Supporting Online Material for

Down-Regulation of a Host microRNA by a *Herpesvirus saimiri* Noncoding RNA

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Supporting Online Material

Materials and Methods

Bioinformatic analysis. Alignment of HSUR sequences was performed using the ClustalW method in TCOFFEE (http://tcoffee.vital-it.ch/cgi-bin/Tcoffee/tcoffee_cgi/index.cgi). Perfectly or highly conserved sequences in HSUR 1 and HSUR 2 (Fig. 1A; fig. S1 and S2) were manually examined for their complementarity to the seed region of human miRNAs expressed in T cells (*1*), and complementarity was further confirmed using RNA22 (http://cbcsrv.watson.ibm.com/rna22.html). Genes up regulated in marmoset T cells transformed by wild-type HVS A11 (*2*) are not predicted to be targets of miR-142-3p, the miR-16 family, or of miR-27 by three algorithms, including TargetScan (http://www.targetscan.org/vert_50/), PicTar (http://pictar.mdc-berlin.de/), and MicroCosm (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/).

Cell culture and transfection. T cells derived from a single marmoset were transformed in parallel with either wild-type HVS A11 or HVS A11 Δ2a (*3*) deleted for the HSUR 1 and 2 genes (*4*) and cultured as described (*3*). Jurkat T cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum. For HSUR knockdown experiments, $1x10^7$ marmoset T cells transformed with wild-type HVS were resuspended in 100 µl solution from the Human T Cell

Nucleofector Kit (Amaxa Biosystems) and mixed with 1 nmol of chimeric antisense oligonucleotides (IDT). The oligonucleotides (oligos) are 20-nt long, contain backbone phosphorothioate to increase stability, and have five nucleotides on each end substituted with 2′-*O*-methoxyethyl ribonucleotides (*5*). Oligos are complementary to regions downstream of the miRNA binding sites in HSUR 1 and HSUR 2 (αHSUR 1 oligo: 5′-GGGUUGTTTGAGACTUUUAG-3′; αHSUR 2 oligo: 5′-AAGCGATACCTCGTGUGUGA-3′; control αGFP oligo: 5′-UCACCTTCACCCTCTCCACU-3′). Transfection was carried out in an electroporation cuvette using a Nucleofector instrument (Amaxa Biosystems). Transfected cells were transferred to complete medium and incubated for 36 hours, split into two, and harvested for both RNA preparation and Western blot analyzes. Data reported are representative of three independent experiments.

Jurkat T cells (1 x 10^6) were nucleofected with 2 µg of plasmid using a Nucleofector Kit V (Amaxa Biosystems) following the manufacturer's instructions. Stable cell lines expressing HSURs were generated by diluting after transfection and culturing in Hygromycin (300 *µ*g/ml, Calbiochem). Stable cell lines created with pCEP-7.4 (see below) were compared to virally transformed cells for the expression of HSUR 1 by Northern blot analyses, and clones showing HSUR 1 expression similar to virally transformed T cells were selected. Stable cell lines expressing mutant versions of HSUR 1 were prepared in the same way and compared to Jurkat cells stably transfected with pCEP-7.4.

For transient expression of HSURs, Jurkat T cells were nucleofected as described above and sorted based on GFP expression with a BD LSRII System

(BD Biosciences). Total RNA was prepared from selected cells with Trizol (Invitrogen) and assayed for miRNA levels.

Plasmids. For preparation of Jurkat T cells stably expressing all seven HSURs, fragments of plasmid pT7.4 (kindly provided by Ronald C. Desrosiers), which contain HVS L-DNA sequences from +22 to approximately +7253, including the genes for HSURs 1 to 7 with their own enhancers, promoters and processing signals, cloned into vector pBR322 (*3*), were used. pCEP-7.4 was constructed by digesting pT7.4 with *Taq*I to obtain the HVS A11 genomic fragment encompassing all seven HSUR genes, and cloning it between the *BamH*I and *Sal*I sites of pCEP∆SaI, a version of pCEP4 (Invitrogen) in which the *Sal*I site at position 1067 was mutated by site-directed mutagenesis. pCEP∆H1 was constructed by first amplifying a genomic region downstream of the HSUR 1 gene (including positions +22 to +1495 of HVS A11 genome) and cloning this fragment into the *BamH*I and *EcoR*I sites of pCEP∆SaI to generate the plasmid pCEP∆H1Int, followed by amplification of the genomic fragment upstream of the HSUR 1 gene (including positions +1720 to +7253 of HVS A11 genome) that contains genes for HSUR 2 through 7, and insertion of this fragment between the *EcoR*I and *Sal*I sites of pCEP∆H1Int. GFP-H3 and GFP-H1 plasmids were constructed by amplifying a fragment containing the U1 promoter-HSUR-U1 3′ box from pUC-U1-HSUR 3 and pUC-U1-HSUR 1, respectively (*6*), and inserting them into a unique *Nsi*I site in the pmaxGFP vector (Amaxa). Mutant versions of

HSUR 1 were obtained by site-directed mutagenesis using a QuickChange Site-Directed Mutagenesis Kit (Stratagene) and confirmed by sequencing. Both transient and stable transfection of Jurkat T cells with plasmids that express either wild-type or a mutant HSUR 1 (H1Mt, Fig. 4A and fig. S8) showed that mutations in the miR-27 binding site of HSUR 1 did not lower its levels in Jurkat T cells (Fig. 4B and fig. S9). Primer sequences are available upon request.

Northern blot analysis. Total RNA, prepared using Trizol (Invitrogen) following the manufacturer's protocol, was typically separated on denaturing 15% polyacrylamide gels for analysis of miRNAs and denaturing 6% polyacrylamide gels for analysis of HSURs, and transferred to Zeta-Probe® GT Genomic membrane (BioRad). Oligonucleotide probes were 5′ end-labeled with [γ-^{32}P]ATP (PerkinElmer Life Sciences) using T4 polynucleotide kinase (New England Biolabs). Probe sequences are available upon request. Hybridization and washes were performed at 30ºC for miRNAs and at 42ºC for snRNAs using ExpressHyb Hybridization Solution (Clontech) according to the manufacturer's instructions. Radioactive signals were quantified using a Storm 840 Phosphorimager (Molecular Dynamics). Membranes were stripped by incubating twice in boiling 1% SDS for 15 minutes and then checked for removal of probe prior to hybridizing with additional probes.

Immunoprecipitation and antibodies. Immunoprecipitation experiments using Y12 (anti-Sm) antibodies (*7*) or non-immune serum were performed as previously

described (*8*) on extracts prepared from either 8 x 10^7 HVS-transformed marmoset T cells, or from 3 x 10^7 Jurkat T cells stably expressing HSURs. For immunoprecipitation with anti-Ago2 antibodies (Upstate, Cat. # 07-590, currently Millipore), 8 x 10^7 HVS-transformed marmoset T cells were resuspended in 500 µl of NET-2 buffer (50 mM Tris [pH 7.5], 150 mM NaCl, and 0.05% NP-40) and sonicated 3 times for 15 sec. Lysates were cleared by centrifugation at 16000g for 10 minutes at 4ºC and incubated with nutation for 3 hours at 4ºC with 5 µl antibody previously immobilized on protein A-sepharose (Amersham). Samples were then washed four times with NET-2 buffer, and RNA was extracted from beads with Trizol following the manufacturer's protocol and analyzed by Northern blot as described above. Antibodies against FOXO1 and GAPDH (loading control) were purchased from Cell Signaling Technology and used for Western blots following the manufacturer's instructions. Antibodies against Cdk6, Cyclin E1, and Bcl2 were purchased from Abcam.

UV cross-linking of living cells with AMT (psoralen) and immunopurification of RNA. Cells (5 x 10^7) were washed in PBS, resuspended in cold PBS with 200 µg/ml 4′-Aminomethyl-trioxsalen hydrochloride (AMT) (Sigma), and irradiated at 365 nm with a Spectroline ENF-280C UV lamp at an intensity of approximately 100 mW/cm^2 for 60 minutes at 4ºC. Total RNA was isolated with Trizol, resuspended in water, denatured by incubation at 95ºC for 5 minutes, and incubated with nutation for 3 hours at 4ºC with 1 µg of Ab-1 anti-m_3G cap antibody (Calbiochem) previously immobilized on protein G-sepharose

(Amersham). Beads were then washed with NET-2 buffer, and RNA was recovered from the beads with Trizol. Cross-linking was reversed by irradiation for 10 minutes with 254 nm UV light at the same intensity as above, and RNA was analyzed by Northern blot.

Quantitative real-time PCR. Mature miRNA and U6 snRNA (endogenous control) levels were assessed by quantitative real-time PCR using Taqman® miRNA Assays (Applied Biosystems) following the manufacturer's instructions. Levels of precursor miRNAs and U6 snRNA (endogenous control) were assessed with specific primers and Fast SYBR® Green Master Mix (Applied Biosystems) detection following the manufacturer's instructions. Primers used were the following:

- U6 snRNA: forward primer: 5′-CGCTTCGGCAGCACATATAC-3′; reverse primer: 5′-AGGGGCCATGCTAATCTTCT-3′
- pre-miR-27a: forward primer: 5′-GCAGGGCTTAGCTGCTTG-3′; reverse primer: 5′-GGGCGGAACTTAGCCACT-3′
- pre-miR-27b: forward primer: 5′-TGCAGAGCTTAGCTGATTGG-3′; reverse primer: 5′-CACTGTGAACAAAGCGGAAA-3′
- pre-miR-23a: forward primer: 5′-CTGGGGATGGGATTTGCT-3′; reverse primer: 5′-TGGAAATCCCTGGCAATG-3′

Amplification was performed with a StepOnePlus Real-Time PCR System (Applied Biosystems) and results were analyzed using StepOne software v2.1. The mean of three replicates, with error bars representing a 99% confidence

interval, is reported.

In experiments in which HSUR 1 was transiently expressed in Jurkat T cells (Fig. 4C), cells were nucleofected with the plasmids shown in fig. S8. GFP-expressing cells were sorted 48 hours after transfection, and total RNA was prepared as described above. MiRNA levels were determined by qRT-PCR as described above. Values were normalized to those of cells transfected with empty vector and are the means ± SD from three independent experiments.

Quantification of miRNAs in virally transformed cells. The total number of molecules of miRNA per cell was determined by Northern blot. Briefly, total RNA from $1x10^7$ cells was loaded together with known amounts of synthetic miRNA on a 15% denaturing polyacrylamide gel, separated by electrophoresis, transferred to a membrane and probed for different miRNAs as described above. Signals obtained were quantified with a Phosphorimager, and the total number of molecules per cell was calculated by comparison of signal obtained from total RNA isolated from cells to a curve of synthetic miRNA of known concentration on the same membrane.

MiRNA decay. Synthetic miRNAs were prepared by 5′-end labeling the miR-27a guide strand (5′-UUCACAGUGGCUAAGUUCCGCdTdT-3′), miR-16 guide strand (5′-UAGCAGCACGUAAAUAUUGGCGdTdT-3′), and miR-20a guide strand (5′-UAAAGUGCUUAUAGUGCAGGUAGdTdT-3’) with [γ-^{32}P]ATP (PerkinElmer Life Sciences) using T4 polynucleotide kinase (New England Biolabs), and annealing

them to an unlabeled miR-27a passenger strand (5′-GCGGAACUUAGCCACUGUGAAdTdT-3′), miR-16 passenger strand (5′-CGCCAAUAUUUACGUGCUGCUAdTdT-3′), and miR-20a passenger strand (5′-CUACCUGCACUAUAAGCACUUUAdTdT-3’), respectively. HVS-transformed marmoset T cells (1 x 10^7) were nucleofected as described above, diluted into 10 ml fresh medium, and transferred to 24-well plates as ten 1 ml aliquots. Cells from each aliquot were harvested at different times; total RNA was prepared, separated on a denaturing 15% polyacrylamide gel, transferred to a membrane, and exposed to a phosphoRimager screen. Membranes were probed for U6 snRNA as a loading control (not shown). Detailed analysis of the decay values suggested that the degradation of miR-27a may follow biphasic kinetics in the Wt cells; therefore relative half-lives are not given. Data reported are representative of three independent experiments.

Legend to supplementary figures

Figure S1. Alignment of sequences from the 5′-end to the Sm binding site of reported genomic sequences (*9-15*) for HSUR 1. MicroRNA binding sites are boxed in blue, the ARE is boxed in red, and Sm binding sites are boxed in grey. Perfectly conserved nucleotides are bold.

Figure S2. Alignment of HSUR 2 sequences. Details are as in Fig. S1.

Figure S3. Coimmunoprecipitation of cellular snRNPs from extracts of virally transformed marmoset T cells with anti-Flag (lane 3) or anti-Ago2 (lane 5) antibody. I: Input (5%); S: Supernatant (5%); P: Pellet (100%).

Figure S4. MiR-27 interacts with HSURs *in vivo*. Marmoset T cells transformed with either wild-type (Wt, lanes 1-4) or a mutant HVS A11 (Mut, lanes 5 and 6) deleted for HSURs 1 and 2 (Mut) were UV crosslinked in the presence (lanes 3-6) or absence (lanes 1 and 2) of AMT. Total RNA was immunoprecipitated with antibodies against the m_3G cap and the crosslinks reversed before Northern blot analysis. I: Input (1%); P: Pellet (100%).

Figure S5. Northern blot analyzes of miRNA expression in marmoset T cells transformed with either wild-type (Wt) or a mutant HVS A11 deleted for HSURs 1 and 2 (Mut). Fifteen micrograms of total RNA were separated on a 15%

denaturing polyacrylamide gel and blotted for indicated miRNAs and snRNAs.

Figure S6. Relative expression of pre-miRNAs in virally transformed marmoset T cells expressing (Wt) or lacking (Mut) HSURs 1 and 2. The levels of the indicated pre-miRNAs were determined by quantitative real-time PCR. The mean of three replicates is shown, with error bars representing a 99% confidence interval.

Figure S7. Pulse-chase assay assessing the decay of radioactively labeled synthetic miRNAs. Total RNA was separated on a denaturing gel and transferred to a membrane to detect the levels of transfected miR-20a at the indicated times after nucleofection of either Wt or Mut marmoset T cell lines.

Figure S8. Plasmid constructs used for transient expression of HSURs in Jurkat T cells (Fig. 4C). The U1 promoter (U1) is the 423-bp human genomic sequence upstream of the U1 transcription initiation site, whereas the 3′ box (U1 3′-box) is the 30-bp transcription termination signal downstream of the U1 coding sequence (*16*). HSUR 1 mutations in the miR-27 binding site (27-BS) in GFP-H1Mt, and in the miR-20 binding site (20-BS) in GFP-H1m20, are described in Fig. 4A. The mutant ARE sequence of HSUR 1 in GFP-H1M1 was previously described [HSUR 1 Mut 1 in (*6*)]. All constructs also contain the GFP gene (GFP) driven by a CMV promoter (CMV).

Figure S9. Northern blot analyzes of HSUR levels in Jurkat T cells. Plasmid

constructs described in Fig. S8 were nucleofected into Jurkat T cells and RNA was analyzed by Northern blot 48 hours after transfection. Numbers below indicate abundance relative to wild-type HSUR 1. U6 snRNA provides a loading control.

Figure S10. HSUR 2 binds miR-16 through base-pairing. **(A)** Partial sequences of HSUR 2 (positions 23 to 45) and its mutants, and their complementarity to miRNAs are shown. Details are as in Figure 4A. **(B)** Coimmunoprecipitation of miRNAs with αSm, as in Fig. 1C, was performed on Jurkat T cells stably transfected with plasmids expressing wild-type HSURs 1 and 3–7, and either wild-type HSUR 2 (Wt, lanes 1-5), no HSUR 2 (ΔH2, lanes 6-10), mutant HSUR 2 H2Mt (lanes 11-15), or mutant HSUR 2 H2m20 (lanes 16-20).

Figure S11. Western blot analysis of Cyclin E1, Cdk6, and Bcl2 in marmoset T cells transformed by HVS expressing (Wt) or lacking (Mut) HSURs 1 and 2.

Table S1

	Cells transformed with Wt HVS A11 (copies/cell)	Reference
HSUR 1	**~20000**	***(8)***
HSUR 2	**~2000**	***(8)***
miR-16	**1963 ± 223**	**This paper**
miR-27	**372 ± 5**	**This paper**
miR-142-3p	**3231 ± 245**	**This paper**

Supplementary references

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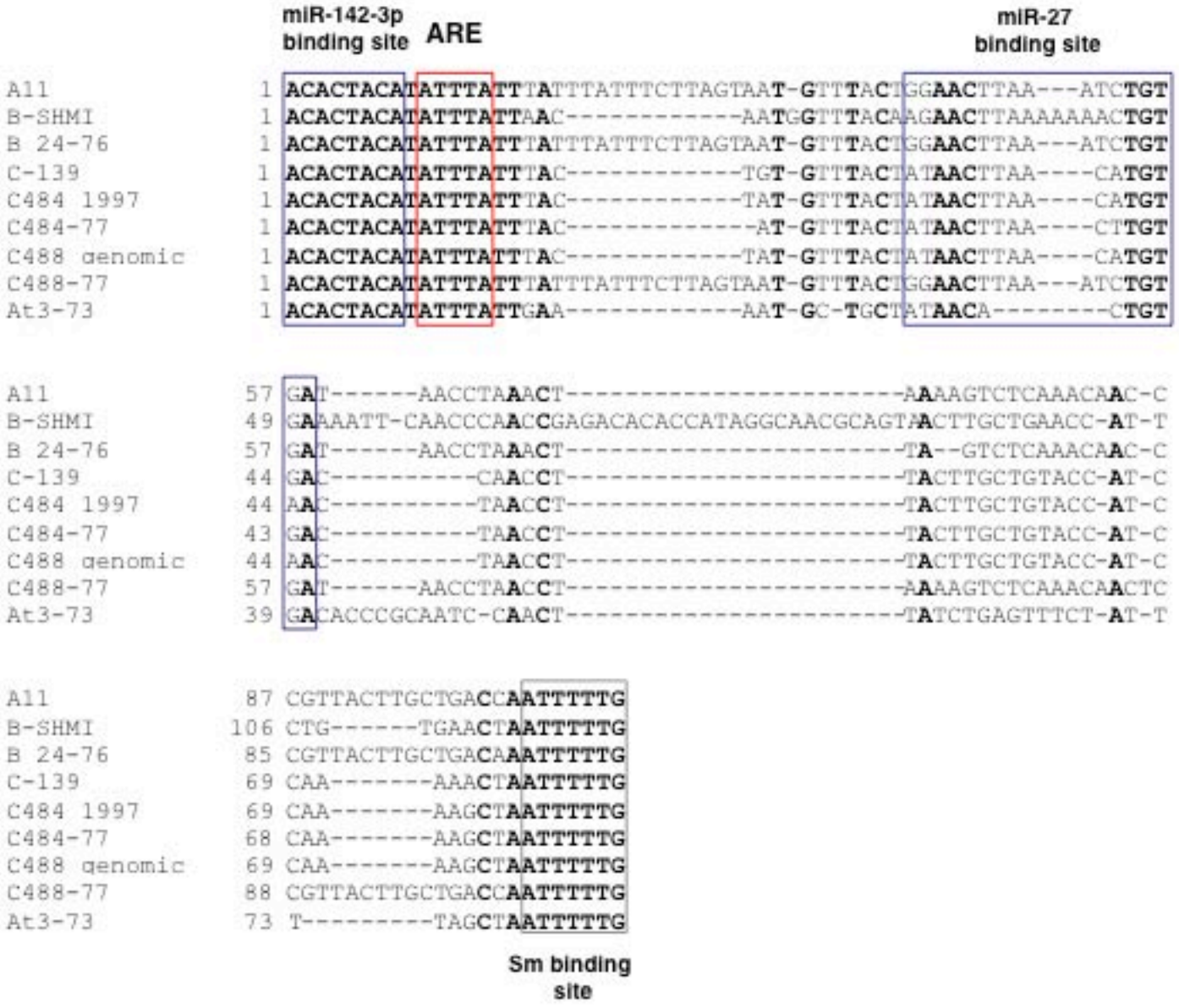
Fig. S1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ARE
miR-27 binding site
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B 24-76 1 ACACTACATATTTATTTATTTATTTCTTAGTAAT-GTTTACTGGAACTTAA---ATCTGT
C-139 1 ACACTACATATTTATTTAC----------TGT-GTTTACTATAACTTAA----CATGT
C484 1997 1 ACACTACATATTTATTTAC----------TAT-GTTTACTATAACTTAA----CATGT
C484-77 1 ACACTACATATTTATTTAC----------AT-GTTTACTATAACTTAA----CTTGT
C488 genomic 1 ACACTACATATTTATTTAC----------TAT-GTTTACTATAACTTAA----CATGT
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At3-73 1 ACACTACATATTTATTGAA----------AAT-GC-TGCTATAACA--------CTGT
A11 57 GAT------AACCTAAACT----------AAAAGTCTCAAACAAC-C
B-SHMI 49 GAAAATT-CAACCCAACCGAGACACACCATAGGCAACGCAGTACTTGCTGAACC-AT-T
B 24-76 57 GAT------AACCTAAACT----------TA--GTCTCAAACAAC-C
C-139 44 GAC----------CAACCT----------TACTTGCTGTACC-AT-C
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C488 genomic 44 AAC----------TAACCT----------TACTTGCTGTACC-AT-C
C488-77 57 GAT------AACCTAACCT----------AAAAGTCTCAAACAACTC
At3-73 39 GACACCCGCAATC-CAACT----------TATCTGAGTTTCT-AT-T
A11 87 CGTTACTTGCTGACCAATTTTTG
B-SHMI 106 CTG------TGAACTAATTTTTG
B 24-76 85 CGTTACTTGCTGACAAATTTTTG
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C488-77 88 CGTTACTTGCTGACCAATTTTTG
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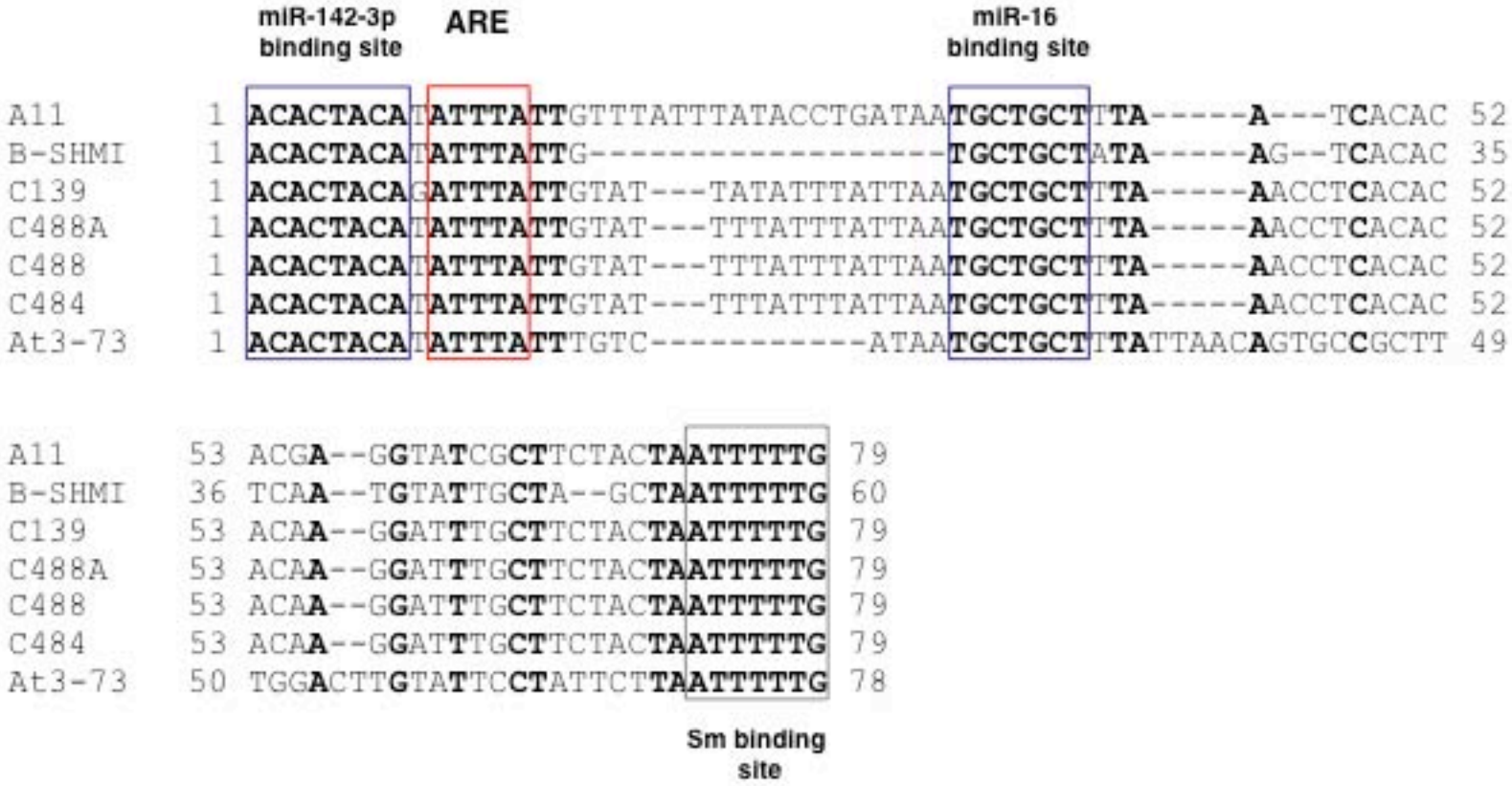
Sm binding site

Fig. S2


miR-142-3p binding site
ARE
miR-16 binding site
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A11 53 ACGA--GGTATCGCTTCTACTAATTTTTG 79
B-SHMI 36 TCAA--TGTATTGCTA--GCTAATTTTTG 60
C139 53 ACAA--GGATTTGCTTCTACTAATTTTTG 79
C488A 53 ACAA--GGATTTGCTTCTACTAATTTTTG 79
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Sm binding site

Fig. S3

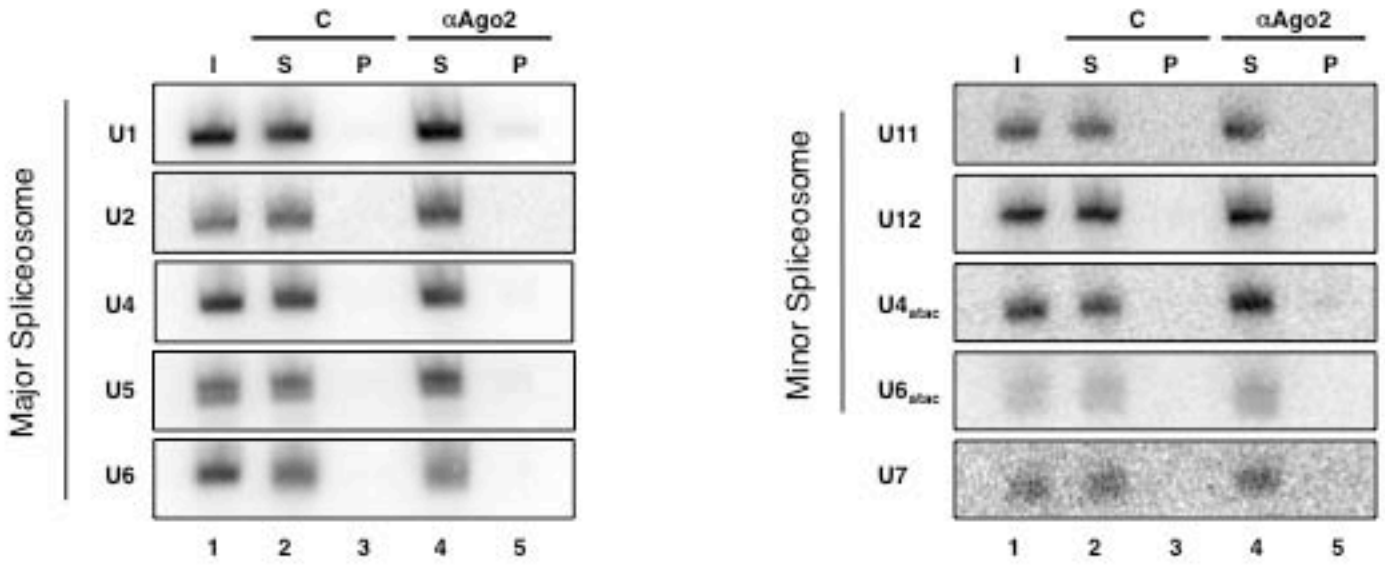
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Fig. S4

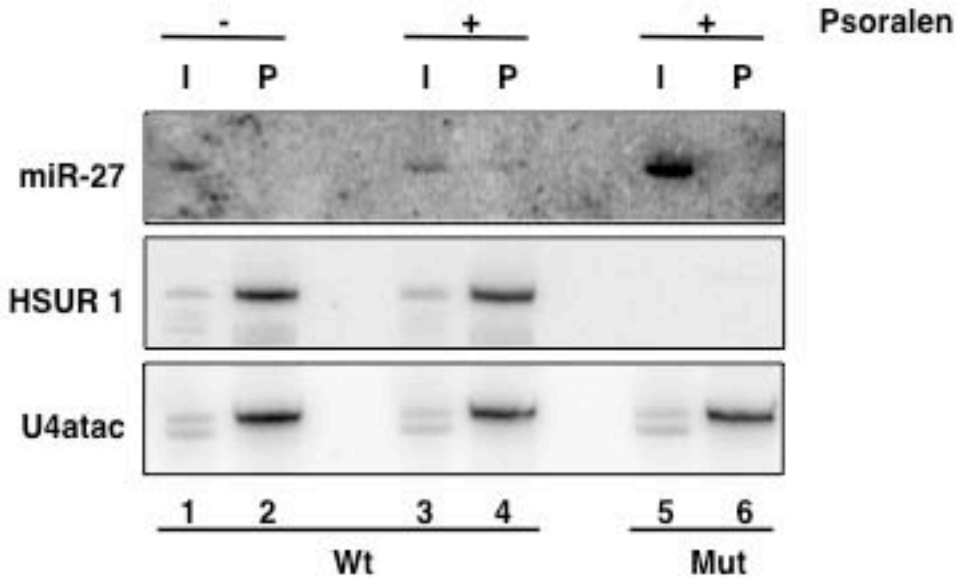
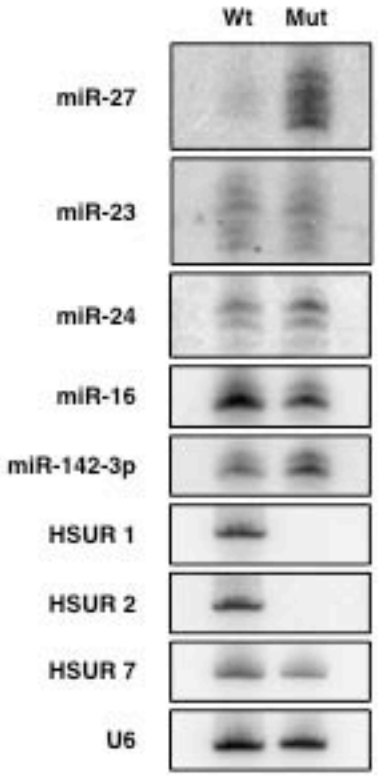


Fig. S5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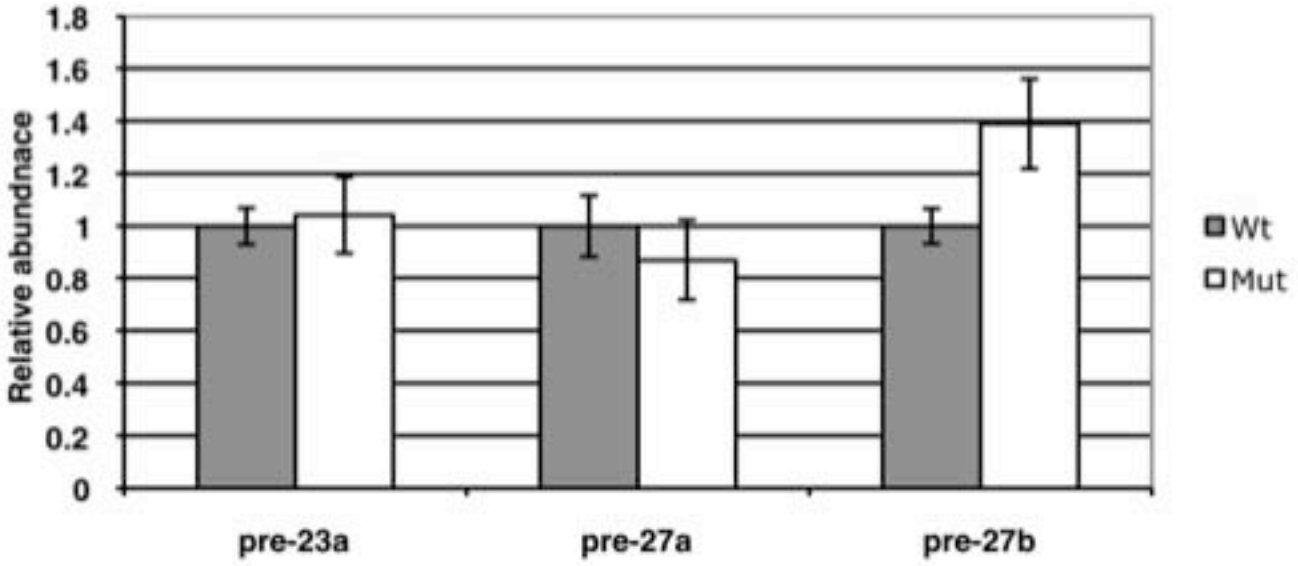
miR-23
miR-24
miR-16
miR-142-3p
HSUR 1
HSUR 2
HSUR 7
U6

Fig. S6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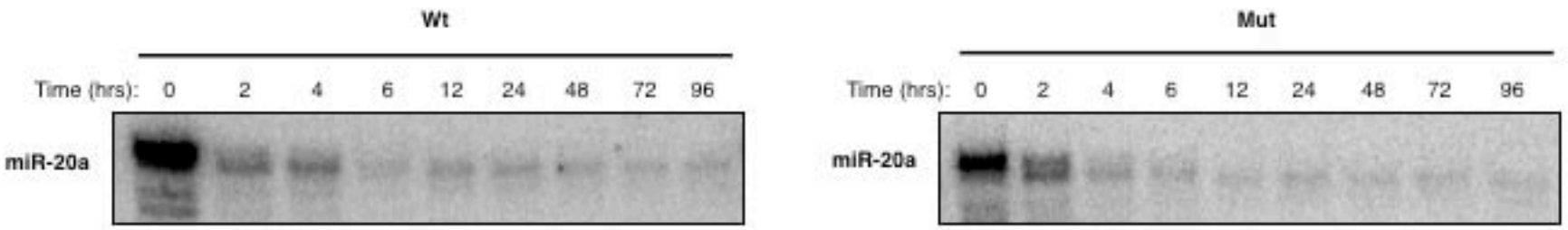
Relative abundnace
pre-23a
pre-27a
pre-27b
Wt
Mut

Fig. S7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miR-20a
Mut
Time (hrs): 0 2 4 6 12 24 48 72 96
miR-20a

Fig. S8

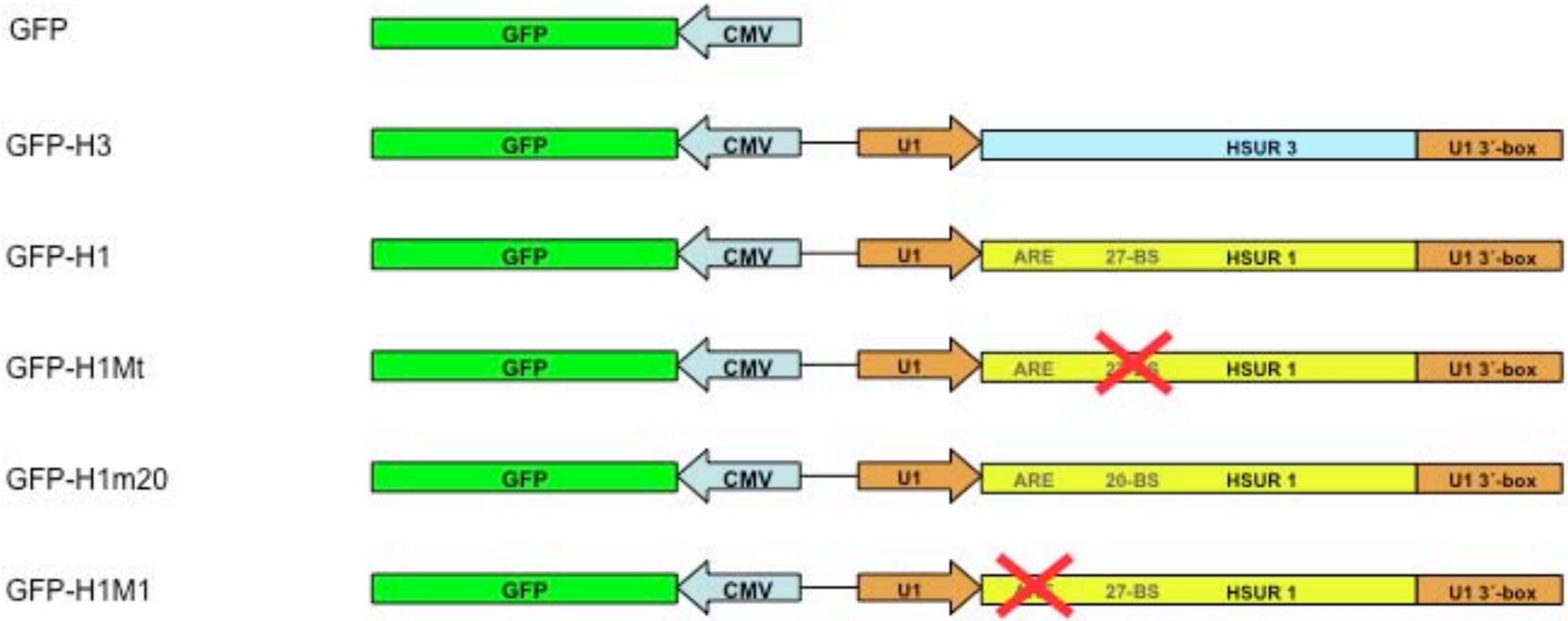
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Fig. S9

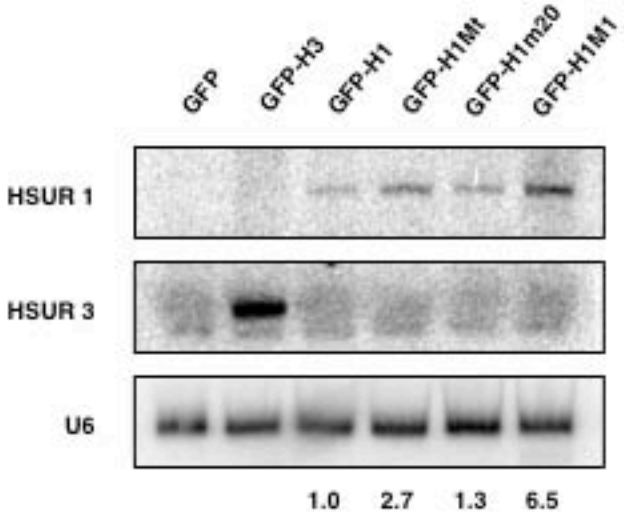
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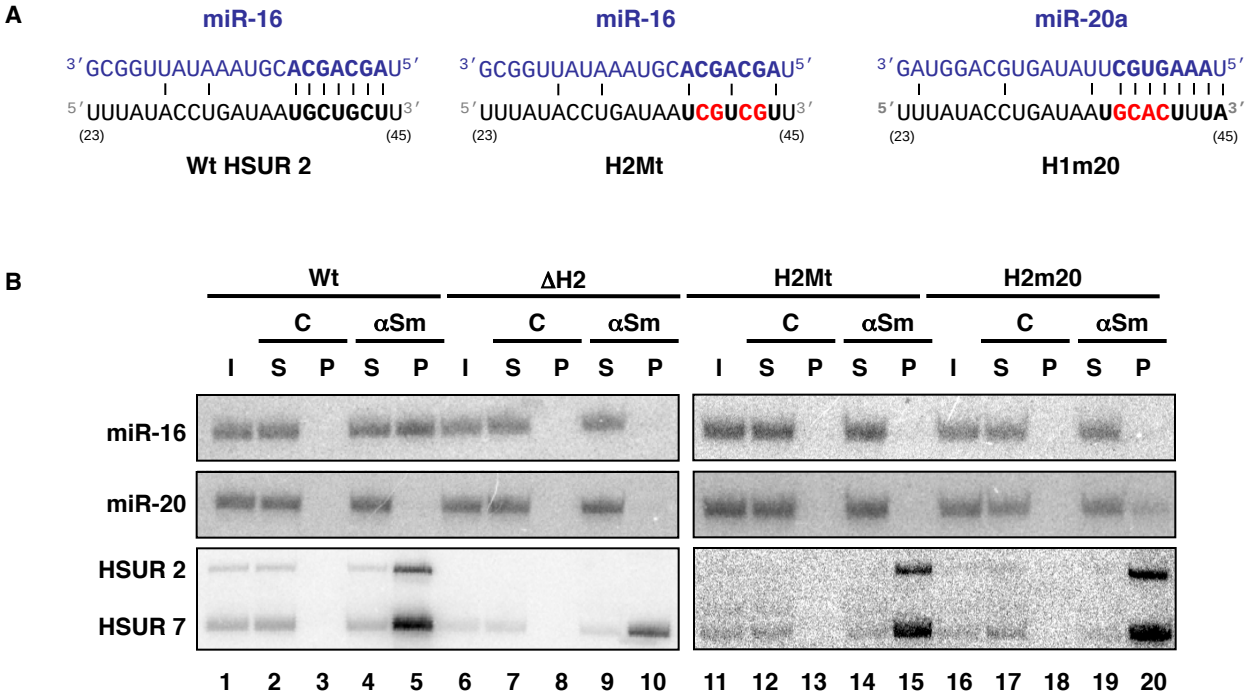
Fig. S10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(23)
(45)
Wt HSUR 2
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miR-20a
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5′UUUAUACCUGAUAAUGCACUUUA3′
(23)
(45)
H1m20
B
Wt
ΔH2
H2Mt
H2m20
C
αSm
I S P S P I S P S P I S P S P I S P S P
miR-16
miR-20
HSUR 2
HSUR 7
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Fig. S11