

SUPPLEMENTAL MATERIAL

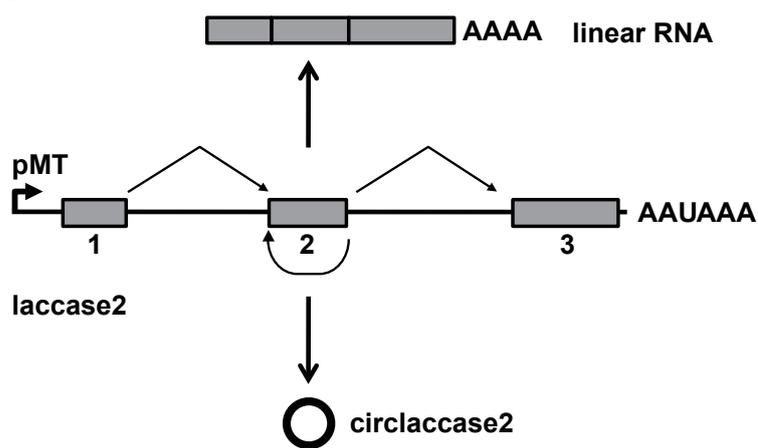
A length-dependent, evolutionarily conserved pathway controls nuclear export of circular RNAs

Chuan Huang, Dongming Liang, Deirdre C. Tatomer, and Jeremy E. Wilusz

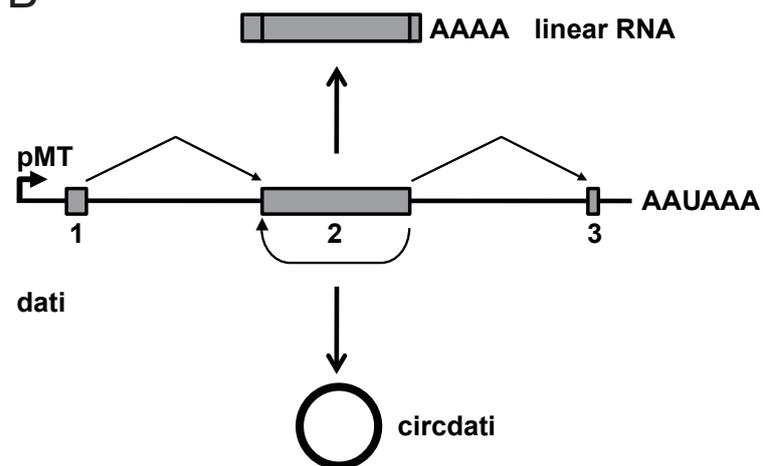
Supplemental Figures S1-S10
Supplemental Tables S1-S5
Supplemental Methods

Supplemental Figure S1

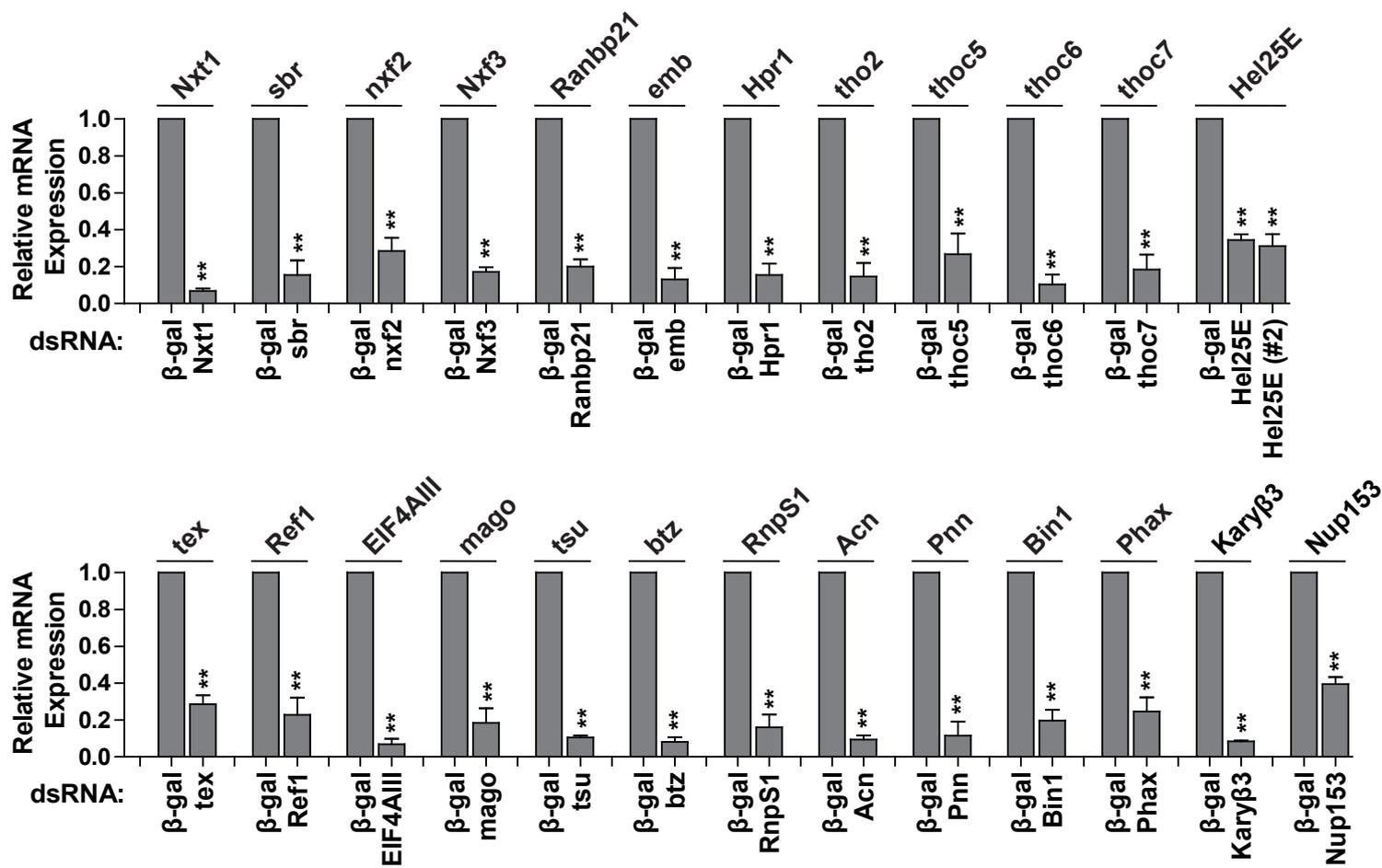
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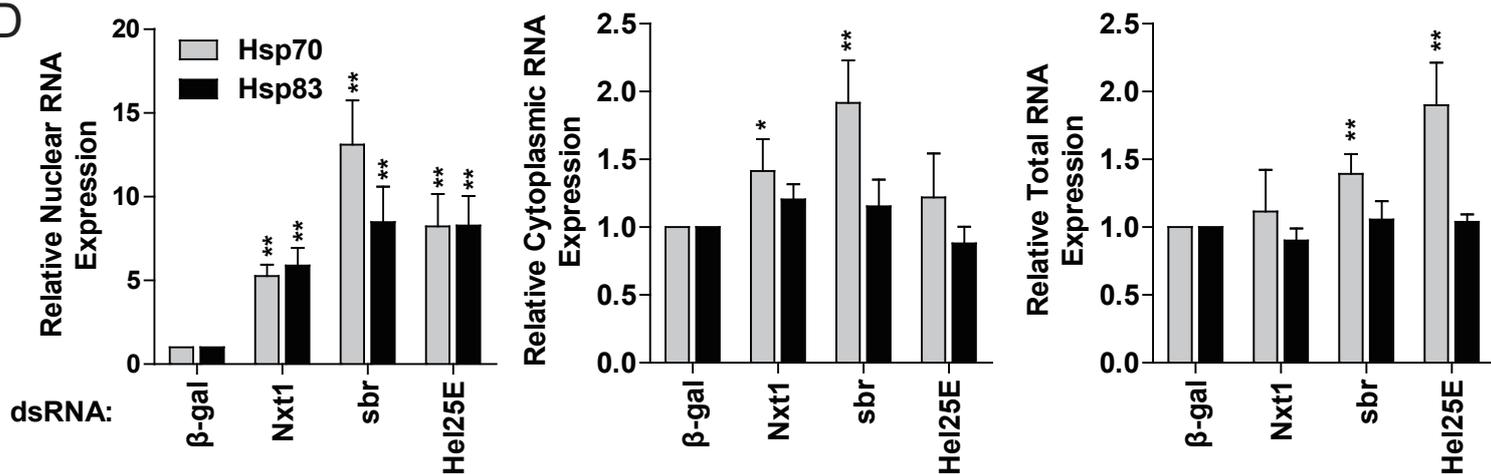
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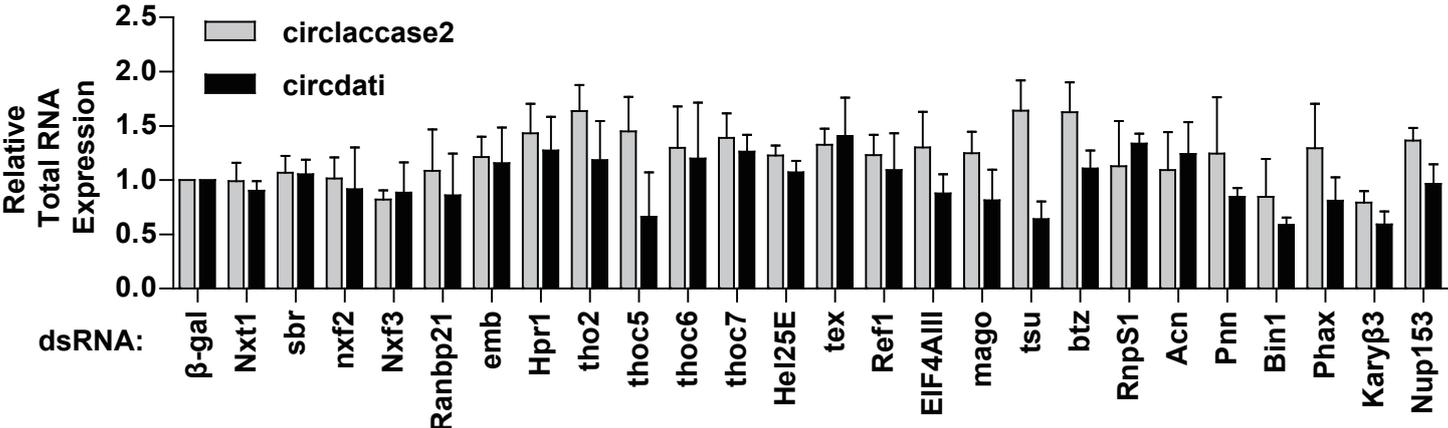
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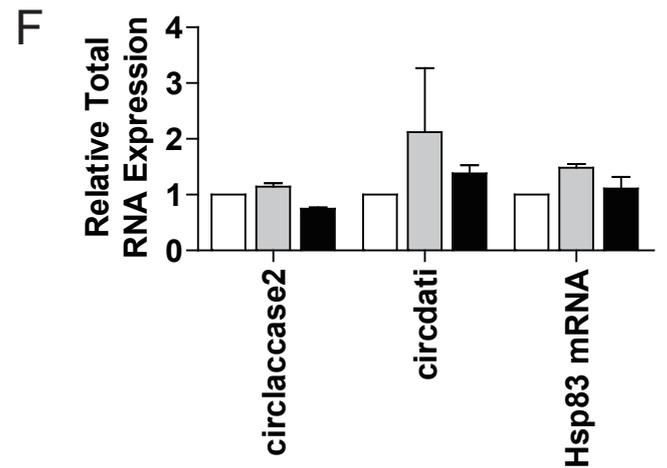
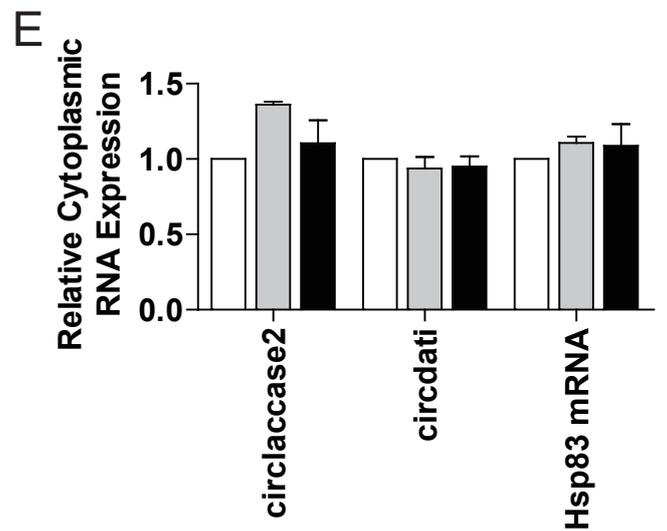
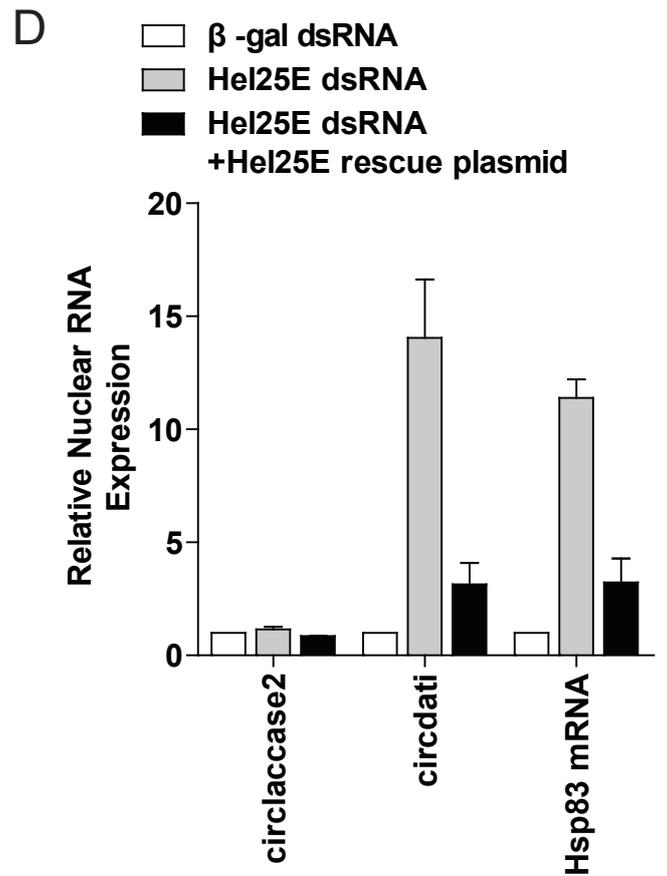
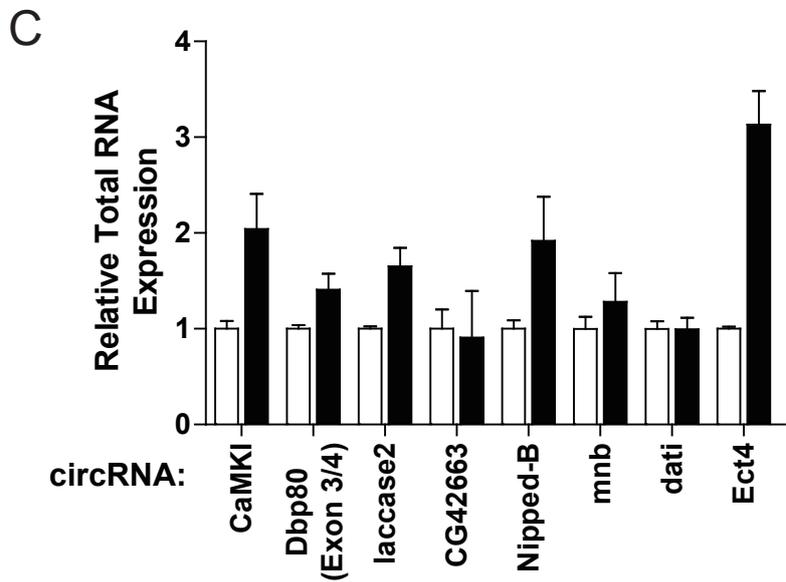
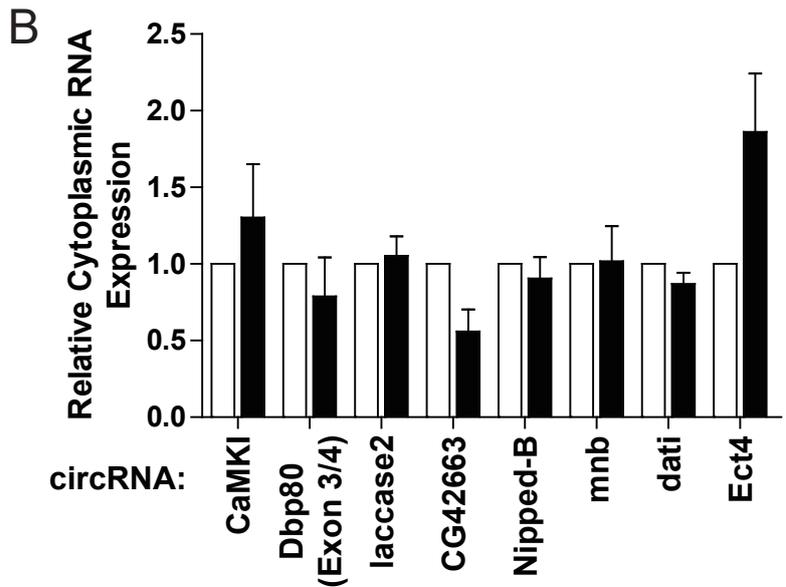
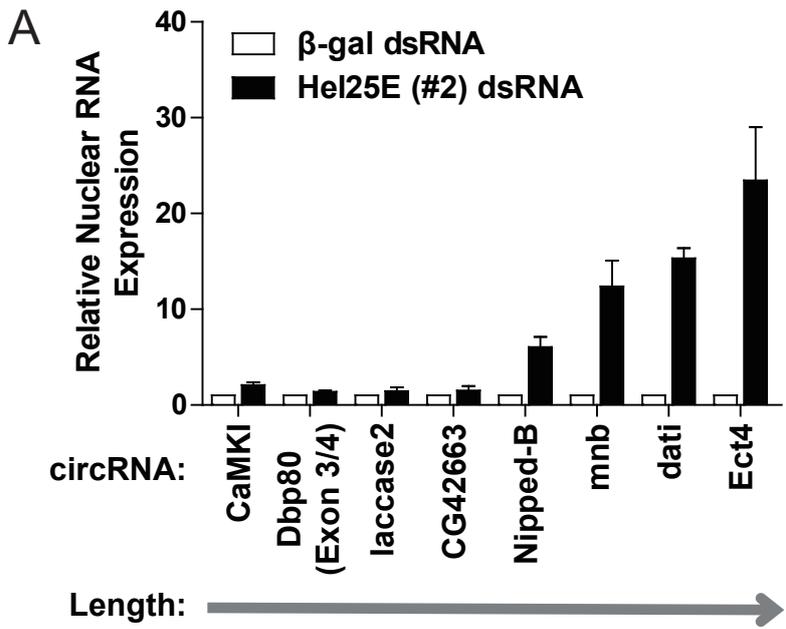
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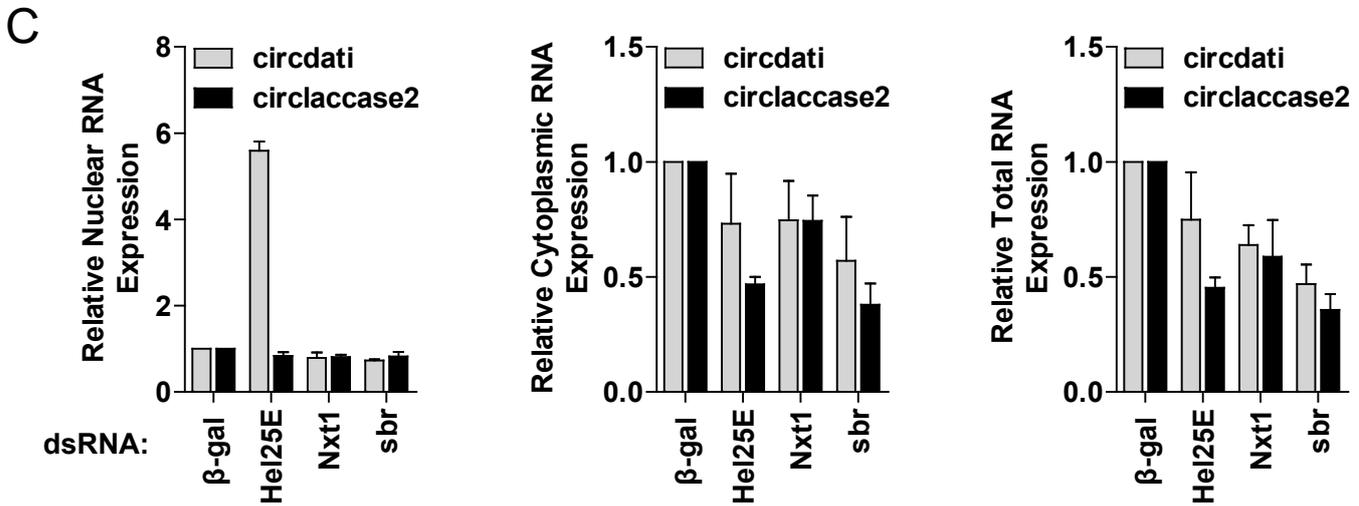
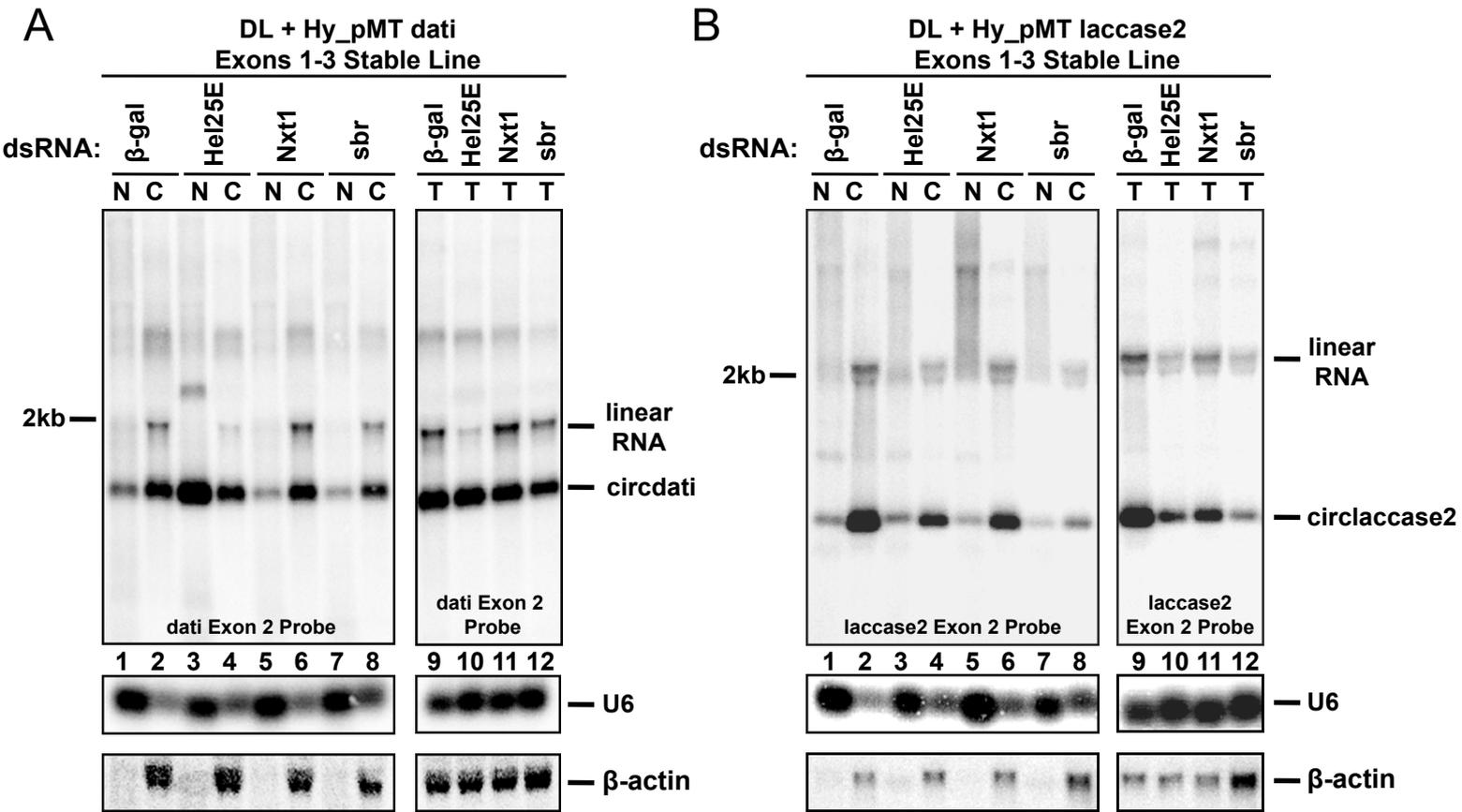
Supplemental Figure S2



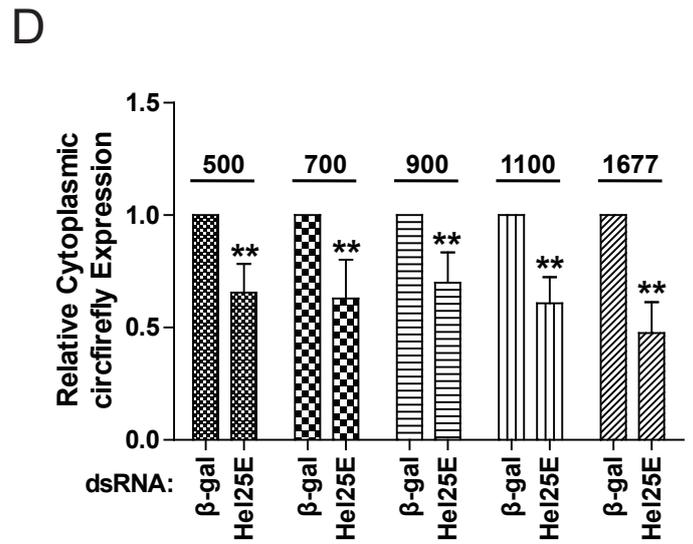
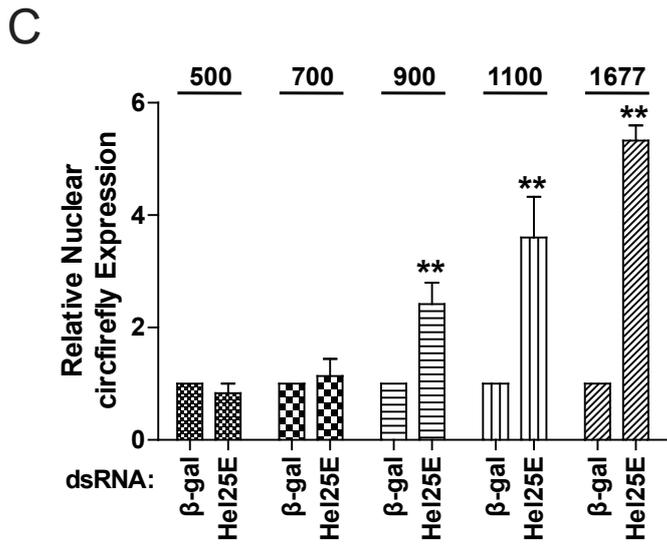
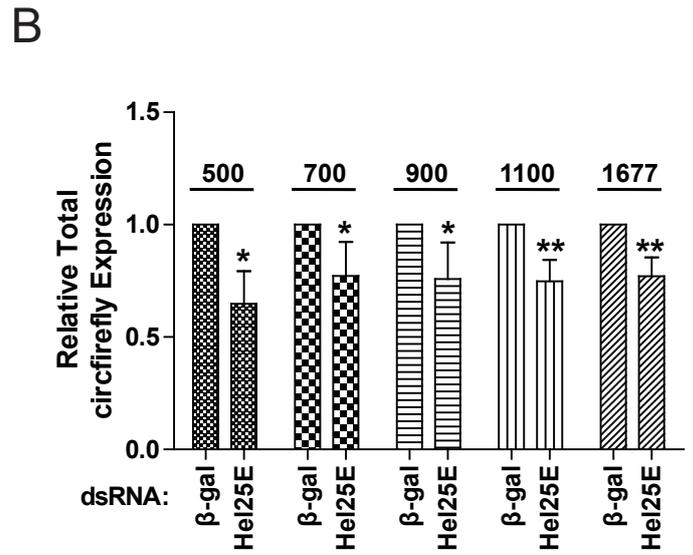
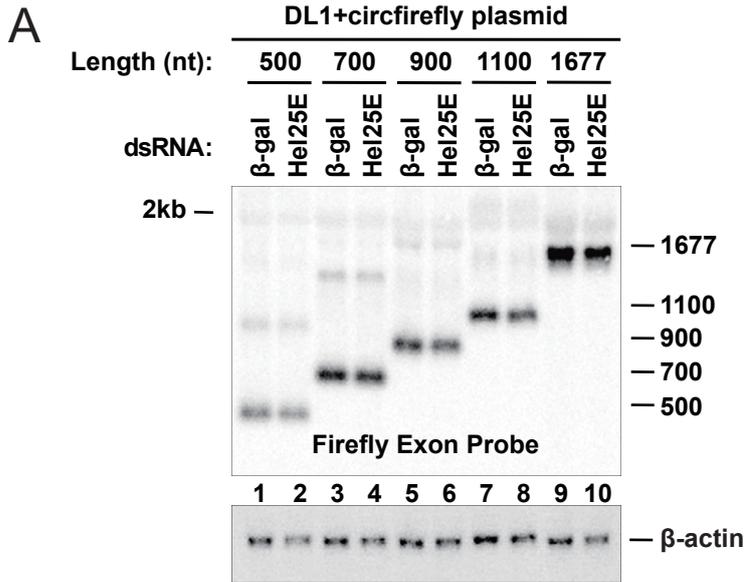
Supplemental Figure S3



Supplemental Figure S4



Supplemental Figure S6



Supplemental Figure S7

Drosophila Hel25E 1 MADND---DLLDYEDEEQTETTAVENQEAP-KKDVKGTYSVSIHSSGFRDFFLLKPEILLRAI
Human UAP56 1 MAENDVDNELLDYEDDEVETAAGGDGAEAPAKKDVKGSYVSIHSSGFRDFFLLKPELLRAI
Human URH49 1 MAEQDVENDLLDYDEEEE-PQAPQESTPAPPKKDIKGSYVSIHSSGFRDFFLLKPELLRAI

Drosophila Hel25E 57 VDCGFEHPSEVQHECIPQAVLGMIDLQAKSGMGKTAVFVLATLQQLEPSDNNTCHVLVM
Human UAP56 61 VDCGFEHPSEVQHECIPQAILGMIDLQAKSGMGKTAVFVLATLQQLEPVTG-QVSVLVM
Human URH49 60 VDCGFEHPSEVQHECIPQAILGMIDLQAKSGMGKTAVFVLATLQQIEPVNG-QVTVLVM

Drosophila Hel25E 117 CHTRELAFAQISKEYERFSKYMP TVKVAVFFGGMAIQKDEETLKS GTPHI VVGTPGRILAL
Human UAP56 120 CHTRELAFAQISKEYERFSKYMP NVKVAVFFGGLSIKKDEEVLKKNCPHI VVGTPGRILAL
Human URH49 119 CHTRELAFAQISKEYERFSKYMP SVKVS VFFGGLSIKKDEEVLKKNCPH VVGTPGRILAL

Drosophila Hel25E 177 IRNKKLNLLKLLKHFVLD ECDKMLEQLDMRRDVQEIFRSTPHGKQVMMFSATLSKDIRPVC
Human UAP56 180 ARNKS LNLLKH I KHFILDECDKMLEQLDMRRDVQEIFRMTPH EKQVMMFSATLSKEIRPVC
Human URH49 179 VRNRSFS LKNV KHFVLD ECDKMLEQLDMRRDVQEIFRLTPHEKQCMMFSATLSKDIRPVC

Drosophila Hel25E 237 K KFMQDPMEVYVDDEAKLTLHGLQQHYVNLKENEKNK KLFELLDVLEFNQVVI FVKS VQR
Human UAP56 240 R KFMQDPMEIFVDDETKLTLHGLQQY YV K LKDN EKNR KLF D LLDVLEFNQVVI FVKS VQR
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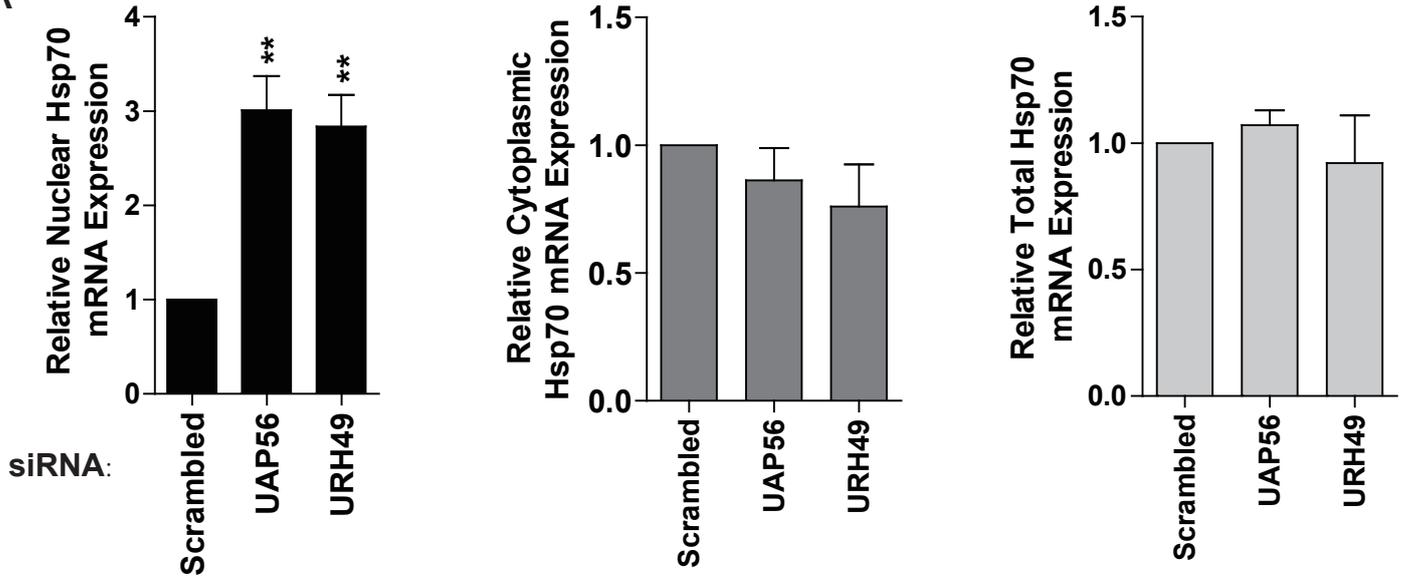
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Human URH49 299 CMA LA QLLVEQNFP AIAIHRGMAQEERLSRYQQFKDFQR RILVATNLFGRGMDIERNIV

Drosophila Hel25E 357 FNYDMPEDSDTYLHRVARAGRFGTKGLAITFVSDENDAKILNEVQDRFDVNI SELPEEID
Human UAP56 360 FNYDMPEDSDTYLHRVARAGRFGTKGLAITFVSDENDAKILNDVQDRFEVNI SELPDEID
Human URH49 359 FNYDMPEDSDTYLHRVARAGRFGTKGLAITFVSDENDAKILNDVQDRFEVNV AELPEEID

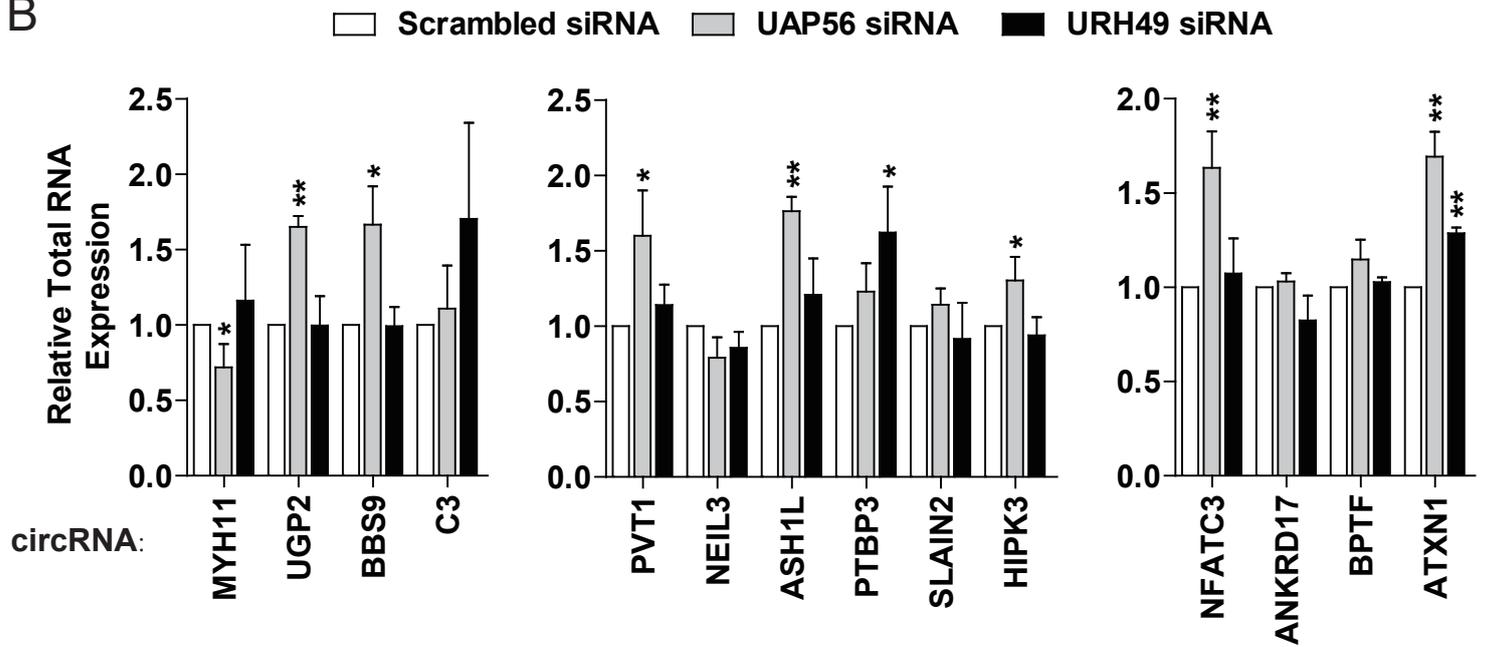
Drosophila Hel25E 417 LSTYIEG-R
Human UAP56 420 ISSYIEQTR
Human URH49 419 ISTYIEQSR

Supplemental Figure S8

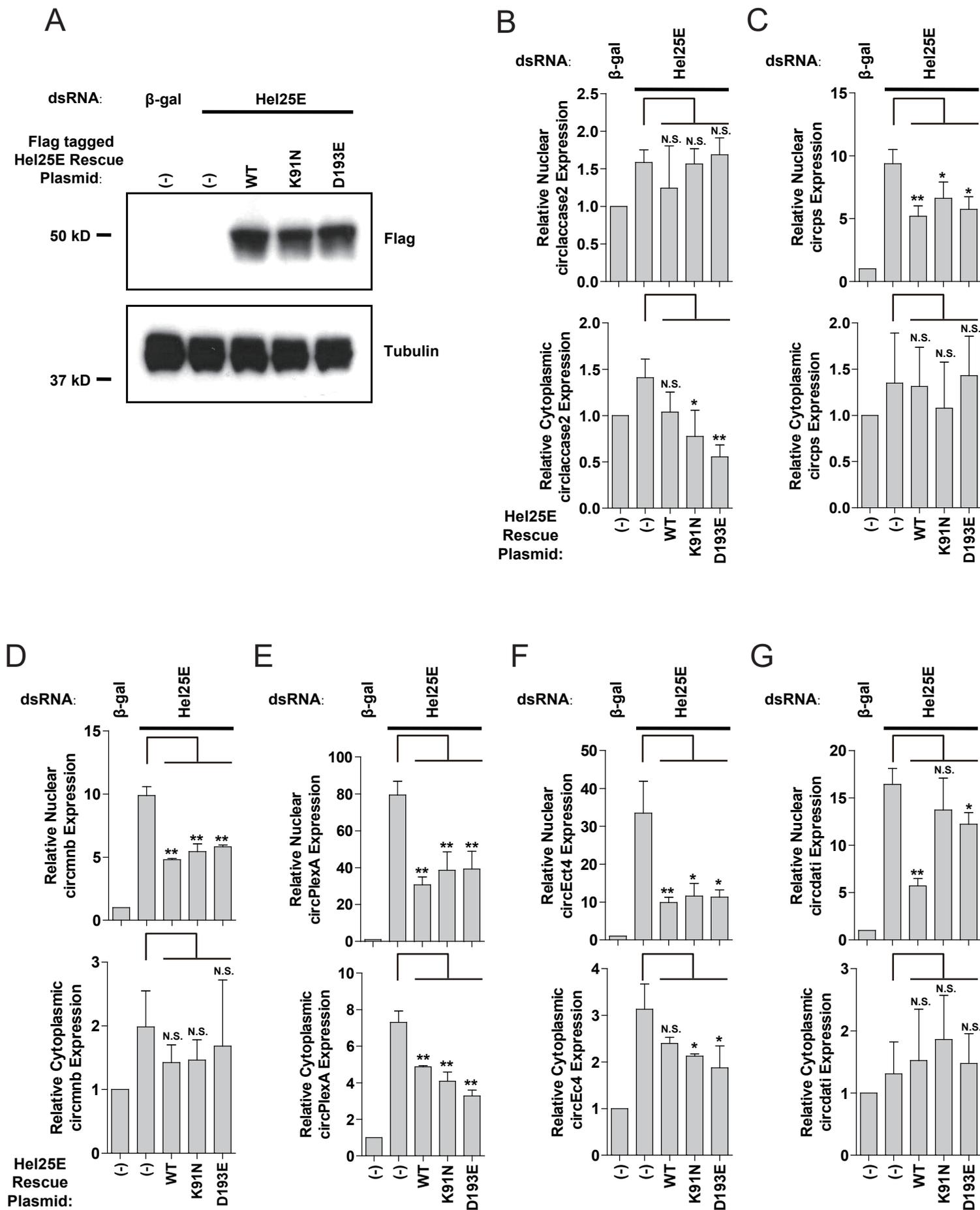
A



B



Supplemental Figure S9

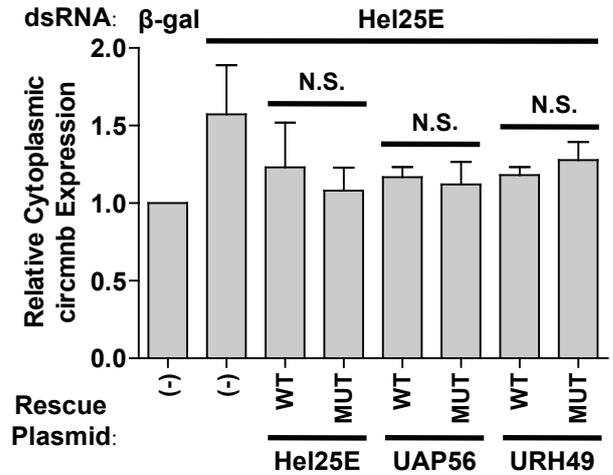
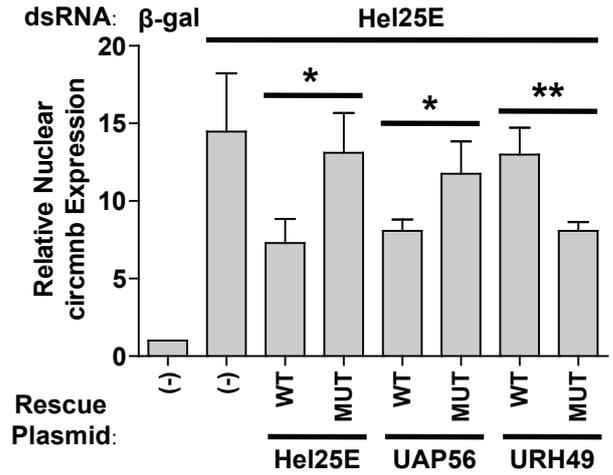


Supplemental Figure S10

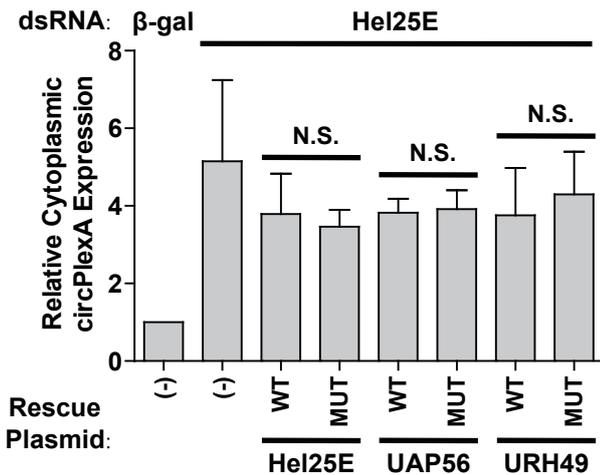
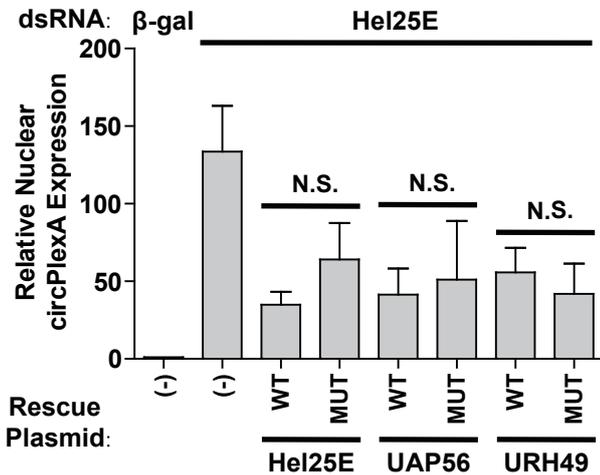
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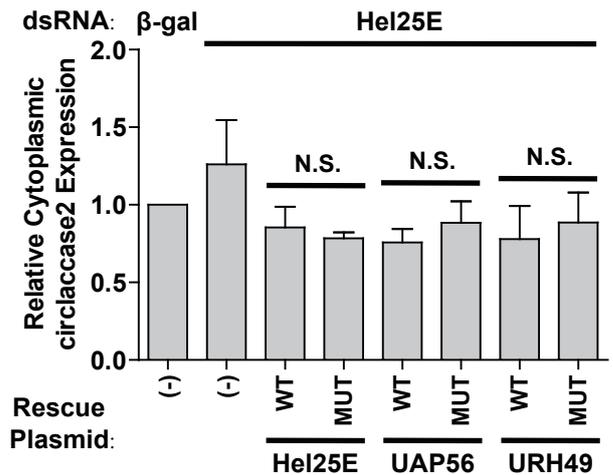
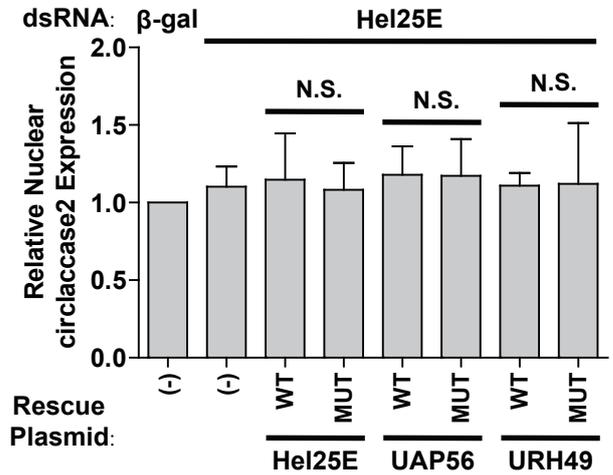
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C



D



Supplemental Figure Legends

Supplemental Figure S1. Depletion of nuclear export factors to identify regulators of linear and circular RNA localization.

(A) Schematic of “Hy_pMT laccase2 Exons 1-3” reporter plasmid. Exons 1, 2, and 3 with shortened intervening introns were cloned downstream from the copper-inducible metallothionein A promoter (pMT), as previously described (Liang et al. 2017). KpnI and XmaI sites were inserted into exon 2 so that plasmid-derived transcripts can be distinguished from endogenous laccase2 transcripts. The plasmid can generate a mature ~1.8-kb linear mRNA that is subsequently polyadenylated (top) or be subjected to backsplicing to generate a 514-nt circular RNA from exon 2 (bottom). (B) Schematic of “Hy_pMT dati Exons 1-3” reporter plasmid, which was cloned similarly to the laccase2 plasmid. Whereas canonical splicing yields an ~1.4-kb linear RNA (top), backsplicing results in production of an 1120-nt circular RNA (bottom). (C) DL1 cells were treated with the indicated dsRNAs for 3 days and qRT-PCR was then used to verify that each dsRNA efficiently depleted its target gene. Data from three independent experiments were normalized to rp49 mRNA and are shown as mean \pm SD. (D) DL1 cells were treated with the indicated dsRNAs for 3 days and then subjected to 37°C heat shock for 20 min. Nuclear (left), cytoplasmic (middle), and total (right) RNA were purified and qRT-PCR was used to quantify steady-state expression of endogenous Hsp70 and Hsp83 transcripts. Data are normalized to the β -gal dsRNA sample and are shown as mean \pm SD, n=3. **, p<0.01; *, p<0.05. These results confirm previous reports that depletion of Nxt1, sbr, or Hel25E results in nuclear accumulation of Hsp70 and Hsp83 mRNAs.

Supplemental Figure S2. The overall expression levels of circlaccase2 and circdati largely do not change with the dsRNA treatments.

DL1 cells were treated with the indicated dsRNAs for 3 days followed by purification of total cellular RNA. qRT-PCR was used to quantify steady-state expression of endogenous circlaccase2 and circdati. Data are normalized to the β -gal dsRNA sample and are shown as mean \pm SD, n=3.

Supplemental Figure S3. Nuclear accumulation of long circular RNAs was also observed using an independent, non-overlapping Hel25E dsRNA and can be rescued with RNAi-resistant Hel25E expression plasmids.

(A-C) DL1 cells were treated for 3 days with a control (β -gal) dsRNA or a dsRNA to deplete Hel25E. RNA was then purified from nuclei (A), cytoplasm (B), or whole cells (C), followed by qRT-PCR to quantify steady-state expression of the indicated circRNAs, which are ordered according to their mature circRNA lengths. Data are normalized to the β -gal dsRNA samples and are shown as mean \pm SD, n=3. (D-F) DL1 cells were treated for 3 days with a control (β -gal) dsRNA, a dsRNA to deplete Hel25E, and/or an inducible Hel25E plasmid that is insensitive to dsRNA treatment (expressed for last 24 h). RNA was then purified from nuclei (D), cytoplasm (E), or whole cells (F), followed by qRT-PCR to quantify steady-state expression of the indicated transcripts. Data are normalized to the β -gal dsRNA samples and are shown as mean \pm SD, n=3.

Supplemental Figure S4. Depletion of Hel25E results in nuclear accumulation of plasmid-derived circdati, but not circlaccase2.

(A, B) DL1 cell lines stably expressing the Hy_pMT dati Exons 1-3 plasmid **(A)** or the Hy_pMT laccase2 Exons 1-3 plasmid **(B)** were treated with the indicated dsRNAs for 3 days and CuSO₄ was added for the last 14 h. RNA was then purified from nuclei (N), cytoplasm (C), or total cells (T) and subjected to Northern blot analysis. U6 snRNA and β -actin mRNA served as controls for fractionation efficiency. Representative blots are shown. **(C)** RNA levels were quantified using ImageJ from three independent Northern blot experiments. Data are normalized to the β -gal dsRNA samples and are shown as mean \pm SD.

Supplemental Figure S5. Effect of Hel25E depletion on localization of nascent Hsp83 transcripts and steady-state circular RNAs.

(A) DL1 cells were treated with dsRNAs for 3 days and nascent RNAs labeled with 4sU for the last 15 min prior to beginning the fractionation procedure. Nascent RNAs in the nuclear (left) and cytoplasmic (right) fractions were purified and qRT-PCR was used to quantify expression of endogenous Hsp83 mRNA. Data are normalized to the β -gal dsRNA sample and are shown as mean \pm SD, n=3. Nascent Hsp83 mRNAs become enriched in the nucleus and fail to efficiently reach the cytoplasm upon Hel25E depletion. **(B-D)** DL1 cells were treated for 3 days with a control (β -gal) dsRNA, a dsRNA to deplete Hel25E, and/or an inducible Hel25E plasmid that is insensitive to dsRNA treatment (expressed for last 24 h). Unlike Fig. 2A,B, no 4sU was added to the cells prior to fractionation. RNA was purified from nuclei **(B)**, cytoplasm **(C)**, or whole cells **(D)**, followed by qRT-PCR to quantify steady-state expression of the indicated circRNAs, which are ordered according to their mature circRNA lengths. Data are normalized to the β -gal dsRNA sample and are shown as mean \pm SD, n=3.

Supplemental Figure S6. Depletion of Hel25E results in nuclear accumulation of long circfirefly transcripts.

(A) Copper-inducible plasmids (driven by the metallothionein A promoter (pMT)) that produce firefly luciferase circular RNAs (circfirefly) of different lengths (500, 700, 900, 1100, and 1677) were generated by cloning the indicated exonic sequences between the circdati introns. Plasmids were then transfected into DL1 cells treated with the indicated dsRNAs. Total RNA was purified and Northern blots used to examine expression of the circfirefly transcripts. Representative blots are shown. (B) Circfirefly RNA levels were quantified using ImageJ from three independent Northern blot experiments. (C, D) As in Fig. 2C, the circfirefly plasmids were transfected into DL1 cells treated with the indicated dsRNAs. RNA was then purified from nuclear (C) or cytoplasmic (D) fractions, analyzed by Northern blotting, and quantified using ImageJ from three independent Northern blot experiments. Data throughout the figure are normalized to the β -gal dsRNA samples and are shown as mean \pm SD, n=3. **, p<0.01; *, p<0.05.

Supplemental Figure S7. Amino acid sequence alignment of *Drosophila* Hel25E, human UAP56, and human URH49.

Amino acids conserved in all three proteins are marked in black. A 4 amino acid region that is similar between Hel25E and UAP56 (which both act on long circular RNAs) but divergent from URH49 (which acts on short circular RNAs) is noted by the box.

Supplemental Figure S8. Effect of UAP56 or URH49 depletion on Hsp70 mRNA and total circular RNA levels.

(A) HeLa cells were treated with siRNAs for 48 h to specifically deplete UAP56 or URH49 and then subjected to 42°C heat shock for 1 h. Nuclear (left), cytoplasmic (middle), and total (right) RNA were purified and qRT-PCR was used to quantify steady-state expression of endogenous Hsp70 transcripts. (B) As in Fig. 3D,E, HeLa cells were treated with siRNAs for 48 h to specifically deplete UAP56 or URH49 and total RNA isolated. qRT-PCR was used to quantify steady-state expression of the indicated circRNAs, which are ordered according to their mature RNA lengths. Data throughout the figure are normalized to the scrambled siRNA samples and are shown as mean \pm SD, n=3. **, p<0.01; *, p<0.05.

Supplemental Figure S9. The ATP binding and helicase domains of Hel25E are largely dispensable for circular RNA localization.

DL1 cells were first treated with a control (β -gal) dsRNA or a dsRNA to deplete endogenous Hel25E. On the next day, inducible plasmids expressing Flag-tagged wildtype (WT) or mutant Hel25E (insensitive to dsRNA treatment) were transfected. K91N, ATP-binding domain mutant. D193E, helicase domain mutant. To obtain equal levels of protein expression, 1 μ g of WT plasmid was transfected while 2 μ g of the K91N and D193E plasmids were transfected. 500 μ M copper sulfate was added after 8 h to induce helicase expression from the plasmids and cells were analyzed after 24 h. (A) Western blotting was used to examine expression of the Flag-tagged Hel25E proteins. α -Tubulin was used as a loading control. Representative blots are shown. (B-G) DL1 cells were fractionated to isolate nuclear and cytoplasmic RNA. qRT-PCR was then used to quantify steady-state expression of the indicated circRNAs: circIaccase2 (B), circps (C), circmnb (D), circPlexA (E), circEct4 (F), and circdati (G). Data throughout the figure are normalized to the β -gal dsRNA samples and are shown as mean \pm SD, n=3. **, p<0.01; *,

$p < 0.05$. Whereas the WT, K91N, and D193E plasmids reduce nuclear accumulation of the circRNAs in **C-F** to similar extents, the ATP-binding and helicase domains are required for proper localization of circdati (**G**). Consistent with Fig 1, circLaccase2 did not accumulate in the nucleus when Hel25E levels were modulated (**B**).

Supplemental Figure S10. A region divergent among the Hel25E homologs controls their circular RNA length preferences.

DL1 cells were first treated with a control (β -gal) dsRNA or a dsRNA to deplete endogenous Hel25E. On the next day, 2 μ g of inducible plasmids expressing Flag-tagged wildtype (WT) or mutant helicases (insensitive to dsRNA treatment) were transfected. 500 μ M copper sulfate was added after 8 h to induce helicase expression from the plasmids and cells were analyzed after 24 h. (**A**) Western blotting was used to examine expression of the Flag-tagged proteins. Discs large was used as a loading control. Representative blots are shown. (**B-D**) As in Fig 4B,C, DL1 cells were fractionated to isolate nuclear and cytoplasmic RNA. qRT-PCR was then used to quantify expression of the indicated circRNAs: circmnb (**B**), circPlexA (**C**), and circLaccase2 (**D**). Data throughout the figure are normalized to the β -gal dsRNA samples and are shown as mean \pm SD, $n=3$. **, $p < 0.01$; *, $p < 0.05$. The divergent 4 amino acids significantly impact circmnb localization (**B**), while circPlexA is only marginally regulated (perhaps because depletion of Hel25E causes a large increase in overall circPlexA levels) (**C**). Consistent with Fig 1, circLaccase2 did not accumulate in the nucleus when Hel25E levels were modulated (**D**).

Supplemental Table Legends

Supplemental Table S1. Endogenous circular RNAs examined in this study.

Supplemental Table S2. Sequences of dsRNAs and siRNAs used in this study.

Supplemental Table S3. qRT-PCR primers used to examine expression of *Drosophila* transcripts.

Supplemental Table S4. qRT-PCR primers used to examine expression of human transcripts.

Supplemental Table S5. Northern blot probes used in this study.

Supplemental Table S1

Drosophila circRNA

circRNA	genomic length	spliced length	exon count
CaMKI	586	259	3
Dbp80(Exon5/6)	353	293	2
Dbp80(Exon3/4)	390	328	2
uex	334	334	1
laccase2	490	490	1
Haspin	557	500	2
CG42663	702	702	1
ps	811	811	1
pan	14846	846	7
Nipped-B	962	907	2
mnb	1096	1096	1
dati	1120	1120	1
PlexA	1439	1439	1
Ect4	1542	1478	2

Human circRNA

circRNA	genomic length	spliced length	exon count
MYH11	173	173	1
UGP2	1631	236	2
BBS9	4479	326	2
C3	451	356	2
PVT1	411	411	1
NEIL3	7370	596	2
ASH1L	742	742	1
ATP5C1	5808	799	6
PTBP3	46988	853	6
SLAIN2	13936	971	5
HIPK3	1099	1099	1
NFATC3	4624	1298	2
ANKRD17	7052	1832	2
BPTF	30550	2026	8
ATXN1	2077	2077	1

Supplemental Table S2

dsRNA target	Forward Primer	Reverse Primer	DRSC
β-gal	TAATACGACTCACTATAGGG CTGGCGTAATAGCGAAGAGG	TAATACGACTCACTATAGGG CATTAAAGCGAGTGGCCAACA	----
Nxt1	TAATACGACTCACTATAGGG TGGACGCCTCTATTTGGAC	TAATACGACTCACTATAGGG ACCTCCTGCATTGCGTAG	04630
sbr	TAATACGACTCACTATAGGG CGACGCAAGGATCGAAAC	TAATACGACTCACTATAGGG CCGTGGTCATGTGCTC	19770
nxf2	TAATACGACTCACTATAGGG GAGAGCTCGTCGCCTAAAGA	TAATACGACTCACTATAGGG ATGTCGAATTCGCAACACAG	26721
Nxf3	TAATACGACTCACTATAGGG GTTTTGCCAGCCTAGAGC	TAATACGACTCACTATAGGG AAGGCAAGCGGCTTAAAG	21749
Ranbp21	TAATACGACTCACTATAGGG CTCTTGGAGAATGCCTCAA	TAATACGACTCACTATAGGG ACTCGGTGCGCTTATCC	19447
emb	TAATACGACTCACTATAGGG AGGAGCACGGCAAACCTG	TAATACGACTCACTATAGGG CCAATGGCCCAGCACAAAC	03528
Hpr1	TAATACGACTCACTATAGGG GAAGACGTGCGCCAAAG	TAATACGACTCACTATAGGG GTGCATCGCCCAATGGAC	12296
tho2	TAATACGACTCACTATAGGG GCAATCGCCTGTATGAGGAT	TAATACGACTCACTATAGGG GGGCCTTAATATCCCGTTGT	28626
thoc5	TAATACGACTCACTATAGGG GCTGCACCTGGAAGAGTG	TAATACGACTCACTATAGGG CGGCGGTGAAGGACTG	04327
thoc6	TAATACGACTCACTATAGGG GAATCTCTGGCCACCAAAC	TAATACGACTCACTATAGGG CTCTCCACCGATTAAGACAC	10528
thoc7	TAATACGACTCACTATAGGG GCGGAAATCGAATCCAGTAA	TAATACGACTCACTATAGGG TGAAGTCATTGCGTCGTTTC	29470
Hel25E(#1)	TAATACGACTCACTATAGGG GCTAGAGCTCGAAGGTGCAT	TAATACGACTCACTATAGGG ATGCCCCAGAAATTTTCACA	30679
Hel25E(#2)	TAATACGACTCACTATAGGG GGGCACCTATGTGTCCATTC	TAATACGACTCACTATAGGG TCGCTCATACTCCTTGCTGA	30680
tex	TAATACGACTCACTATAGGG GCCGATGGTCGTTACCTG	TAATACGACTCACTATAGGG CGTGGGTCCGAACTCAAT	16517
Ref1	TAATACGACTCACTATAGGG TGGACAAAATTGAAATGAGCC	TAATACGACTCACTATAGGG GGAAGTTGGCCTTCTGAAC	12497
EIF4AIII	TAATACGACTCACTATAGGG CATGATGAATGTCCAGTGCC	TAATACGACTCACTATAGGG AGCTGAGATGAGCACACCCT	26402
mago	TAATACGACTCACTATAGGG CACGGAGGACTTTTACCTAC	TAATACGACTCACTATAGGG ATATGGGCTTGATCTTGAATG	04703
tsu	TAATACGACTCACTATAGGG CGATGTGTTGGACATTGACA	TAATACGACTCACTATAGGG GACGCTTTTCGGACTTTTT	07265
btz	TAATACGACTCACTATAGGG ATCCGCAGTACATCCCAAAG	TAATACGACTCACTATAGGG GCTGTTGCTTCTCTGGAACC	23613
RnpS1	TAATACGACTCACTATAGGG CAAAGACAAAGACAAGGATAAG	TAATACGACTCACTATAGGG ATCCTCGGGTGTGGCATA	15154
Acn	TAATACGACTCACTATAGGG CCAAGAAGAGGCACAAGGAG	TAATACGACTCACTATAGGG TCCAGTTGCACATCGCTTAG	29245
Pnn	TAATACGACTCACTATAGGG TCCCCGAAATGCAACAC	TAATACGACTCACTATAGGG TGCACCTTTGGCCTAAAGAA	16415
Bin1	TAATACGACTCACTATAGGG ATCTATGATTGTGGAGGAAAAG	TAATACGACTCACTATAGGG GGACGCTGGCGCCTG	16843
Phax	TAATACGACTCACTATAGGG CATTTCGGCGCACGGTAA	TAATACGACTCACTATAGGG GTCCTTTTCCTCGTACAGTTT	07066
Karyβ3	TAATACGACTCACTATAGGG GACTGCCCGAAAAATATATTGA	TAATACGACTCACTATAGGG GTTCGCATCACTGGTCCC	12355
Nup153	TAATACGACTCACTATAGGG ATCGATTCAAGAAATCGACG	TAATACGACTCACTATAGGG CGCGTGAAGGAAATATCAAA	19904

siRNA target	siRNA sequence
control	QIAGEN, AllStars Negative Control siRNA, Cat. No. 1027280
huUAP56	5'AAGGGCUJGGCUAUCACAUUUU3'
huURH49	5'AAA GGCCUAGCCAUCACUUUU3'

Supplemental Table S3

qRT-PCR primers for *Drosophila* genes

Primer	Sequence
Nxt1(qPCR)-F	ATGGACAGCGATTTGAAAGCC
Nxt1(qPCR)-R	TTTGTGGCGTCGGTTGTCC
sbr(qPCR)-F	ACGACGAGACCAACAATCCC
sbr(qPCR)-R	CGTTGCGGATACAAAAGCCA
nx12(qPCR)-F	GCATCCTGATGAACGTGTGC
nx12(qPCR)-R	CGTCAAGGGCCGTATATCCC
Nxf3(qPCR)-F	ACTTCAAGCGAAGTGGACCG
Nxf3(qPCR)-R	ACTTCCTCTTGCAAGCGTTTT
Ranbp21(qPCR)-F	CATGGCCTGTGAGCGGTTTA
Ranbp21(qPCR)-R	CAAAGTGTGCGACTTGCTGG
emb(qPCR)-F	CTGGACAAGATCGTCGAGGT
emb(qPCR)-R	GTCCATGCCTCTGGATGCTC
Hpr1(qPCR)-F	CGCCGTAGAAGTGGCTAACA
Hpr1(qPCR)-R	GACCAACAGCTCCACTTTGC
tho2(qPCR)-F	ACGACATGCCCTTTGTGCAAC
tho2(qPCR)-R	TTCTTGACGATTCGCCCTC
thoc5(qPCR)-F	GCTCGATTGCAACCGTCAAC
thoc5(qPCR)-R	GGCTTAAACGCTGCCAAGAG
thoc6(qPCR)-F	ACGCGCTTACAACAATGTCC
thoc6(qPCR)-R	TCGGAACCCCTGTCCAGTTC
thoc7(qPCR)-F	TGGAAGTGGAGAGGATCGGA
thoc7(qPCR)-R	GAATTGGGCCATCAGTCGGT
Hel25E(qPCR)-F	GTCCATTCACAGTTCCGGCT
Hel25E(qPCR)-R	GAATGCACTCGTGTCAACC
tex(qPCR)-F	CGGACGCACAAGTCTCCTT
tex(qPCR)-R	ATTCGCTCGTCGTGGCTAAA
Ref1(qPCR)-F	CGGCTTCAAACGTCCGGT
Ref1(qPCR)-R	GTATGAGTCCAGTTCGGCGT
EIF4AIII(qPCR)-F	TTTCGGACGCAAAGGTGTTG
EIF4AIII(qPCR)-R	TGGGCATCTCGTCAATTTGT
mago(qPCR)-F	GGTCAAAGATCCCGAGGGC
mago(qPCR)-R	TGGGCTTGATCTTGAAATGCAG
tsu(qPCR)-F	CAAAGAGGCCCTGAACGGTG
tsu(qPCR)-R	TTATCTGCGACGCTTTTCGG
btz(qPCR)-F	GAGGGCATCTCGACAACAT
btz(qPCR)-R	TTCTTTGTTCCCCACCTG
RnpS1(qPCR)-F	TTACGGTATCCCGGTTGTC
RnpS1(qPCR)-R	ACCTATTGAACTGGGGTGGG
Acn(qPCR)-F	CACGACAAACACAGCAACGA
Acn(qPCR)-R	CCTCTGTCCCTAGAACGC
Pnn(qPCR)-F	GGCGCAAGAATTGGAAGTT
Pnn(qPCR)-R	AACTGCTTTCGTGTCAGG
Bin1(qPCR)-F	AAGAGCAAAGAGAAGCCGGA
Bin1(qPCR)-R	CGTTGGCCATGTCTCCCTAT
Phax(qPCR)-F	AAACTCAGCGCATTGAAGCC
Phax(qPCR)-R	TGAGTGATGTTATCGTGCTGCT

Primer	Sequence
Karyβ3(qPCR)-F	ATTTGCGAGGTAGTTGCCGA
Karyβ3(qPCR)-R	AGGCGAGTTAGCGCATTGAA
Nup153(qPCR)-F	CCGTCGTCCAAAGATGGGAA
Nup153(qPCR)-R	CCTCGTTTCCGCTTGGTAGT
rp49(qPCR)-F	TACAGGCCCAAGATCGTGAAG
rp49(qPCR)-R	GACGCACTCTGTTGTCGATACC
18S(qPCR)-F	GTGCTGAAGCTTATGTAGCCT
18S(qPCR)-R	TGGGACAAACCAACAGGTACG
β-actin-F	CTCCGTCCACCATGAAGATT
β-actin-R	TTCGAGATCCACATCTGCTG
U6(qPCR)-F	GTTCTTGCTTCGGCAGAACATATACT
U6(qPCR)-R	TGTGGAACGCTTCACGATTTTGC
U7(qPCR)-F	GAAATTTGTCTTGGTGGG
U7(qPCR)-R	AACGGGAACACTCAATG
Hsp70(qPCR)-F	CAAGAACCCTAAGGGTGAAGC
Hsp70(qPCR)-R	GCCGGTTGTCAAAGTCCTC
Hsp83(qPCR)-F	CATTCCAGGCTGAGATTGCTC
Hsp83(qPCR)-R	ATCGGAAGCGTTCGAGATCA
circuex(qPCR)-F	ATTCGCAATGGTTCGCCGTG
circuex(qPCR)-R	GCAACGGATTTTCAGCACTTTACT
circDbp80(Exon5/6)(qPCR)-F	AGCCACCCAAGGTATCATG
circDbp80(Exon5/6)(qPCR)-R	AATTGTCCCATTTCGCGCAGC
circDbp80(Exon3/4)(qPCR)-F	GCCATGCTTAGCCGAGTCAAC
circDbp80(Exon3/4)(qPCR)-R	GCTTTGTGTTCAACAGCCCC
circIaccase2(qPCR)-F	GCCTCGAGAATTTGCTACTATCA
circIaccase2(qPCR)-R	ACATGTTGCTGCCAGAAGGAC
circHaspin(qPCR)-F	CGATGTCTATCGAATGATGCGGA
circHaspin(qPCR)-R	CCGGATGGTCATTTTCAGATCCC
circCaMKI(qPCR)-F	GGGTCTACACAGAAAAGGACG
circCaMKI(qPCR)-R	CATTTGCCATCGAAATGATTTGCA
circCG42663(qPCR)-F	AGAAGCCAAAATGTTCCGCGG
circCG42663(qPCR)-R	ATTTGCGCTCCACGTTGACA
circCps(qPCR)-F	TCCGATCCGGAAAATGAACAA
circCps(qPCR)-R	GACAGTTCGTGTACGTGCT
circNipped-B(qPCR)-F	AAAGGTTTGGCTTTTCAGGAAGT
circNipped-B(qPCR)-R	TCAAATGTGCGCTAAGATACTGT
circMnb(qPCR)-F	GAGGATACGAATAGCGGCGG
circMnb(qPCR)-R	AATTGTCGCCACGCTTTGTT
circDati(qPCR)-F	GGTGCCAACTGTGCGAAGTT
circDati(qPCR)-R	CGACGCCCGACATCAAATACAAT
circPan(qPCR)-F	AAACAAGAATGCGGTGTTCCAGG
circPan(qPCR)-R	CTCGAAAACTTCTTTGCACTGCA
circPlexA(qPCR)-F	ATTTCTTTGCGTTGGTGTCAAT
circPlexA(qPCR)-R	CCCAGCATGCCATGTGTTCTA
circEtc4(qPCR)-F	TTCAGCCTCAGTCTTTCGAG
circEtc4(qPCR)-R	TTTTCTGACTTTGCGTGGG

Supplemental Table S4

qRT-PCR primers for Human genes

Primer	Sequence
huHsp70(qPCR)-F	ATGTCGGTGGTGGGCATAGA
huHsp70(qPCR)-R	CACAGCGACGTAGCAGCTCT
hucircMYH11(qPCR)-F	GCGAGGTGAACGCACTCAA
hucircMYH11(qPCR)-R	GCTGGGACTCCTCCTCTGC
hucircUGP2(qPCR)-F	CTTCTGTGGATTGGGGAAAA
hucircUGP2(qPCR)-R	TGTGATGATGCTGTGGTGAG
hucircBBS9(qPCR)-F	CGCCGGCTACTAGCAAGATT
hucircBBS9(qPCR)-R	CTTGAAGGCCGAAGAATAAGCTC
hucircC3(qPCR)-F	GGAAGTCCCTGAAGGTCTGTG
hucircC3(qPCR)-R	TTTCCACCTGCTCGTTTCG
hucircPVT1(qPCR)-F	TTCAGCACTCTGGACGGACTT
hucircPVT1(qPCR)-R	TATGGCATGGGCAGGGTAG
hucircNEIL3(qPCR)-F	TGCATTCTCCGAGTTGTGGG
hucircNEIL3(qPCR)-R	CACGGGTACTTCATTAAGTGGCTA
hucircASH1L(qPCR)-F	TTTCTTTAATTCTCTTGGACCC
hucircASH1L(qPCR)-R	ACCCTCATCACCAGCCTTG
hucircPTBP3(qPCR)-F	CCTTGAACCCCTATGGCTG
hucircPTBP3(qPCR)-R	TCCCATTAGCATACACACCTG
hucircSLAIN2(qPCR)-F	TCAAGTGCCAAACGGAGGAA
hucircSLAIN2(qPCR)-R	ATCCAAAACCTTGCCTGCACC
hucircHIPK3(qPCR)-F	TATGTTGGTGGATCCTGTTCCGGCA
hucircHIPK3(qPCR)-R	TGGTGGGTAGACCAAGACTTGTGA
hucircNFATC3(qPCR)-F	AACTCATCATCGAGCCCATT
hucircNFATC3(qPCR)-R	TGGTAAGCAAAGTGGTGTGG
hucircANKRD17(qPCR)-F	CAACATTTTTCCCGCTTAG
hucircANKRD17(qPCR)-R	CAATTTCTTTGTCTGGATCCTTG
hucircBPTF(qPCR)-F	TCCAAGTGACTCCCCATTTT
hucircBPTF(qPCR)-R	GTACCTGCATCTGGGGTGAC
hucircATXN1(qPCR)-F	GATTGAAGACAGCCATAGCC
hucircATXN1(qPCR)-R	CTGATAAACGGAAAGTCACATT
huUAP56(qPCR)-F	GACAGCAGCTGGGGGAGATG
huUAP56(qPCR)-R	CTCATGCTGGACTTCTGACG
huURH49(qPCR)-F	GCCCCAGGCTCCTCAAGAGA
huURH49(qPCR)-R	CTCATGCTGGACCTCAGAAG
huMALAT1(qPCR)-F	GTCATAACCAGCCTGGCAGT
huMALAT1(qPCR)-R	GCTTATTCCCAATGGAGGT
hu18S(qPCR)-F	CTCAACACGGGAAACCTCAC
hu18S(qPCR)-R	CGCTCCACCAACTAAGAACG

Supplemental Table S5

Probe	Sequence
laccase2 Exon 2	GCTGAGCTCCCCGGG
dati Exon 2	TCCATCTCCTGCTTGGGCTTG
Firefly Exon	TAGCGCTTCATGGCTTTGTG
U6	TGTGGAACGCTTCACGATTTTGC
β -actin	AGCACAGTGTTGGCGTACAG

Supplemental Methods

Metabolic labeling of nascent RNAs with 4sU and nascent RNA purification

After bathing DL1 cells with dsRNAs for 3 days, 250 μ M 4sU (Sigma T4509) was added to the media for 15 min followed by the cellular fractionation procedure. 20 μ g of nuclear or cytoplasmic 4sU-labeled RNA was then incubated in a total volume of 500 μ L at room temperature for 1.5 h with rotation in biotinylation buffer (10 mM Tris pH 7.4, 1 mM EDTA) and 10 μ g/mL MTSEA biotin-XX (Biotium 90066; dissolved in dimethylformamide). To verify that there was not significant variation across the biotinylation reactions, 2 ng of a custom synthetic RNA (Dharmacon) was additionally included in each reaction.

Synthetic RNA (as originally described by Russo et al. 2017):

5' -AUUUAGGUGACACUAUAGGAUCCUCUAGAGUCGACCUUCUCCCUAUAUGUGAGUCGUAUUAGCA[4-S-U]CAG-3' .

Excess MTSEA biotin-XX was removed by extracting with an equal volume of chloroform:isoamyl alcohol (Sigma C0549-1PT), and RNA was precipitated by centrifuging at 12,000 x g for 30 min at 4°C with 1:10 volume of 5 M NaCl and an equal volume of isopropanol. The RNA pellet was washed with 80% ethanol and resuspended in 100 μ L DEPC-treated water. 4sU-labeled and unlabeled RNA were then separated using streptavidin-coated magnetic beads (Thermo Fisher Scientific 65602). This was done by incubating the biotinylated RNA with 100 μ L streptavidin beads in hybridization buffer (5 mM Tris pH 7.5, 0.5 mM EDTA, 1 M NaCl) at room temperature for 1.5 h. Beads were washed four times with high salt washing buffer (100 mM Tris pH 7.4, 10 mM EDTA, 1 M NaCl, 0.1% Tween 20). Nascent RNA was then eluted with 100 μ L 0.1 M dithiothreitol (DTT) twice, purified using the RNeasy MinElute Cleanup Kit (Qiagen 74204), and reverse transcribed using random hexamers and SuperScript III (ThermoFisher Scientific 18080051).

To verify equal pulldown of the custom synthetic RNA across reactions, the following primers were used for qRT-PCR: 5'-ATTTAGGTGACACTATAGGATCCTCTAG-3' and 5'-GCTAATACGACTCACTATAGGGAGAAG-3' (Russo et al. 2017).

Expression Plasmids

All *Drosophila* circular RNA expression plasmids were generated from the **Hy_pMT EGFP SV40 pA Sense** plasmid (<https://www.addgene.org/69911/>), which is a modified form of the pMK33/pMthHy plasmid. In brief, the metallothionein promoter (marked in blue) drives expression of the EGFP ORF (marked in green) that terminates in the SV40 polyadenylation signal (marked in pink). HygroR (marked in red) is driven by a copia transposon LTR promoter and terminates with an SV40 late poly(A) signal (marked in gray). An Amp selectable marker is also present. The full plasmid sequence is as follows:

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GATCAATCGTTGCAGGACAGGATGTGGTGCCCGATGTGACTAGCTCTTTGCTGCAGGCCGTCCTATCCTCTGGTTCGGATAAGAGACCCAGAAGCTC  
CGGCCCCACCACCGCCACCGCCACCCCCATACATATGTGGTACGCAAGTAAGAGTGCCTGCGCATGCCCCATGTGCCCCACCAAGAGCTTTGCATCC  
CATACAAGTCCCAAGTGGAGAACCGAACCAATTCTTCGCGGGCAGAACAAAAGCTTCTGCACACGTCTCCACTCGAATTTGGAGCCGGCCGGCGT  
GTGCAAAAGAGGTGAATCGAACGAAAGACCCGTGTGTAAAGCCGCGTTCCAAAATGTATAAAACCGAGAGCATCTGGCCAATGTGCATCAGTTGTG  
GTCAGCAGCAAAATCAAGTGAATCATCTCAGTGCAACTAAAGGGGGATCTCGAGGTGCGACGGTATCGATAAGCTTGATATCACATGGTGAGCAAG
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GGCGAGGAGCTGTTACCGGGGTGGTGCCATCCTGGTTCGAGCTGGACGGCGACGTAACGGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCG
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AAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGG
ACGGCAACATCCTGGGGCACAAGCTGGAGTACAACATAACAGCCACAACGTCTATATCATGCGCCACAAGCAGAAGAACGGCATCAAGGTGAAGT
CAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCCTACCAGCAGAACACCCCATCGCGCAGCGCCCGTGTGCTGCCCGAC
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Hy_pMT dati MCS Exon **Note: This plasmid was used for subsequent cloning**
(Exon containing multiple cloning site [MCS] in red)

CCTCTGCCATGACTATCGTAGAGTAAAAAGGGTTaagcatttccgaccatataaagtataaataactcttgatcagaatcaatagccgagtcgaactg
gccatgtccgctcaacctatgaggattgaaacaagcattcattctgaaatagacgcagcgaagttgttgactcatattgtcacactcactc
taacgcccacaaagcgccttaacctgcccacggacaccttttgaatcgaaatattcggtatcttttcatatttttattagttttgtaaatat
atcgagttgcaaaaaaactattccacgccaactataactcccacaaaccgccccaaaaatgcccacaccactcttttaagaaatgattcaattttgt
tggatataaataagtcgtttcccttatttcaatctatatgcccgaacacatttgaccacgcccactctgacccctaaaaccgccccaaTTTTAAAAATT
CAAAAAACCCTTTTACGCTTAACTTTTTTTGTTTTACAGTcAGGTACCACCGGTTAATTAAGGTAACCATGCATGCTAGCCCGGGGAGCTC
agGTATTGTTTTGAAGATTTTTCTGCTTATCTATATACTTTAAAGGGAACCTGGTAATCGAATATTCGATGCAGTCCAAACAAGAGTAGTAAAAAT
CAACAGAGAACGCTATTCGAGTTTTTAGACTAGACTATATGTACACCCGATATTTAGCTGTTACGAGCGCAACAATGAACTTTCAAAATTTGTGTGAC
TGTTTTGGACGTTTTGCAGGTGTAAGTAGGGCGTGGCCAAAGTGTTTTTGGTATGTCAATAAAAAATGGCAAGACAATGAAAACGAAGATTAATCA
AAACATTTTTTGTGGgggctggaacacagtttttggcaaatcgattttatctatcgatgaataatataatgaatacattttgcaagagcgtgggag
tggcagttctggaagcgtttgtggcgcttagaggatgctgtagcaacaaacttgcgctgcatctatgcttaactcctcaactttctagcttttagttaga
tagtttctgaaatctcgacgttcatacagacggatattggccagttcgactcgtcttttgatgctaatcaagaatataatgtagtttatatggtcgga
acgcttcccttcaactgttacatacctttaaacaaatctagtagatacactttccctctacagatgaatgggtatgaAAACAAAATCAACAGCATCAA
CGCATTTTTGAAAGTTGAAGCGTGATAGT

Hy_pMT Flag MCS SV40 **Note: This plasmid was used for subsequent cloning**
ACCATGGACTACAAAGACCATGacggtgattataaagatcatgacatcgattacaagatgacgatgacaagACCGGTggtaccGCTAGCTTAATTA
AGAGCTCCCCGGG

The following five plasmids were generated by inserting the indicated sequences into the KpnI and XmaI sites of Hy_pMT dati MCS Exon.

Hy_pMT dati MCS circfirefly_1677

atggaagatgccccaaacattaagaaggccagcgcattctaccactcgaagacgggaccgcccggcgagcagctgcacaaagccatgaagcgct
acgccctgggtgcccggaaccatcgcccttaccgacgcacatatcgaggtggacattacctacgccagtagtactcgagatgagcgttcggctggcaga
agctatgaagcgctatgggctgaatacaaacaccatcggtatcgtggtgtgcagcgagaaatagcttgcagttcttcatgcccgtgttgggtgccctgttc
atcgggtgtggctgtggccccagctaaccgacatctacaacgagcgcgagctgctgaacagcatgggcatcagccagccaccgctcgtatctcgtgagca
agaaagggctgcaaaagatcctcaacgtgcaaaagaagctaccgatcatacaaaagatcatcatcatggatagcaagaccgactaccagggttcca
aagcatgtacaccttcgtgacttccatttggccaccggcttcaacgagtagcacttctgctgcccagagcttgcagccgggacaaaaccatcgccctg
atcatgaacagtagtggcagtagccgatttgcccaagggcgtagccctaccgacccgacccgcttgtgtccgattcagtcagccccgacccccatct
tcggcaaccagatcatccccgacaccgctatcctcagcgtggtgcccatttcaaccacgcttcggcatgttcaaccacgctgggtacttgcctgagg
ctttcgggtcgtgctcatgtaccgcttcgaggaggagctattcttgcgcagcttgaagactataagattcaatctgcccctgttgggtgccacacta
tttagcttcttcgctaagagcactctcctcagcaagtagcagcactaagcaacttgcacgagatcgccagcggcggggcggcggcctcagcaaggaggtag
gtgaggccgtggccaaacgcttccacctaccaggcatccgcccagggtacggcctgcagaaacaaccagcgcattctgatcacccccgaagggga
cgacaagctggcgcagtaggcaaggtgggtgcccttctcagagctaaaggtggtagcttggacaccgtaagacactgggtgtgaaccagcggc
gagctgtcgtcgtggccccatgatcatgagcggctacgtttaacaaccccagggtacaaaacgctctcatcgacaaggacggctggctgcacagcg
ggacatcgccactctgggacgagcagcacttctcctcgtggacggcgtgaagagcctgatcaaatcaagggctaccaggtagccccagccga
actggagagcatcctgctgcaacacccccacatcttgcagcggggctcggcggcctgcccagcagcagatgcccggcagctgcccggcagctcgtc
gtgctggaacacggtaaaaccatgaccgagaaggagatcgtggactatgtggccagcagggtacaaccccaagaagctgcccgggtgggtgtggt
tcgtggacgaggtgcctaaaggactgaccggcaaggtggacgcccgaagatccgcgagattctcattaaggccaagaagggcggcaagatcgccct
gtaa

Hy_pMT dati MCS circfirefly_1100

atggaagatgccccaaacattaagaaggccagcgcattctaccactcgaagacgggaccgcccggcgagcagctgcacaaagccatgaagcgct
acgccctgggtgcccggaaccatcgcccttaccgacgcacatatcgaggtggacattacctacgccagtagtactcgagatgagcgttcggctggcaga
agctatgaagcgctatgggctgaatacaaacaccatcggtatcgtggtgtgcagcgagaaatagcttgcagttcttcatgcccgtgttgggtgccctgttc
atcgggtgtggctgtggccccagctaaccgacatctacaacgagcgcgagctgctgaacagcatgggcatcagccagccaccgctcgtatctcgtgagca
agaaagggctgcaaaagatcctcaacgtgcaaaagaagctaccgatcatacaaaagatcatcatcatggatagcaagaccgactaccagggttcca
aagcatgtacaccttcgtgacttccatttggccaccggcttcaacgagtagcacttctgctgcccagagcttgcagccgggacaaaaccatcgccctg
atcatgaacagtagtggcagtagccgatttgcccaagggcgtagccctaccgacccgacccgcttgtgtccgattcagtcagccccgacccccatct
tcggcaaccagatcatccccgacaccgctatcctcagcgtggtgcccatttcaaccacgcttcggcatgttcaaccacgctgggtacttgcctgagg
ctttcgggtcgtgctcatgtaccgcttcgaggaggagctattcttgcgcagcttgaagactataagattcaatctgcccctgttgggtgccacacta
tttagcttcttcgctaagagcactctcctcagcaagtagcagcactaagcaacttgcacgagatcgccagcggcggggcggcggcctcagcaaggaggtag
gtgaggccgtggccaaacgcttccacctaccaggcatccgcccagggtacggcctgcagaaacaaccagcgcattctgatcacccccgaagggga
cgacaagcc

Hy_pMT dati MCS circfirefly_900

atggaagatgccccaaacattaagaaggccagcgcattctaccactcgaagacgggaccgcccggcgagcagctgcacaaagccatgaagcgct
acgccctgggtgcccggaaccatcgcccttaccgacgcacatatcgaggtggacattacctacgccagtagtactcgagatgagcgttcggctggcaga
agctatgaagcgctatgggctgaatacaaacaccatcggtatcgtggtgtgcagcgagaaatagcttgcagttcttcatgcccgtgttgggtgccctgttc
atcgggtgtggctgtggccccagctaaccgacatctacaacgagcgcgagctgctgaacagcatgggcatcagccagccaccgctcgtatctcgtgagca
agaaagggctgcaaaagatcctcaacgtgcaaaagaagctaccgatcatacaaaagatcatcatcatggatagcaagaccgactaccagggttcca
aagcatgtacaccttcgtgacttccatttggccaccggcttcaacgagtagcacttctgctgcccagagcttgcagccgggacaaaaccatcgccctg
atcatgaacagtagtggcagtagccgatttgcccaagggcgtagccctaccgacccgacccgcttgtgtccgattcagtcagccccgacccccatct
tcggcaaccagatcatccccgacaccgctatcctcagcgtggtgcccatttcaaccacgcttcggcatgttcaaccacgctgggtacttgcctgagg
ctttcgggtcgtgctcatgtaccgcttcgaggaggagctattcttgcgcagcttgaagactataagattcaatctgcccctgttgggtgccacacta
ttt

Hy_pMT dati MCS circfirefly_700

atggaagatgccccaaacattaagaaggccagcgcattctaccactcgaagacgggaccgcccggcgagcagctgcacaaagccatgaagcgct
acgccctgggtgcccggaaccatcgcccttaccgacgcacatatcgaggtggacattacctacgccagtagtactcgagatgagcgttcggctggcaga
agctatgaagcgctatgggctgaatacaaacaccatcggtatcgtggtgtgcagcgagaaatagcttgcagttcttcatgcccgtgttgggtgccctgttc
atcgggtgtggctgtggccccagctaaccgacatctacaacgagcgcgagctgctgaacagcatgggcatcagccagccaccgctcgtatctcgtgagca
agaaagggctgcaaaagatcctcaacgtgcaaaagaagctaccgatcatacaaaagatcatcatcatggatagcaagaccgactaccagggttcca
aagcatgtacaccttcgtgacttccatttggccaccggcttcaacgagtagcacttctgctgcccagagcttgcagccgggacaaaaccatcgccctg
atcatgaacagtagtggcagtagccgatttgcccaagggcgtagccctaccgacccgacccgcttgtgtccgattcagtcagccccgacccccatct
tcggcaaccagatcatccccgacaccgctatcctcagcgtggtgcccatttcaaccacgcttcggcatgttcaaccacgctgggtacttgcctgagg
ctttcgggtcgtgctcatgtaccgcttcgaggaggagctattcttgcgcagcttgaagactataagattcaatctgcccctgttgggtgccacacta
ttt

Hy_pMT dati MCS circfirefly_500

atggaagatgccccaaacattaagaaggccagcgcattctaccactcgaagacgggaccgcccggcgagcagctgcacaaagccatgaagcgct
acgccctgggtgcccggaaccatcgcccttaccgacgcacatatcgaggtggacattacctacgccagtagtactcgagatgagcgttcggctggcaga
agctatgaagcgctatgggctgaatacaaacaccatcggtatcgtggtgtgcagcgagaaatagcttgcagttcttcatgcccgtgttgggtgccctgttc
atcgggtgtggctgtggccccagctaaccgacatctacaacgagcgcgagctgctgaacagcatgggcatcagccagccaccgctcgtatctcgtgagca
agaaagggctgcaaaagatcctcaacgtgcaaaagaagctaccgatcatacaaaagatcatcatcatggatagcaagaccgactaccagggttcca
aagcatgtacaccttcgtgacttccatttggccaccggcttcaacgagtagcacttctgctgcccagagcttgcagccgggacaaaaccatcgccctg
atcatgaacagtagtggcagtagccgatttgcccaagggcgtagccctaccgacccgacccgcttgtgtccgattcagtcagccccgacccccatct
tcggcaaccagatcatccccgacaccgctatcctcagcgtggtgcccatttcaaccacgcttcggcatgttcaaccacgctgggtacttgcctgagg
ctttcgggtcgtgctcatgtaccgcttcgaggaggagctattcttgcgcagcttgaagactataagattcaatctgcccctgttgggtgccacacta
ttt

The following four plasmids were generated by inserting the indicated sequences into the AgeI and NotI sites of Hy_pMT Flag MCS SV40.

Hy_pMT Flag-Hel25E WT

```
ATGGCCGACAATGACGATCTTTTGGACTACGAGGATGAGGAGCAGACCAGACCACTGCGGTGAAAAACCAGGAGGCCCCCAAGAAGGATGTCAAGG
GCACCTATGTGTCCATTACAGTTCCGGCTTCCGCGATTTCCCTCTGAAACCGGAGATCCTGCGCGCCATCGTGGACTGCGGGCTTCGAGCATCCCTC
GGAGGTTACAGCAGAGTGCATTCGCGAGGCCGACTGGGCATGGACATCCTCTGTGTCAGGCCAAGTCCGGCATGGGTAAAGACCCCGCTTTCGTTCTG
GCCACGCTGCAGCAGCTGGAGCCGTCGGACAACAACACCTGCCACGTCCTGGTCATGTGCCACACCCGCGAGCTGGCCTTCCAGATCAGCAAGGAGT
ATGAGCGATTCTCCAAGTACATGCCACAGTCAAGGTGGTGTCTTCTTTGGCGGAATGGCTATTCAAAGGACGAGGAGACCCTCAAGAGCGGCAC
CCCGCATATTGTGGTGGCACCCTGGCCGAATTTCTCGCCCTCATTGCAACAAGAACTTAATCTGAAGCTTTTGAAGCACTTTGTGCTCGACGAG
TGGCACAAGATGCTGGAGCAGCTGGATATGCGTCGTGACGTTCAAGAGATTTTCCGTAGCACGCCGCACGGCAACAAGTGATGATGTTCTCTGCCA
CATTGAGCAAGGACATTCGTCGCCGTTTGC AAAAAGTTTCATGCAAGATCCCATGGAGGTTCTACGTCGACGATGAGGCCAAGCTGACGCTGCACGGACT
GCAGCAGCACTACGTCATCTGAAGGAGAACGAGAAGAACAAGAACTGTTTCAACTGCTCGACGTGCTCGAGTTCAATCAGTGGTGCATCTTTGTG
AAGTCTGTGCAACGTTGCGTGGCTCTGTGCGAGCTGCTGACGGAGCAGAACTTCCCGCCATCGGCATCCATCGTGGGATGACCCAGGAGGAGCGTC
TGAATCGTACCAGCAGTTCAAGGACTTCCAGAAGCGCATTTCTGGTGGCCACCAATCTCTTTGGCCGCGGCATGGACATCGAGCGTGTGAACATCGT
GTTCAACTACGACATGCCCGAGGATTCGACACCTACTTGCATCGCGTGGCCCGTGGCCGTCGCTTCGGCACCAGGGACTGGCAATCACATTCGTT
TCGGACGAGAACGACGCCAAGATACTTAACGAAGTACAGGATCGTTTCGATGTGAACATCAGTGAGCTGCCCGAGGAAATCGATCTCTACATACA
TTGAGGGACGCTAG
```

Hy_pMT Flag-Hel25E MUT

(Desired mutations noted in red)

```
ATGGCCGACAATGACGATCTTTTGGACTACGAGGATGAGGAGCAGACCAGACCACTGCGGTGAAAAACCAGGAGGCCCCCAAGAAGGATGTCAAGG
GCACCTATGTGTCCATTACAGTTCCGGCTTCCGCGATTTCCCTCTGAAACCGGAGATCCTGCGCGCCATCGTGGACTGCGGGCTTCGAGCATCCCTC
GGAGGTTACAGCAGAGTGCATTCGCGAGGCCGACTGGGCATGGACATCCTCTGTGTCAGGCCAAGTCCGGCATGGGTAAAGACCCCGCTTTCGTTCTG
GCCACGCTGCAGCAGCTGGAGCCGTCGGACAACAACACCTGCCACGTCCTGGTCATGTGCCACACCCGCGAGCTGGCCTTCCAGATCAGCAAGGAGT
ATGAGCGATTCTCCAAGTACATGCCACAGTCAAGGTGGTGTCTTCTTTGGCGGAATGGCTATTCAAAGGACGAGGAGACCCTCAAGAGCGGCAC
CCCGCATATTGTGGTGGCACCCTGGCCGAATTTCTCGCCCTCATTGCAACAAGAACTTAATCTGAAGCTTTTGAAGCACTTTGTGCTCGACGAG
TGGCACAAGATGCTGGAGCAGCTGGATATGCGTCGTGACGTTCAAGAGATTTTCCGTAGCACGCCGCACGGCAACAAGTGATGATGTTCTCTGCCA
CATTGAGCAAGGACATTCGTCGCCGTTTGC AAAAAGTTTCATGCAAGATCCCATGGAGGTTCTACGTCGACGATGAGGCCAAGCTGACGCTGCACGGACT
GCAGCAGCACTACGTCATCTGAAGGAGAACGAGAAGAACAAGAACTGTTTCAACTGCTCGACGTGCTCGAGTTCAATCAGTGGTGCATCTTTGTG
AAGTCTGTGCAACGTTGCGTGGCTCTGTGCGAGCTGCTGACGGAGCAGAACTTCCCGCCATCGGCATCCATCGTGGGATGACCCAGGAGGAGCGTC
TGAATCGTACCAGCAGTTCAAGGACTTCCAGAAGCGCATTTCTGGTGGCCACCAATCTCTTTGGCCGCGGCATGGACATCGAGCGTGTGAACATCGT
GTTCAACTACGACATGCCCGAGGATTCGACACCTACTTGCATCGCGTGGCCCGTGGCCGTCGCTTCGGCACCAGGGACTGGCAATCACATTCGTT
TCGGACGAGAACGACGCCAAGATACTTAACGAAGTACAGGATCGTTTCGATGTGAACATCAGTGAGCTGCCCGAGGAAATCGATCTCTACATACA
TTGAGGGACGCTAG
```

Hy_pMT Flag-Hel25E K91N

(Desired mutation noted in red)

```
ATGGCCGACAATGACGATCTTTTGGACTACGAGGATGAGGAGCAGACCAGACCACTGCGGTGAAAAACCAGGAGGCCCCCAAGAAGGATGTCAAGG
GCACCTATGTGTCCATTACAGTTCCGGCTTCCGCGATTTCCCTCTGAAACCGGAGATCCTGCGCGCCATCGTGGACTGCGGGCTTCGAGCATCCCTC
GGAGGTTACAGCAGAGTGCATTCGCGAGGCCGACTGGGCATGGACATCCTCTGTGTCAGGCCAAGTCCGGCATGGGTAAAGACCCCGCTTTCGTTCTG
GCCACGCTGCAGCAGCTGGAGCCGTCGGACAACAACACCTGCCACGTCCTGGTCATGTGCCACACCCGCGAGCTGGCCTTCCAGATCAGCAAGGAGT
ATGAGCGATTCTCCAAGTACATGCCACAGTCAAGGTGGTGTCTTCTTTGGCGGAATGGCTATTCAAAGGACGAGGAGACCCTCAAGAGCGGCAC
CCCGCATATTGTGGTGGCACCCTGGCCGAATTTCTCGCCCTCATTGCAACAAGAACTTAATCTGAAGCTTTTGAAGCACTTTGTGCTCGACGAG
TGGCACAAGATGCTGGAGCAGCTGGATATGCGTCGTGACGTTCAAGAGATTTTCCGTAGCACGCCGCACGGCAACAAGTGATGATGTTCTCTGCCA
CATTGAGCAAGGACATTCGTCGCCGTTTGC AAAAAGTTTCATGCAAGATCCCATGGAGGTTCTACGTCGACGATGAGGCCAAGCTGACGCTGCACGGACT
GCAGCAGCACTACGTCATCTGAAGGAGAACGAGAAGAACAAGAACTGTTTCAACTGCTCGACGTGCTCGAGTTCAATCAGTGGTGCATCTTTGTG
AAGTCTGTGCAACGTTGCGTGGCTCTGTGCGAGCTGCTGACGGAGCAGAACTTCCCGCCATCGGCATCCATCGTGGGATGACCCAGGAGGAGCGTC
TGAATCGTACCAGCAGTTCAAGGACTTCCAGAAGCGCATTTCTGGTGGCCACCAATCTCTTTGGCCGCGGCATGGACATCGAGCGTGTGAACATCGT
GTTCAACTACGACATGCCCGAGGATTCGACACCTACTTGCATCGCGTGGCCCGTGGCCGTCGCTTCGGCACCAGGGACTGGCAATCACATTCGTT
TCGGACGAGAACGACGCCAAGATACTTAACGAAGTACAGGATCGTTTCGATGTGAACATCAGTGAGCTGCCCGAGGAAATCGATCTCTACATACA
TTGAGGGACGCTAG
```

Hy_pMT Flag-Hel25E D193E

(Desired mutation noted in red)

```
ATGGCCGACAATGACGATCTTTTGGACTACGAGGATGAGGAGCAGACCAGACCACTGCGGTGAAAAACCAGGAGGCCCCCAAGAAGGATGTCAAGG
GCACCTATGTGTCCATTACAGTTCCGGCTTCCGCGATTTCCCTCTGAAACCGGAGATCCTGCGCGCCATCGTGGACTGCGGGCTTCGAGCATCCCTC
GGAGGTTACAGCAGAGTGCATTCGCGAGGCCGACTGGGCATGGACATCCTCTGTGTCAGGCCAAGTCCGGCATGGGTAAAGACCCCGCTTTCGTTCTG
GCCACGCTGCAGCAGCTGGAGCCGTCGGACAACAACACCTGCCACGTCCTGGTCATGTGCCACACCCGCGAGCTGGCCTTCCAGATCAGCAAGGAGT
ATGAGCGATTCTCCAAGTACATGCCACAGTCAAGGTGGTGTCTTCTTTGGCGGAATGGCTATTCAAAGGACGAGGAGACCCTCAAGAGCGGCAC
CCCGCATATTGTGGTGGCACCCTGGCCGAATTTCTCGCCCTCATTGCAACAAGAACTTAATCTGAAGCTTTTGAAGCACTTTGTGCTCGAAGGAG
TGGCACAAGATGCTGGAGCAGCTGGATATGCGTCGTGACGTTCAAGAGATTTTCCGTAGCACGCCGCACGGCAACAAGTGATGATGTTCTCTGCCA
CATTGAGCAAGGACATTCGTCGCCGTTTGC AAAAAGTTTCATGCAAGATCCCATGGAGGTTCTACGTCGACGATGAGGCCAAGCTGACGCTGCACGGACT
GCAGCAGCACTACGTCATCTGAAGGAGAACGAGAAGAACAAGAACTGTTTCAACTGCTCGACGTGCTCGAGTTCAATCAGTGGTGCATCTTTGTG
AAGTCTGTGCAACGTTGCGTGGCTCTGTGCGAGCTGCTGACGGAGCAGAACTTCCCGCCATCGGCATCCATCGTGGGATGACCCAGGAGGAGCGTC
TGAATCGTACCAGCAGTTCAAGGACTTCCAGAAGCGCATTTCTGGTGGCCACCAATCTCTTTGGCCGCGGCATGGACATCGAGCGTGTGAACATCGT
GTTCAACTACGACATGCCCGAGGATTCGACACCTACTTGCATCGCGTGGCCCGTGGCCGTCGCTTCGGCACCAGGGACTGGCAATCACATTCGTT
TCGGACGAGAACGACGCCAAGATACTTAACGAAGTACAGGATCGTTTCGATGTGAACATCAGTGAGCTGCCCGAGGAAATCGATCTCTACATACA
TTGAGGGACGCTAG
```

The following four plasmids were generated by inserting the indicated sequences into the KpnI and NotI sites of Hy_pMT Flag MCS SV40.

Hy_pMT Flag-UAP56 WT

```
atggcagagaacgatgtggacaatgagctcttggactatgaagatgatgaggtggagacagcagctgggggagatggggctgaggccctgccaaga
aggatgtcaagggctcctatgtctccatccacagctctggcttctcgtgacttctctgctcaagccagagtgtgctccgggccattgtcgaactgtggctt
tgagcatccgtcagaagtcagcatgagtgcatccctcaggccattctgggaaatggatgtcctgtgcccaggccaagtccgggatgggaaagacagca
gtgtttgtcctggccacactgcaacagctggagccagttactgggcaaggtgtcgtgactggatgtgtcacactcgggagttggctttcagatca
gcaaggaatatgagcgcttctctaaatacatgcccattgtcaaggttggctgttttttttgggtggtctgtctatcaagaaggatgaagaggtgctgaa
gaagaactgcccgcataatcgtcgtggggactccagggcgtatcctagccctggctcgaataaagagcctcaacctcaaacacataaacactttatt
ttggatgaatgtgataagatgcttgaacagctcgacatgctcgggatgtccaggaattttctgcatgacccccacgagaagcaggtcatgatgt
tcagtgctaccttgagcaaaagatccgtccagctctgcccgaagttcatgcaagatccaatggagatctcctggtgatgagacgaagttgacgct
gcatgggttgcagcagtaactcgtgaaactgaaggacaacgagaagaaccggaagctctttgacctctggatgtccttgagttaaccaggtgggtg
atcctttgtgaagctgtgagcagcgtgcaattgacctggcccagctactgagggagcagaacttcccagccattgccatccaccggtgggatgcccagg
aggagaggtcttctcggatcagcagtttaaagattttcaacgacgaattctgtggctaccaacctatttggccgaggtatggacatcgagcgggt
gaacattgcttttaattatgacatgctgaggattctgacacctacctgcatcgggtggccagagcagggccggtttggcaccgaagggcttggctatc
acatttgtgtccgatgagaatgatgccaagatcctcaatgatgtgcaggatcgctttgaggtcaatatagtgagctgctgatgagatagacatct
cctcctacattgaacagacacggtag
```

Hy_pMT Flag-UAP56 MUT

(Desired mutations noted in red)

```
atggcagagaacgatgtggacaatgagctcttggactatgaagatgatgaggtggagacagcagctgggggagatggggctgaggccctgccaaga
aggatgtcaagggctcctatgtctccatccacagctctggcttctcgtgacttctctgctcaagccagagtgtgctccgggccattgtcgaactgtggctt
tgagcatccgtcagaagtcagcatgagtgcatccctcaggccattctgggaaatggatgtcctgtgcccaggccaagtccgggatgggaaagacagca
gtgtttgtcctggccacactgcaacagctggagccagttactgggcaaggtgtcgtgactggatgtgtcacactcgggagttggctttcagatca
gcaaggaatatgagcgcttctctaaatacatgcccattgtcaaggttggctgttttttttgggtggtctgtctatcaagaaggatgaagaggtgctgaa
gaagaactgcccgcataatcgtcgtggggactccagggcgtatcctagccctggctcgaataaggagcttcaagctcaaacacataaacactttatt
ttggatgaatgtgataagatgcttgaacagctcgacatgctcgggatgtccaggaattttctgcatgacccccacgagaagcaggtcatgatgt
tcagtgctaccttgagcaaaagatccgtccagctctgcccgaagttcatgcaagatccaatggagatctcctggtgatgagacgaagttgacgct
gcatgggttgcagcagtaactcgtgaaactgaaggacaacgagaagaaccggaagctctttgacctctggatgtccttgagttaaccaggtgggtg
atcctttgtgaagctgtgagcagcgtgcaattgacctggcccagctactgagggagcagaacttcccagccattgccatccaccggtgggatgcccagg
aggagaggtcttctcggatcagcagtttaaagattttcaacgacgaattctgtggctaccaacctatttggccgaggtatggacatcgagcgggt
gaacattgcttttaattatgacatgctgaggattctgacacctacctgcatcgggtggccagagcagggccggtttggcaccgaagggcttggctatc
acatttgtgtccgatgagaatgatgccaagatcctcaatgatgtgcaggatcgctttgaggtcaatatagtgagctgctgatgagatagacatct
cctcctacattgaacagacacggtag
```

Hy_pMT Flag-URH49 WT

```
atggcagagaacgatgtggaaaaagatcctttggattacgatgaagaggaagagcccaggctcctcaagagagcacaccagctccccctaagaaa
acatcaagggatcctacgttttccatccacagctctggcttccgggacttctcgtgaaagccggagctcctgcccggccatcgtggactgtggcttga
gcatcctctgaggtccagcatgagtgcatcccccaggccatcctgggcatggagctcctgtgccaggccaagtccgggatgggcaagacagcggctc
tctcgtgctggccaccctacagcagattgagcctgtcaacggacaggtgacggtcctggtcatgtgccacacagagggagctggccttcagatcagca
aggaatatgagcgcttttccaagtacatgcccagcgtcaaggtgtctgtgttctcgtggtctctccatcaagaaggatgaagaaggtgttgaagaa
gaactgtccccatgtcgtggtgggggaccccggcgcacatcctggcgtcgtgcggaataggagcttcaagcctaagaatgtgaagcatttggctg
gacgaggtgacaagatgctggagcagctggacatgcccggggatgtgagggatcttccgctgacaccacagagaagcagtgcatgatgttca
gcgccacctgagcaagacatccggcctgtgtgcaggaagttcatgaggatcccatggaggtgtttgtggacgagagccaagctcacgctgca
cggctgcagcagtaactcgtcaaaactcaaagacagtgagaagaaccgcaagctctttgatctcttggatgtgctggagtttaaccaggtgataatc
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```

Hy_pMT Flag-URH49 MUT

(Desired mutations noted in red)

```
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```