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Supplemental Information

HSV1 MicroRNA Modulation of GPI Anchoring and Downstream Immune Evasion

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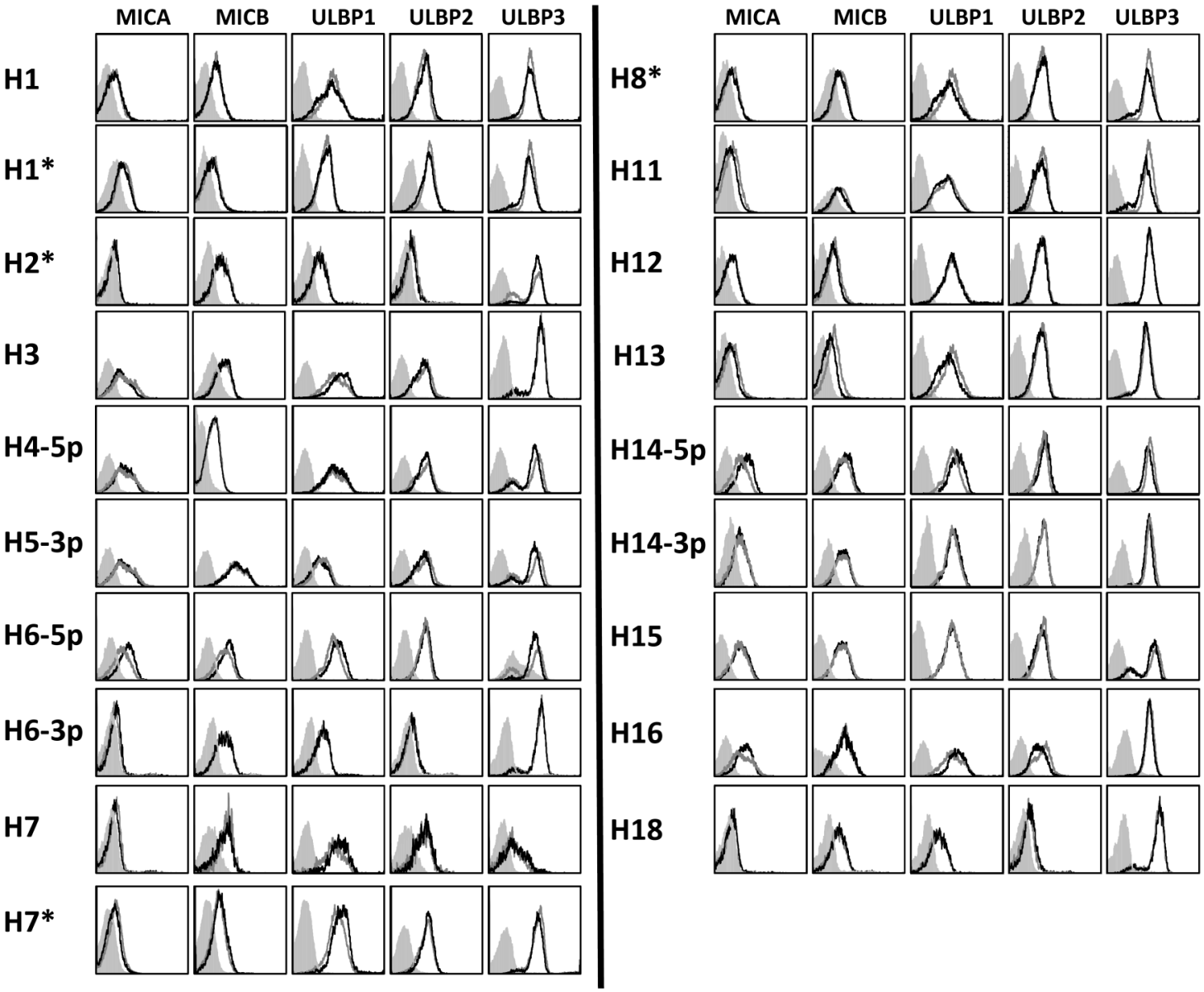
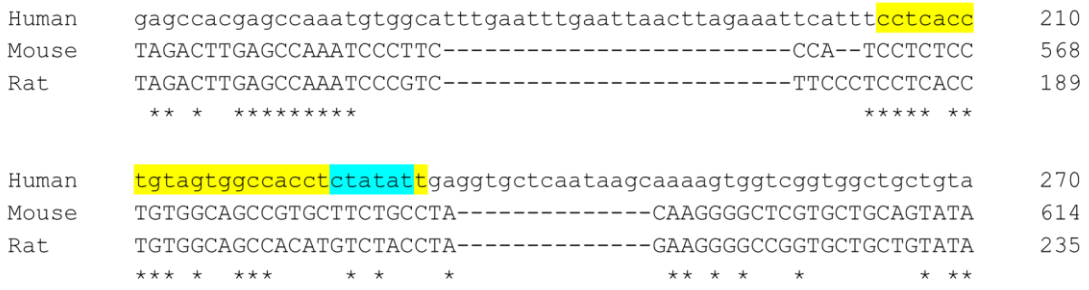


<u>Supplementary Figure 1</u>. Most HSV1 microRNAs do not affect NKG2D ligand expression. Related to Figure 1.

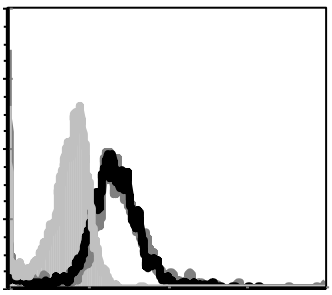
BJAB cells were stably transduced with lentiviral vectors encoding for the indicated HSV1 encoded microRNAs. Cells were stained for the expression of the different stress induced ligands of NKG2D: MICA, MICB, ULBP1, ULBP2 & ULBP3, and expression levels were analyzed by FACS. All stains were performed at least twice. In all histograms, the black histograms represent the staining of the cells expressing miRNA of interest, the empty gray histograms represent staining of control SINGFP expressing cells and the full gray histograms represent staining of the SINGFP cells

with secondary antibody only. The background of cells expressing each of the viral miRNAs was similar to that of SINGFP and is not shown in the figure. Figure combines staining from several experiments.



<u>Supplementary Figure 2</u>. Multiple sequence alignments of human, mouse and rat PIGT-3'UTRs. Related to Figure 1.

The sequences of human, mouse and rat PIGTs were aligned using Clustal Omega (Sievers et al. 2011). Displayed are the alignments of the area targeted by miR H8 in the Human PIGT-3'UTR (yellow). Although some conservation is seen, the seed binding site (turquoise) is not conserved, attesting to the specificity of HSV1 miR H8 to human PIGT. The mouse and rat sequences were examined because these animals serve as models for experimental HSV1 reactivation (see main text).



<u>Supplementary Figure 3</u>. PIGT staining B16 cells expressing miR H8. Related to Figure 2.

Intracellular FACS staining for the expression of PIGT in Murine melanoma B16 cells transduced to express miR H8 (black hitogram) or a control miR (empty gray histogram). No differences were seen in PIGT expression levels. Full gray histogram- isotype control.

HSV1 microRNA	Forward primer	Reverse primer
H1	GATCCCC**GATGGAAGGACGGGA AGTGGA**TTCAAGAGATCCACTTCC CGTCCTTCCATCTTTTTGGAAA	AGCTTTTCCAAAAAGATGGAAGGAC GGGAAGTGGATCTCTTGAATCCACTT CCCGTCCTTCCATCGGG
H1*	GATCCCC**TACACCCCCCTGCCTTC CACCCT**TTCAAGAGAAGGGTGGA AGGCAGGGGGGTGTATTTTTGGA AA	AGCTTTTCCAAAAATACACCCCCCTGC CTTCCACCCTCTCTTGAAAGGGTGGA AGGCAGGGGGGTGTAGGG
H2*	GATCCCC**TCGCACGCGCCCGGCA CAGACT**TTCAAGAGAAGTCTGTG CCGGGCGCGTGCGATTTTTGGAA A	GATCCCCTCGCACGCGCCCGGCACAG ACTTTCAAGAGAAGTCTGTGCCGGGC GCGTGCGATTTTTGGAAA
H3	GATCCCCC**TGGGACTGTGCGGTT GGGAC**TTCAAGAGAGTCCCAACC GCACAGTCCCAGTTTTTGGAAA	GATCCCCCTGGGACTGTGCGGTTGG GACTTCAAGAGAGTCCCAACCGCACA GTCCCAGTTTTTGGAAA
H4-5p	GATCCCC**GGTAGAGTTTGACAGG CAAGCA**TTCAAGAGATGCTTGCCT GTCAAACTCTACCTTTTTGGAAA	GATCCCCGGTAGAGTTTGACAGGCA AGCATTCAAGAGATGCTTGCCTGTCA AACTCTACCTTTTTGGAAA
H5-3p	GATCCCC**GTCAGAGATCCAAACC CTCCGG**TTCAAGAGACCGGAGGG TTTGGATCTCTGACTTTTTGGAAA	AGCTTTTCCAAAAAGTCAGAGATCCA AACCCTCCGGTCTCTTGAACCGGAGG GTTTGGATCTCTGACGGG
H6-5p	GATCCCC**GGTGGAAGGCAGGGG GGTGTA**TTCAAGAGATACACCCC CCTGCCTTCCACCTTTTTGGAAA	AGCTTTTCCAAAAAGGTGGAAGGCA GGGGGGTGTATCTCTTGAATACACCC CCCTGCCTTCCACCGGG
H6-3p	GATCCCC**CACTTCCCGTCCTTCCAT CCC**TTCAAGAGAGGGATGGAAGG ACGGGAAGTGTTTTTGGAAA	GATCCCCCACTTCCCGTCCTTCCATCC CTTCAAGAGAGGGATGGAAGGACGG GAAGTGTTTTTGGAAA
H7	GATCCCC**AAAGGGGTCTGCAACC AAAGG**TTCAAGAGACCTTTGGTT GCAGACCCCTTTTTTTTGGAAA	AGCTTTTCCAAAAAAAAGGGGTCTGC AACCAAAGGTCTCTTGAACCTTTGGT TGCAGACCCCTTTGGG
H7*	GATCCCC**TTTCGACCCCTCTTC**TTC AAGAGAGAAGAGGGGTCGGGAT CCAAATTTTTGGAAA	AGCTTTTCCAAAAATTTGGATCCCGA CCCCTCTTCTCTCTTGAAGAAGAGGG GTCGGGATCCAAAGGG
H8	GATCCCC**TATATAGGGTCAGGGG GTTC**TTCAAGAGAGAACCCCCTGA CCCTATATATTTTTGGAAA	AGCTTTTCCAAAAATATATAGGGTCA GGGGGTTCTCTCTTGAAGAACCCCCT GACCCTATATAGGG
Precursor-miR-H8	GATCCCCGTCCCTGTA**TATATAGG GTCAGGGGGTTC**CGCACCCCCTA ACATGGCGCCCCCGGTCCCTGTAT ATATAGTTGTCTTTTTGGAAA	AGCTTTTCCAAAAAGACAACTATATA TACAGGGACCGGGGGCGCCATGTTA GGGGGTGCGGAACCCCCTGACCCTA TATATACAGGGACGGG
H8*	GATCCCC**GCCCCCGGTCCCTGTAT ATA**TTCAAGAGATATATACAGGG ACCGGGGGCTTTTTGGAAA	AGCTTTTCCAAAAAGCCCCCGGTCCC TGTATATATCTCTTGAATATATACAGG GACCGGGGGCGGG
H11	GATCCCC**TTAGGACAAAGTGCGA ACGC**TTCAAGAGAGCGTTCGCAC TTTGTCCTAATTTTTGGAAA	AGCTTTTCCAAAAATTAGGACAAAGT GCGAACGCTCTCTTGAAGCGTTCGCA CTTTGTCCTAAGGG
H12	GATCCCC**TTGGGACGAAGTGCGA ACGCTT**TTCAAGAGAAAGCGTTC GCACTTCGTCCCAATTTTTGGAAA	AGCTTTTCCAAAAATTGGGACGAAGT GCGAACGCTTTCTCTTGAAAAGCGTT CGCACTTCGTCCCAAGGG

H13	GATCCCC**TTAGGGCGAAGTGCGAGCACTGG**TTCAAGAGACCAGTGCTCGCACTTCGCCCTAATTTTTGGAAA	AGCTTTTCCAAAAATTAGGGCGAAGTGCGAGCACTGGTCTCTTGAACCAGTGCTCGCACTTCGCCCTAAGGG
H14-5p	GATCCCC**AGTCGCACTCGTCCCTGGCTCAGG**TTCAAGAGACCTGAGCCAGGGACGAGTGCGACTTTTTTGGAAA	AGCTTTTCCAAAAAAGTCGCACTCGTCCCTGGCTCAGGTCTCTTGAACCTGAGCCAGGGACGAGTGCGACTGGG
H14-3p	GATCCCC**TCTGTGCCGGGCGCGTGCGAC**TTCAAGAGAGTCGCACGCGCCCGGCACAGATTTTTGGAAA	AGCTTTTCCAAAAATCTGTGCCGGGCGCGTGCGACTCTCTTGAAGTCGCACGCGCCCGGCACAGAGGG
H15	GATCCCC**GGCCCCGGGCCGGGCCGCCACG**TTCAAGAGACGTGGCGGCCCGGCCCGGGGCCTTTTTGGAAA	AGCTTTTCCAAAAAGGCCCCGGGCCGGGCCGCCACGTCTCTTGAACGTGGCGGCCCGGCCCGGGGCCGGG
H16	GATCCCC**CCAGGAGGCTGGGATCGAAGGC**TTCAAGAGAGCCTTCGATCCCAGCCTCCTGGTTTTTGGAAA	AGCTTTTCCAAAAACCAGGAGGCTGGGATCGAAGGCTCTCTTGAAGCCTTCGATCCCAGCCTCCTGGGGG
H18	GATCCCC**CCCGCCCGCCGGACGCCGGGACC**TTCAAGAGAGGTCCCGGCGTCCGGCGGGCGGGTTTTTGGAAA	AGCTTTTCCAAAAACCCGCCCGCCGGACGCCGGGACCTCTCTTGAAGGTCCCGGCGTCCGGCGGGCGGGGGG

Supplemental Table 1.- Oligonucleotides used to generate artificial miRNA hairpins. Related to Figure 1.

The above oligonucleotides were used for generating artificial short hairpins (the miRNA sequence is marked by bold letters). For the precursor miR H8, these primers represent the full length pre-miR with the addition of cloning sites; they were annealed and cloned into the SIN expression vector.

Primer identity	*Primer 5'-3'*
PIGT-FW	CCCGCGGCCGCGCCGCCACCATGGCGGCGGCTATGCCG
PIGT-REV	CCCCTCGAGTCAGAGTGGGGGGACACCTCG
PIGT3'-FW	GATCTAGATTCTTGCCCTTTCCAGCAGCT
PIGT3'-REV	GATCTAGAAACCACGGAAACAGCCGTTTTT
PIGT3'-seed mut-FW	CCTCACCTGTAGTGGCCACCTCTCGATT
PIGT3'-seed mut-REV	AATCGAGAGGTGGCCACTACAGGTGAGG

Supplemental Table 2 – PIGT primers used for cloning PIGT and the PIGT 3'UTR. Related to Figure 1.

The above primers were used to clone human PIGT for overexpression experiments and the 3'UTR of hPIGT (and the two base mutations in the miR H8 seed binding region of this 3'UTR) for the dual luciferase assays.

Primer identity	**Primer 5'-3'**
hHPRT FW	TGACACTGGCAAAACAATGCA
hHPRT REV	GGTCCTTTTCACCAGCAAGCT
hUBC FW	ATTTGGGTCGCGGTTCTTG
hUBC REV	TGCCTTGACATTCTCGATGGT
hPIGT FW	ACTGGATGGAAACCTTGGTG
hPIGT REV	CAAGGGCAAGGAGAACAAAC

Supplemental Table 3 - qPCR primers used in the paper. Related to Figure 2.

The above primers were used for qPCR, using SYBER GREEN reagents, as described in the materials and methods section.

Supplemental experimental procedure

Cell culture, Lentiviral Constructs, Production and Transduction

The BJAB and YTS eco cells were kept in RPMI-1640, A549 and Vero cells were kept in DMEM. Media were supplemented with 10% FCS (Sigma-Aldrich) and with 1% each of pen-strep, sodium pyruvate, L-glutamine and non-essential amino acids (Biological Industries). RNA artificial hairpins that function as hairpins were generated by using the pTER vector (van de Wetering et al., 2003) and two complementary specific oligonucleotides (Table S1). The artificial hairpin and H1 RNA polymerase III promoter were excised from the vector and cloned into the lentiviral vector SIN18-pRLL-hEFIap-E-GFP-WRPE. Lentiviruses were generated in 293T cells using a transient three-plasmid transfection protocol as previously described(Stern-Ginossar et al., 2007).

Flow Cytometry

FACS analysis was performed using a FACSCalibur (Becton Dickinson) and the FACS Express program (De Novo software). At least 5000 cells were gated and counted in each experiment. In all experiments using cells transduced with a GFP-expressing lentivirus, the histograms are gated on the GFP+ population. For intracellular FACS staining, cells were counted and incubated overnight as above. Cells were then washed in PBSx1, incubated in 100% Methanol at -20˚C for 1hour; PBSx1 was then added to the Methanol at a 1:1 concentration and the cells were centrifuged and re-suspended in PBSx1 for rehydration at room temperature for 1hour. Cells were then incubated with primary antibodies at a concentration of 0.5 µg/well for 1 hr, washed with PBSx1, to remove excess antibodies and then incubated with the appropriate secondary antibody for 1h at room temperature.

Antibodies used in FACS analyses

The following primary antibodies were used for flow cytometry: anti-MICA (clone 159227, R&D Systems), anti-MICB (clone 236511, R&D Systems), anti-ULBP1 (clone 170818, R&D Systems), anti-ULBP2/5/6 (clone165903, R&D Systems), anti-ULBP3 (clone 166514, R&D Systems), anti-PIGT (clone 2A2, catalog number-H00051604-M01, Abnova), mIgG2a isotype control (clone MOPC173, Biolegend). The fusion proteins: NKp46-Ig, NKp44-Ig, NKp30-Ig & KIR2DS4-Ig were prepared as previously described (Stanietsky et al., 2013), (Arnon et al., 2004), (Mandelboim et al., 2001), (Arnon et al., 2001), (Katz et al., 2001). These fusion proteins were used to stain the cells at a concentration of 4µg/well, for 1hr on ice, secondary antibody was then applied as above. The following secondary antibodies were used for flow cytometry: anti-mouse AlexaFluor 647 and anti-human AlexaFluor 647, both were purchased from Jackson Laboratories.

Lentivirus preparation

The pMDG envelope expression cassette (3.5 µg), the gag-pol packaging construct (6.5 µg), and the relevant vector construct (10 µg) were transfected into 293T cells using the LT1 transfection reagent (Mirus Bio LLC, Madison, WI). Two days after transfection, the supernatants containing viruses were collected and filtered. These viruses where then used to transduce cells in the presence of polybrene (5 µg/ml). PIGT was amplified from cDNA derived from BJAB cells and cloned into the lentiviral vector pHAGE-DsRED(−)- eGFP(+) which also contains GFP. ShRNAs for PIGT were obtained from Sigma-Aldrich in the pLKO.1 plasmid. Lentiviral vectors were produced by using the three-plasmid transient transfection method as described above. The ShRNA sequences directed against PIGT were as follows:

clone#1,CCGGCGGAAGACCTATGCCATCTATCTCGAGATAG

ATGGCATAGGTCTTCCGTTTTTTG; clone #2,CGGCACTGTCACTGATGTGGA TAACTCGAGTTATCCACATCAGTGACAGTG TTTTTG.

qRT-PCR

Total RNA was isolated by using the Quick RNA Miniprep kit (Zymo) according to the manufacturer's instructions. Total RNA (0.25-2 μg) was reverse transcribed with mMLV Reverse Transcriptase (Invitrogen) and with 0.5 μg of poly(T) 3′ rapid amplification of complementary DNA ends (RACE) adaptor using the FirstChoice RLM-RACE kit (Ambion), according to the manufacturer's instructions. Quantitative PCR was used to measure mRNA expression was as follows: cDNA was mixed with 150 μM of both the forward and reverse primers in a final volume of 5 μl and mixed with 5 μl of SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen). hUBC and hHPRT were used as endogenous reference genes for PCR quantification. PCR was performed on QuantStudio12K Flex Real Time PCR System (Applied Biosystems). For a list of the primers used for the qPCR, see supplemental table 2. For identification of the mature miR H8, custom-designed mature miRNA-Taqman primers (Thermo Fisher Scientific) and probes (Applied Biosystems) were used, in accordance with the manufacturer's instruction.

Luciferase assay

The generation of the Firefly luciferase constructs was as described (Stern-Ginossar et al., 2007). Mutations in the PIGT 3′UTR were generated by PCR extension of mutated complementary primers using the RedTaq PCR reaction mix (Sigma-Aldrich). For a list of primers used see supplemental table 3. HeLa and 293T cells, plated in 24-well plates, were transfected with the LT1 transfection reagent (Mirus) with 100 ng of a Firefly-luciferase reporter vector and 5 ng of the control Renilla luciferase pRL-CMV (Promega) at a final volume of 0.5 ml. Firefly and Renilla

luciferase activities were measured consecutively with the Dual-Luciferase Assay System (Promega) 48 hr after transfection. Firefly luciferase activity was normalized to Renilla luciferase activity and then normalized to the average activity of the control reporter.

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