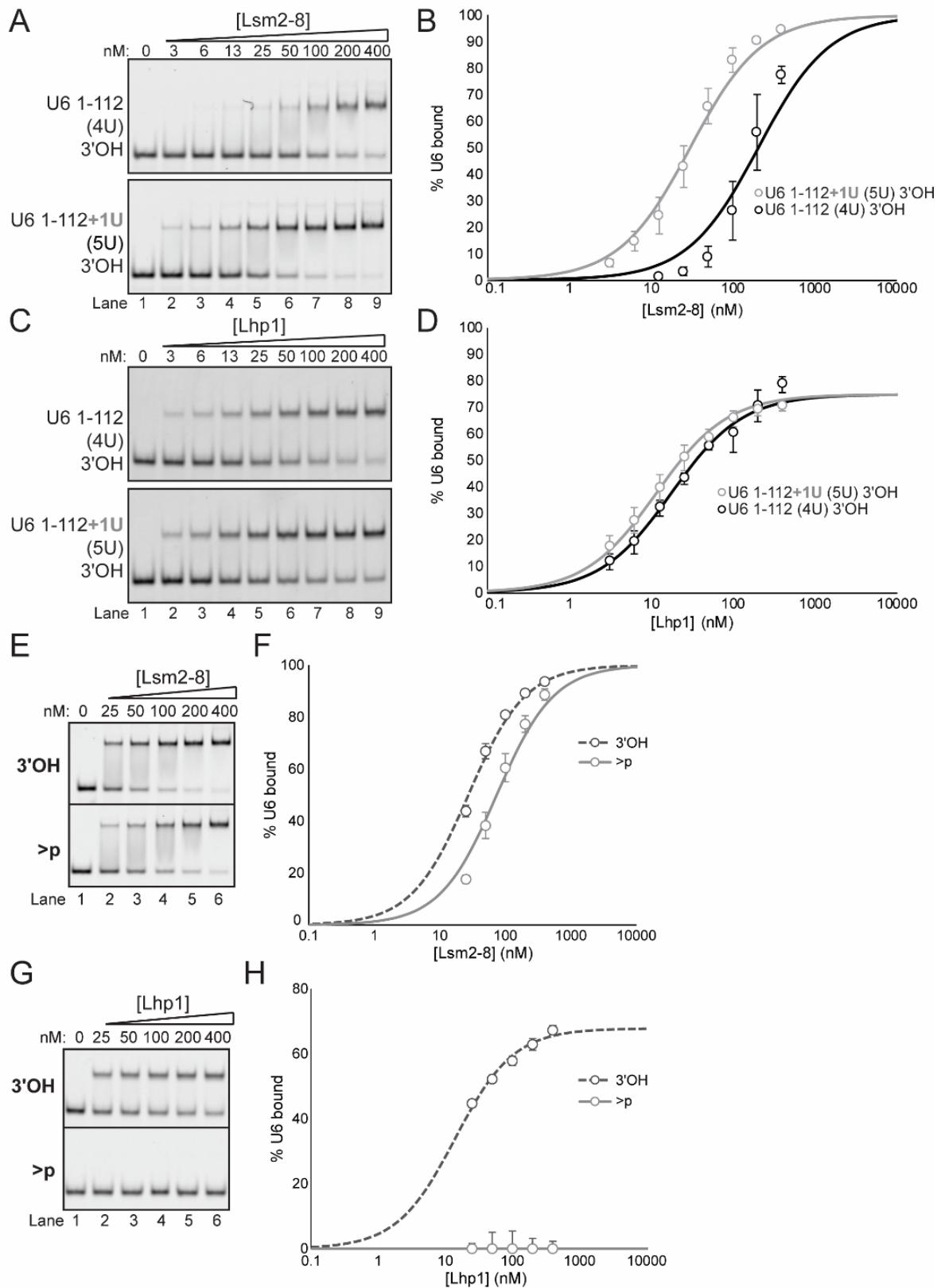
Supplementary Figure 4. yUsb1 and hUsb1 have different pH optima.

(a) yUsb1 and hUsb1 have different pH optima (indicated by an asterisk) and different tolerances for low and high pH. Single time-point comparison of yUsb1 (top; 30 minute time point) and hUsb1 (bottom; 5 minute time point) in conditions with pH ranging from 4.0 - 10.0. yUsb1 is most active at pH ~6.5 and is active over a range from pH 4.0 - 8.5. hUsb1 is most active at pH ~7.5 and is active over a range from pH 5.5 - 10.0. (b) Quantification of (a) showing the amount of substrate depleted as a function of pH.



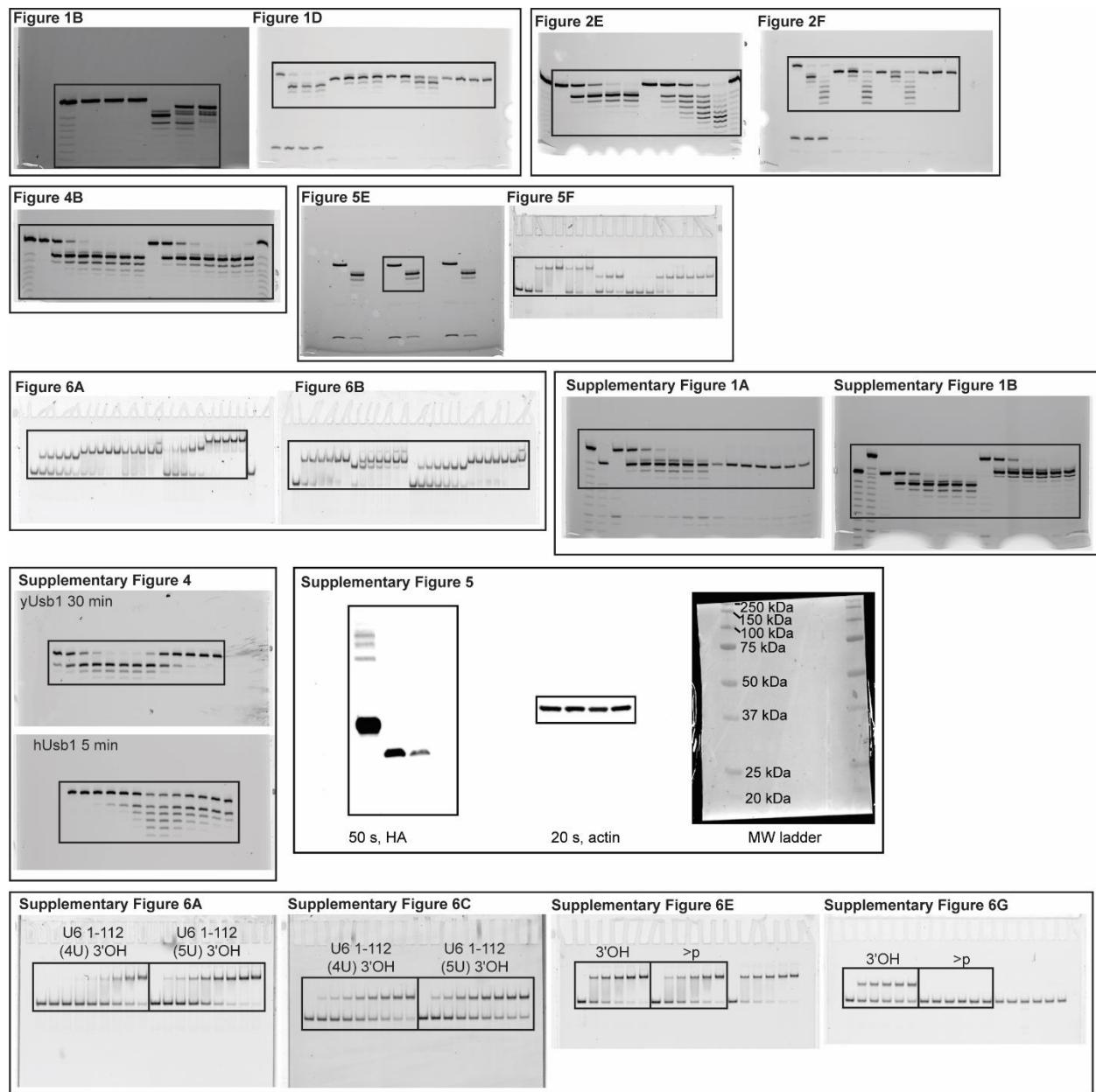
Supplementary Figure 5. The N-terminal domain is important for *yUsb1* expression or stability.

- (a) Serial dilutions of yeast containing full-length (FL) or N-terminally truncated ($\Delta 42$, $\Delta 44$, and $\Delta 70$) *yUsb1* on a high-copy p425-GPD plasmid. *yUsb1* is tagged with a single N-terminal HA tag.
- (b) SDS-PAGE and western blotting analysis of *Usb1* protein expression detected using a primary antibody to the genetically encoded N-terminal HA epitope tag. Actin was used as a loading control.



Supplementary Figure 6. Lsm2-8 preferentially binds full-length U6 RNA with an additional uridine.

(a) Native gel analysis comparing Lsm2-8 affinity to full-length fluorescently labeled U6 1-112 with a 2',3'-cis diol versus U6 1-112 with an additional uridine at the 3' end (U6 1-112+1U) and a 2',3'-cis diol. (b) Quantification of Lsm2-8 binding to U6 in (a). Plotted data points represent the average of three technical replicates \pm s.d. (c) Native gel analysis comparing Lhp1 affinity to RNAs as described in (A). (d) Quantification of Lhp1 binding to U6 in (c). Plotted data points represent the average of three technical replicates \pm s.d. (e) Native gel analysis comparing Lsm2-8 affinity to full-length fluorescently labeled U6 with an additional uridine at the 3' end (U6 1-112+1U) and different 3' modifications. Lsm2-8 binds U6 with a 2',3'-cis diol better than U6 with a 2',3'-cyclic phosphate. (f) Quantification of Lsm2-8 binding to U6 in (e). Plotted data points represent the average of three technical replicates \pm s.d. (g) Native gel analysis comparing Lhp1 affinity to RNAs as described in (e). Lhp1 binds U6 with a 2',3'-cis diol, but not a 2',3'-cyclic phosphate. (h) Quantification of Lhp1 binding to U6 in (g). Plotted data points represent the average of three technical replicates \pm s.d.



Supplementary Figure 7. Uncropped gel scans from Figures 1, 2, 5, 6, and Supplementary Figures 1, 4-6.

Supplementary Table 1. Summary of the effect of mutations and truncations of yUsb1 *in vivo*.

USB1 allele	USB1Δ complementation
<u>pRS414 backbone</u>	
Vector	-
wild type	+++
Point mutations	
W75A	+++
F78A	+
F78H	+++
Y80A	+++
H133A*	-
H133N*	-
H231A*	-
H231N*	-
N-terminal truncations	
usb1Δ1-20	+++
usb1Δ1-41	+++
usb1Δ1-42	+++
usb1Δ1-43	+++
usb1Δ1-44	-
usb1Δ1-45	-
usb1Δ1-46	-
usb1Δ1-47	-
usb1Δ1-48	-
usb1Δ1-49	-
usb1Δ1-50	-
usb1Δ1-50 + SV40 NLS	-
usb1Δ1-70 + SV40 NLS	-

Mutations or truncations were introduced into pRS414-Usb1 and plated on media lacking TRP and containing 5-FOA. Ability to complement loss of the *URA3* marked wild-type Usb1 plasmid is listed in the right hand column, where “-” indicates no growth and “+++” indicates growth similar to wild-type Usb1. * indicates strains that were not tested by serial dilution.

Supplementary Table 2. DALI search of unique structural homologs to yUsb1 catalytic domain.

PDB ID	Z-score	rmsd	% identity	Name	Activity
4H7W	16.2	3.1	14	<i>HsUsb1</i>	RNA exonuclease, 3'-5' phosphodiesterase, leaves >p
1VDX	12	3.9	13	<i>PhRNL</i>	unknown, putative RNA 2'-5' ligase/2',3' CPDase
4QAK	12	3.3	11	<i>EcThpR</i>	2',3' CPDase, leaves 2'P
5JJ2	11.6	3.8	11	<i>HsAKAP18</i>	binds NMPs, unknown activity, not a 3',5' CPDase
2VFL	11.5	4	10	<i>RnAKAP18</i>	binds NMPs, unknown activity, not a 3',5' CPDase
1IUH	11.3	3.6	12	<i>TtRNL</i>	unknown, putative RNA 2'-5' ligase/2',3' CPDase
2D4G	10.7	4.3	13	<i>BsYjcG</i>	unknown, putative 2'-5' ligase/2',3' CPDase
2FYH	9.8	3.9	14	PF0027	putative RNA 2'-5' ligase/known 2',3' CPDase, leaves 2'P
4Z5V	9.4	3.9	16	MHV ns2	2'-5' phosphodiesterase (cleaves 5'AMP from 2' termini of 2-5A)
2FSQ	9	4.4	5	ATU0111	unknown
5AF2	8.7	3.3	11	VP3	2'-5' phosphodiesterase (cleaves 5'AMP from 2' termini of 2-5A)
1JH7	8.6	4.5	9	<i>AtCNPase</i>	ADP-ribose 1",2" CPDase, leaves PO ₃ on 1" position
2YP0	5.6	3.8	9	<i>MmCNPase</i>	2',3'-cyclic nucleotide 3'- phosphodiesterase, leaves 2'P
1WOJ	5.3	3.9	9	<i>HsCNPase</i>	2',3'-cyclic nucleotide 3'- phosphodiesterase, leaves 2'P
2ILX	5.2	5.1	13	<i>RnCNPase</i>	2',3'-cyclic nucleotide 3'- phosphodiesterase, leaves 2'P
2I3E	4.3	4.4	11	CaRICH	2',3' CPDase, leaves 2'P

Supplementary Table 3. List of synthetic DNA oligonucleotides.

Oligos to make pET28b-HT-yUsb1 1-290, truncation, and mutations	
BamHI-pET28b-USB1	5'-CGGGATCCAATGGAATTCATATCTGCAGACTATTC-3'
Xhol-pET28b-USB1	5'-TGCTCGAGTTAGTAAAGGGATTCTAATGGAGTG-3'
Usb1-1-for	5'-ATGGAATTCATATCTGCAGACTATTCTAGCAGTGACGG-3'
Usb1-71-for	5'-ATGAGTCGATTTGGCGTCGTTCACATATTTGAGTG GC-3'
His-TEV-rev	5'-GCCCTGGAAGTACAGGTTCTCGCTGCTGTGATGATGA TGATGATGGC-3'
Usb1_F78A_for	5'-GCGACATATTGAGTGGCGTCCGACTCCAGCG-3'
Usb1_F78H_for	5'-CATACATATTGAGTGGCGTCCGACTCCAGCG-3'
Usb1_F78_rev	5'-CGAACGCCAAAATCGACTCATATTCATATCC-3'
Oligos to make p3HT-HT-hUsb1 79-265	
hUsb1-D78N-for	5'-GGAAACTGGGCTACACATGTGTACGTTCC-3'
hUsb1-TEV-rev	5'-CATATGAGCGCCCTGGAAATACAGGTTTC-3'
Oligos to make pET28b-Smt3-TEV-AtRNL(677-1104)	
Trunc-AtRNL-for	5'-GCTAGCGAAAACCTGTATTCCAGGGCGCTGTGAAGG AAGCTGTCCAAAAGGATGAG-3'
Trunc-AtRNL-rev	5'-GTGATGATGATGATGATGATGATGATGGCCATGGT ATATCTCCTTCTAAAGTTAA-3'
Oligos to amplify <i>hph</i> gene flanked by Usb1 up and downstream region for <i>USB1</i> deletion strain:	
Usb1_deletion_for	5'-CGAACCCAAACTATCTGTACATCAGTTGCTGTGCTC TTCTGGTCCCTTATTGCGGTTTAGAGACATTATCATGATG GCGCCAGATCTGTTAGCTGCCTTG-3'
Usb1_deletion_rev	5'-CGGGTGGGGGTATGGTAGGATTGACATTATCATGATG TTGAACATATCCTCATCTTGAGTGTCTTCGCGAAGCGA GCTCGTTTCGACACTGGATGGC-3'
Usb1_del_con_for	5'-GTTTCCCTAACAAAGGGAATCTC-3'
Usb1_del_con_rev	5'-TACCTAACAAAGTTATCGAGG-3'
Oligos to create pRS414-Usb1 and pRS416-Usb1:	
BamHI-Usb1+500-for:	5'-ACGGGATCCATAGTTGCACACCCGCTACTCCCTCATT GACAGC-3'
Xhol-Usb1+500-rev	5'-CAGCTCGAGCTGAGGGATTATATACCACGGATCTAC-3'
Cloning oligos to introduce mutations into pRS414-Usb1:	
Usb1_W75A_for	5'-GCGCGTTCGTTCACATATTGAGTGGCGTCCG-3'
Usb1_W75A_rev	5'-AAATCGACTCATATTGAGTGGCGTCCG-3'
Usb1_F78A_for	5'-GCGACATATTGAGTGGCGTCCGACTCCAGCG-3'
Usb1_F78H_for	5'-CATACATATTGAGTGGCGTCCGACTCCAGCG-3'
Usb1_F78_rev	5'-CGAACGCCAAAATCGACTCATATTGAGTGGCGTCCG-3'
Usb1_Y80A_for	5'-GCTTTGAGTGGCGTCCGACTCCAGCGATTC-3'
Usb1_Y80A_rev	5'-TGTGAACGAAACGCCAAAATCGACTCATATTGAGTGGCGTCCG-3'
Usb1_H133A_for	5'-GCGGTTCCCTAACTCGATCGTTGTTGAAAC-3'
Usb1_H133N_for	5'-AACGTTCCCTAACTCGATCGTTGTTGAAAC-3'
Usb1_H133_rev	5'-TAAAGGTTGGGGCTCCAAAGTGTGAGATGAAC-3'
Usb1_H231A_for	5'-GCGGTTCAATTGCCATCGCTAGCAACCCCTCAAAG-3'
Usb1_H231N_for	5'-AACGTTCAATTGCCATCGCTAGCAACCCCTCAAAG-3'
Usb1_H231_rev	5'-TAAATTTGCCTACTTACAATTAAATCCTGATAG-3'

Usb1+500_START-rev	5'-CATAGGCCAGTCTTCTTAAGCGATTGGTAACC-3'
Usb1+500_SV40-rev	5'-AACTTTCTTTTTTTGGTGGCATAGGCCAGTCTTCT TCTAAGCGATTGGTAACC-3'
Usb1-21_for	5'-AGTAACAAAAGTGAGGTCCAATCGAATAACACG-3'
Usb1-42_for	5'-ACTGATTTACCTGCAATACCGGATTCAATTATATTG AAG-3'
Usb1-43_for	5'-GATTACCTGCAATACCGGATTCAATTATATTG-3'
Usb1-44_for	5'-TTACCTGCAATACCGGATTCAATTATATTGAAG-3'
Usb1-45_for	5'-CCTGCAATACCGGATTCAATTATATTGAAGTAC-3'
Usb1-46_for	5'-GCAATACCGGATTCAATTATATTGAAGTACAC-3'
Usb1-47_for	5'-ATACCGGATTCAATTATATTGAAGTACACATC-3'
Usb1-48_for	5'-CCGGATTCAATTATATTGAAGTACACATCCCT-3'
Usb1-49_for	5'-GATTCAATTATATTGAAGTACACATCCCTCCG-3'
Usb1-50_for	5'-TCAATTATATTGAAGTACACATCCCTCCGAAT-3'
Usb1-51_for	5'-ATTATATTGAAGTACACATCCCTCCGAATTTAC-3'
Usb1-71_for	5'-ATGAGTCGATTTGGCGTTCGTTCACATATTTGAGT GGC-3'
Oligos to make p425-GPD-yUsb1 and hUsb1, truncations, and mutations	
BamHI-yUsb1-for	5'-CGGGATCCAATGGAATTCATATCTGCAGACTATT-3'
Sall-yUsb1-rev	5'-GGCGTCGACTTAGTAAAAGGGATTCTAATGGAGTGA-3'
BamHI-hUsb1-for	5'-ACGGGATCCATATGAGTGCAGCCCCCTTAGTAGGG-3'
Sall-hUsb1-rev	5'-GGCGTCGACTCATTCAGCGGCATGGAAAAAAATTG-3'
p425-up-HA-r	5'-AGCGTAATCTGGTACGTCGTATGGGTACATATGGAT CCACTAGTTCTAGAACCTCGTCG-3'
Usb1-1-for	5'-ATGGAATTCATATCTGCAGACTATTCTAGCAGTGACGG-3'
Usb1-43_for	5'-GATTACCTGCAATACCGGATTCAATTATATTG-3'
Usb1-45_for	5'-CCTGCAATACCGGATTCAATTATATTGAAGTAC-3'
Usb1-71_for	5'-ATGAGTCGATTTGGCGTTCGTTCACATATTTGAGT GGC-3'
hUsb1-H84F-for	5'-TTCGTCTACGTCCCGTATGAAGCCAAGGAGGAA-3'
hUsb1-H84-rev	5'-AGTTGCCAGTCCCACGCTCGTGAGGAAA-3'
hUsb1-wt-79-for	5'-GGGAACTGGGCAACTCATGTCTACGTCCCG-3'
hUsb1-H84F-79-for	5'-GGGAACTGGGCAACTTCGTCTACGTCCCG-3'
Oligos to make HH-U6 95-112+3U-HDV plasmid	
HH-U6_95-112(UUU)-for	5'-AACACGTTCGCGTGTCAAGAGATTATTCGTTTTTG GGTCGGCATGGCATCTCCACCTCCTCGCGGTCCG-3'
HH-U6_95-112(UUU)-rev	5'-CGGCCCTAACGGCCTCATCAGAGAGATTATCTCCCTAT AGTGAGTCGTATTAGAACAGAGC-3'
Oligos to make HH-U6 13-112-HDV plasmid	
U6_13-112_for	5'-CGAAACACGTTCGCGTGTCAACCCTCGTGGACATTGGT CAATTGAAACAATACAGAGA-3'
HH-U6_13-112_rev	5'-GCCTAACGGCCTCATCAGACCCCTCGTGGCTCCCTATAGT GAGTCGTATTAAAG-3'

Supplementary Table 4. Nucleotide sequence of synthetic genes.

hUsb1, codon optimized for <i>S. cerevisiae</i> (used for <i>in vivo</i> complementation assays)	5'-GGATCCATATGAGTGCAGCCCCTTAGTAGGGTACAGCTAA GCGGGAGTGAAGATGAGAGCGAGGACGGAATGAGGACCAGACGG GTGATGGAAGTCATAGAAGAGGCCAGAGCCCCCTGCCGAGGCAAC GTTTCCCCGTACCCGACAGTGTTGAATATGTTCCCAGGTACCGAA GAGGGACCCGAAGATGACTCTACCAAACACGGAGGGAGGGTCCGT ACTTTCTCACGAGCGTGGAACTGGCAACTCATGTCTACGTCC CGTATGAAGCCAAGGGAGGAATTGGATCTGGATGTGCTACTT CCCCACGCCAACCTACGTACCGAGACTGGTAAGGATGAAAGTCT TCCACCTGAGTCTGAGTCAGAGCGTTGCTGAGGCATCACTGGAT ACTGCCTTCGTACAGGCGCTAACGGCTCGTATGACCAGTTTCACA GGTTCTTTTACTGCCAATCAGGTGAAGATATATAACAAACCAAGAAA AAACAAGGACCTTATTGGCCTAGAGGTTACTTCCGGTCACGCACAA TTTTGGACCTAGTCTCGAGGTTGACCGTGTGATGGAAGAATTAA CCTGACCACTTTTATCAAGACCCCTCATTCCATCTGTCACTTGCATG GTGTGTCGGAGATGCTCGTCTGCAACTAGAAGGGCAATGTCTCAA GAGCTGCAAGCCATAGTCGATGGTTCGAGGATGCTGAGGTCTGC TTAGAGTCCACACAGAGCAGGTGCGTTGCAAGAGTGGCAACAAATT TTTTCCATGCCGCTGAAATGAGTCGAC-3'
hUsb1, codon optimized for <i>E. coli</i> (used for protein expression and crystallization)	5'-CATATGTCAGCTGCACCATTAGTCGGGTATTCATCATCGGGTTC TGAGGATGAGTCCGAGGACGGGATGCGTACACGTCCTGGCGACGG ATCGCACCGTCGTGGCAGTCCCCATTGCCCTGCCAGCGCTTCCCT GTGCCGATTGGTCTGAACATGTTCCAGGGACCGAGGAAGGAC CGGAAGATGATAGCACTAACGATGGAGGTGCGTACGCACCTTCCC ACACGAGCGCGGAAACTGGGCTACACATGTGTACGTTCCCTACGAA GCGAAGGAAGAATTCTGGATTGCTGATGTCCTGCTTCCACATGC GCAGACTTACGTGCCGCGCTTAGTCCGCATGAAAGTCTTCACCTG TCCTTGTCCCAATCGGTAGTTCTGCCATCATTGGATTGCTTT GTGCAGGCCTTGAAGGCCCGTATGACATCGTCCACCGCTTTTT CACCGCCAATCAGGTAAAGATTATAACAAATCAAGAGAAGACACGCA CATTCAATTGGCTTGGAGGTAACCTCAGGGCACGCTCAATTGGAC TTAGTAAGTGAAGTAGACCGCGTGTGGAGGAGTTAACCTTACAA CTTTCTATCAAGATCCGTCTTCCACCTGAGCTTAGCCTGGTGCCTG GGAGATGCTCGCCTTCAGTTGAAGGCCATGCCCTCAAGAACTGC AAGCAATCGTAGACGGGTCGAGGACGCCAGGTACTGCTCGTGT ACACACCGAGCAGGTTCGCTGCAAATCAGGTAAATAAGTTTCTCCA TGCCACTTAAATGACTCGAGGATCC-3'
Lhp1 from <i>S. cerevisiae</i> , codon optimized for <i>E. coli</i> (used for protein expression)	5'-CATATGTCGAAAAACCGCAGCAGGAAGAACAGGAAAAACCGC AGTCTCGTCGTAACTCTTCGCTGTTATCGAATTCACCCCGGAAGTT CTGGACCGTTGCCTGAAACAGGTTGAATTCTACTTCTCTGAATTCAA CTTCCCGTACGCCGTTCTCGTACCCACCGCTGAAAAAAACGAC GGTTGGGTTCCGATCTCTACCATCGTACCTCAACCGTATGAAAAAA ATACCGTCCGGTTGACAAAGTTATCGAAGCTCTGCCTTCTGAAA TCCTGGAAGTTCTGCTGACGGTGAACACGTTAACACGTCGTGTTCCG CTGGACCTGACCGCTGCTCGTAACGCTCGTATCGAACAGAACCGC GTACCCCTGGCTGTTATGAACCTCCGCCACGAAGACGTTGAAGCTT CAGATCCCGAAGTGCAGGAAAACCTGGAAGGCTTCTCAAAAAACT GGGTGAAATCAACCAAGGTTCGCTGCGTCGTGACCACCGTAACAAA AAATTCAACGGTACCGTTCTGGTTGAATTCAAAACCATCCCGGAATG CGAAGCTTCTGAATCTTACTCTAACGACGACGAATCTAACGAAA

	TCCTGTCTTACGAAGGTAAAAACTGTCTGTTCTGACCAAAAACAG TTCGACCTGCAGCGTGAAGCTTCTAAATCTAAAAACTTCTCTGGTCG TTCTCGTTCTTCAACGGTCACAAAAAAAAACCTGCCGAAATTCC CGAAAAACAAAAACGGTAAAGAAGAACACAAAGAACATGATGACTCG TCTGCTATCGCTGACGACGACGAAGAACACAAAGAACATGATGACTCG AGGATCC-3'
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Supplementary Table 5. List of synthetic RNA oligonucleotides.

FAM-U6 95-112	5'-/56-FAM/rArGrArGrArUrUrUrArUrUrCrGrUrUrU-3'
FAM-U6 95-112-3P	5'-/56-FAM/rArGrArGrArUrUrUrArUrUrCrGrUrUrU/3Phos/-3'
FAM-U6 95-112+1U	5'-/56-FAM/rArGrArGrArUrUrUrArUrUrCrGrUrUrU-3'
FAM-U6 95-112+1U-3P	5'-/56-FAM/rArGrArGrArUrUrUrArUrUrCrGrUrUrU/3Phos/-3'
FAM-U6 95-112+3U	5'-/56-FAM/rArGrArGrArUrUrUrArUrUrCrGrUrUrU-3'
FAM-U6 95-112+3U-3P	5'-/56-FAM/rArGrArGrArUrUrUrArUrUrCrGrUrUrU/3Phos/-3'
FAM-U6 95-112+6U	5'-/56-FAM/rArGrArGrArUrUrUrArUrUrCrGrUrUrU-3'
FAM-U6 95-111dUrU	5'-/56-FAM/rArGrArGrArUrUrUrArUrUrCrGrUrUrU/ideoxyU/rU-3'
FAM-U6 84-94	5'-/56-FAM/rCrCrGrUrUrUrArCrArA-3'
FAM-U6 84-112+3U-3P	5'-/56-FAM/rCrCrGrUrUrUrArCrArArGrArGrArA/rUrUrUrArUrUrCrGrUrUrUrUrU/3Phos/-3'
5'Cy3-U6 1-12	5'-/5Cy3/rGrUrUrCrGrCrArArGrUrA-3'

SUPPLEMENTARY REFERENCES

1. Hilcenko, C. *et al.* Aberrant 3' oligoadenylation of spliceosomal U6 small nuclear RNA in poikiloderma with neutropenia. *Blood* **121**, 1028-1038 (2013).