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**A 2.5-kilobase deletion containing a cluster of cine microRNAs in the latency-associated-transcript locus of the pseudorabies virus affects the host response of porcine trigeminal ganglia during established latency**

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1 **A 2.5 kb deletion containing a cluster of nine microRNAs in the**  
2 **LAT locus of the pseudorabies virus affects the host response**  
3 **of porcine trigeminal ganglia during established latency.**

4

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14

15 Running Head: The establishment of PrV latency in the pig host

16

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19

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24 **Abstract**

25 The alphaherpesvirus Pseudorabies virus (PrV) establishes latency primarily in neurons of  
26 trigeminal ganglia when only transcription of the latency-associated transcript (LAT) locus  
27 is detected. Eleven microRNAs (miRNAs) cluster within LAT, suggesting a role in  
28 establishment and/or maintenance of latency.

29 We generated a mutant PrV (M) deleted of nine miRNA genes which displayed almost  
30 identical properties with the parental PrV (WT) during propagation *in vitro*. Fifteen pigs  
31 were experimentally infected with either WT, M or mock infected.

32 Similar levels of virus excretion and host antibody response were observed in all infected  
33 animals. At 62 days post infection trigeminal ganglia were excised and profiled by deep  
34 sequencing and RT-qPCR.

35 Latency was established in all infected animals without evidence of viral reactivation  
36 demonstrating that miRNAs are not mandatory for this process. Lower levels of Large  
37 Latency Transcript (LLT) were found in ganglia infected by M compared to WT PrV. All PrV  
38 miRNAs were expressed, with highest expression found for prv-miR-LLT1, prv-miR-LLT2  
39 (in WT-ganglia) and prv-miR-LLT10 (in both WT and M-ganglia). No evidence of  
40 differentially expressed porcine miRNAs was found. Fifty-four porcine genes were  
41 differentially expressed between WT, M and control ganglia. Both viruses triggered a  
42 strong host immune response, but in M- ganglia gene upregulation was prevalent.

43 Pathway analyses indicated that several biofunctions, including those related to cell-  
44 mediated immune response and migration of dendritic cells, were impaired in M- ganglia.

45 These findings are consistent with a function of the LAT locus in the modulation of host  
46 response for maintaining a latent state.

47 **Importance**

48 This study provides a thorough reference on the establishment of latency by PrV in its  
49 natural host, the pig. Our results corroborate the evidence obtained from the study of  
50 several LAT mutants of other alphaherpesviruses encoding miRNAs from their LAT  
51 regions. Neither PrV miRNA expression nor high LLT expression levels are essential to  
52 achieve latency in trigeminal ganglia. Once latency is established by PrV the only  
53 remarkable differences are found in the pattern of host response. This indicates that, LAT  
54 functions as an immune evasion locus.

55



## 56 **Introduction**

57 Pseudorabies virus (PrV) is a porcine alphaherpesvirus. The genome of PrV is more than  
58 142 kb in size and is characterized by the presence of 70 different coding genes plus the  
59 Latency Associated Transcript (LAT) locus (1, 2). PrV is the aetiological agent of  
60 Aujeszky's disease causing neurological, respiratory and reproductive disease in the pig,  
61 its' natural host. Despite successful vaccination campaigns and eradication of the virus  
62 from various countries, Pseudorabies outbreaks still occur in swine populations worldwide,  
63 as recently reported in China (3). Because latent infection persists for the lifetime after  
64 recovery from acute disease, pigs latently infected by PrV are a constant danger for  
65 reactivation and virus shedding and spreading in susceptible populations (4-6).

66 A particular feature of herpesviruses is their ability to establish and maintain latent  
67 infections wherein the virus genome circularizes and persists as an episome. As for other  
68 alphaherpesviruses, neurons in the trigeminal ganglia are the primary site of PrV latency  
69 (7). Over this period, the transcription of viral lytic genes is repressed and transcription of  
70 the viral genome is restricted to the LAT locus overlapping the internal repeat sequence  
71 (IRS) (8-10). RNAs of multiple sizes are transcribed from the strand opposite that encoding  
72 EP0 and IE180 which can be detected in infected swine trigeminal ganglia (8, 10, 11). The  
73 largest is the 8.4-kb Large Latency Transcript (LLT). Transcription from the LAT region is  
74 active also during lytic infection of cultured mammalian cells although a different set of  
75 transcripts is expressed (12).

76 MicroRNAs (miRNAs) are small non-coding RNAs approximately 22 nt long that regulate  
77 gene expression post-transcriptionally. By complete or partial hybridization, miRNAs  
78 induce target mRNA degradation and/or translational repression, and thus serve key roles  
79 in the regulation of almost every important cellular process in multicellular eukaryotes (13-  
80 15). Given their small size, their lack of antigenicity and their ability to inhibit translation of

81 specific mRNA species, miRNAs are thought to represent ideal tools for viruses to  
82 establish conditions permissive for viral replication, for establishment of latency, or to allow  
83 rapid responses to changes in the environment, such as those that trigger reactivation  
84 from latency (16-18). The first viral miRNA was identified in Epstein–Barr virus (EBV), a  
85 gammaherpesvirus (19). With the advances in sequencing technologies, identification of  
86 miRNAs in human and animal herpesviruses rapidly followed (17, 20).

87 Several alphaherpesvirus have been reported to encode miRNAs which are often  
88 clustered in the viral genome, map within the LAT locus or in adjacent regions, and are  
89 encoded on both strands (20, 21). In PrV, a cluster of eleven miRNA genes has been  
90 identified by deep sequencing in porcine immature dendritic cells (22) and in a porcine  
91 kidney (PK15) cell line (23) during lytic infection. This cluster is entirely contained within  
92 the ~4.6 kb intron of the large latency transcript (LLT) which functions as a primary miRNA  
93 precursor (23).

94 Here, we report the results of an experimental infection to assess the importance of a  
95 miRNA-containing region for the establishment of PrV latency in its natural porcine host.  
96 To this end, we generated a PrV clone deleted of a 2.5 kb portion of the LLT intron  
97 harboring nine miRNA genes. We adopted a deep sequencing approach to characterize  
98 the transcriptional profiles of trigeminal ganglia focusing on miRNAs and coding genes.

99

## 100 **Materials and Methods**

### 101 **Construction of virus mutants**

102 The virus generated in this study was derived from the GFP-expressing mutant pPrV-  
103  $\Delta$ gGG (24), which contains the genome of PrV strain Kaplan (PrV-Ka) (25) cloned as a  
104 bacterial artificial chromosome (BAC).

105 To delete the miRNA cluster, pPrV- $\Delta$ gGG (Fig. 1A) was mutagenized in *E. coli* using the  
106 Counter-Selection BAC Modification Kit (Gene Bridges). The provided selection cassette  
107 conferring streptomycin sensitivity (RpsL) and kanamycin resistance (KanR) was amplified  
108 by PCR (Pfx DNA polymerase, Life technologies) with primers PDMIRN-F (5'-  
109 CGGTGGGTCGACGGCTCCTGGGGCTGAAAGCGGCGCTGCGGATCCCCGCggcctggt  
110 gatgatggcgggatcg-3' and PDMIRN-R (5'-GTGTGCGTGTGCGAGAGAGAA  
111 GAGATGCGGGGGAGGGCGGCGGGCGCTTGtcagaagaactcgtcaagaaggcg-3'), which  
112 contained 5'-extensions (upper case letters) corresponding to nucleotides 98050 to 98099,  
113 and the reversal of nucleotides 100571 to 100620 of the PrV-Ka genome sequence,  
114 respectively (GenBank accession # JQ809328) (26). The 1419 bp PCR product was used  
115 for Red/ET-mediated recombination with pPrV- $\Delta$ gGG resulting in pPrV- $\Delta$ miRN (Fig. 1B).  
116 Correct insertion of the selection markers, and precise deletion of PrV sequences were  
117 confirmed by restriction analyses and Southern blot hybridization, as well as by PCR  
118 amplification and sequencing of the mutated genome region (results not shown). Infectious  
119 PrV was rescued after transfection (FuGENE HD reagent, Promega) of rabbit kidney  
120 (RK13) cells with BAC DNA.

### 121 **Propagation, titration and growth kinetics of pPrV- $\Delta$ gGG and pPrV- $\Delta$ miRN**

122 Rabbit (RK13) and porcine (PK15) kidney cells were used for productive virus replication.  
123 RK13 cells were grown in minimum essential medium (MEM) supplemented with 10% fetal  
124 bovine serum (FBS). For determination of one-step growth kinetics cells were infected on  
125 ice with pPrV- $\Delta$ miRN or pPrV- $\Delta$ gGG at a multiplicity of infection (MOI) of 5 and shifted to  
126 37°C after 1 h. After an additional hour, non-penetrated virus was inactivated by low-pH  
127 treatment (27) and the inoculum was replaced by fresh medium. At different times of  
128 culture at 37°C (Fig. 2), the infected cells were lysed by freeze-thawing, and progeny virus  
129 titers were determined by plaque assays overlaid with semi-solid MEM containing 5% FBS

130 and 6 g/l methylcellulose. Mean titers of three independent experiments, and mean  
131 diameters of 30 plaques per virus mutant as well as standard deviations were calculated.

132 PK15 cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented  
133 with 10 % FBS and 100 U/ml Penicillin and 100µg/ml Streptomycin at 37 °C in presence of  
134 5% CO<sub>2</sub>. PK15 cells were grown in 6 well culture plates. After reaching 90 to 100% of  
135 confluence, cells were infected with either pPrV-ΔgGG or pPrV-ΔmiRN at a MOI of 10 and  
136 incubated for 45 min at room temperature. The inoculum was then aspirated, cells were  
137 washed several times and incubated with Dulbecco's Modified Eagle's Medium (DMEM)  
138 supplemented with 10% FBS. Supernatants and cells were harvested at different times  
139 and used respectively i) for viral titrations and growth kinetics as for RK13 cells (Fig. 2),  
140 and ii) for total RNA extractions followed by RT-qPCR of viral genes and miRNAs.

#### 141 **Establishment of PrV latency *in vivo***

142 The *in vivo* animal experiment was approved by an independent ethical committee  
143 (7221.3-1. 1-016/12). Fifteen 60 day-old pigs (German Landrace) were used for  
144 experimental infection. Animals were housed in the BSL3 facility of the Friedrich-Loeffler-  
145 Institut, Germany and tested for absence of PrV antibodies prior to the start of the  
146 experiment. Three groups of five animals each were infected intranasally with 10<sup>5</sup> plaque  
147 forming units (pfu) of pPrV-ΔgGG (animal no. WT 54-58), pPrV-ΔmiRN (animal no. M 49-  
148 53) or mock infected (control group; animal no. C 21-25). The pigs were allowed to recover  
149 in the following 62 days to ensure establishment of latency. During this time pigs were  
150 monitored for clinical symptoms. In order to check for virus shedding, nasal swabs were  
151 collected every two days after infection until virus excretion ceased. Blood samples were  
152 collected at 4, 7, 10, 15, 20, 30, 45 and 62 days post infection (p.i.) using a V-trough  
153 device.

154 The host antibody response was assessed by ELISA using PrV gB as antigen. DNA

155 samples from nasal swabs were analyzed by quantitative Real-Time PCR targeting the gB  
156 gene (28).

157 Animals were slaughtered at 62 days p.i. trigeminal ganglia were excised, rinsed with ice-  
158 cold physiological saline solution, frozen in liquid nitrogen within 30 minutes after excision,  
159 and stored at -80°C until processed.

#### 160 **Nucleic acid extraction and purification**

161 Total RNAs from infected PK15 cells were extracted using QIAzol Reagent and purified  
162 with the RNeasy Mini Kit according to the manufacturer's instructions (Qiagen).

163 Frozen trigeminal ganglia were homogenized in ice cold TRIzol Reagent using an Ultra-  
164 Turrax (IKA-WERK). RNA extraction was performed according to the manufacturer's  
165 instructions (Invitrogen). Genomic DNA was obtained upon phase separation for RNA  
166 extraction by adding a back extraction buffer containing 4 M guanidine thiocyanate, 50 mM  
167 sodium citrate and 1 M Tris pH 8.0 (free base) to the interphase-organic phase mixture.  
168 After centrifugation at 12000 x g for 15 min at 4°C, the upper aqueous phase containing  
169 DNA was transferred to a clean tube and DNA was precipitated by adding 0.8 volumes of  
170 isopropanol per 1 ml of TRIzol, followed by centrifugation at 12000 x g for 5 min at 4°C and  
171 pellet washing with 75% ethanol.

172 Yields and purity of nucleic acids were measured with a NanoDrop ND-1000  
173 spectrophotometer. To remove unwanted residual DNA, all RNA samples were treated  
174 with TURBO DNase (Ambion). PK15 RNAs were treated with DNase twice and further  
175 checked by qPCR of viral genes to ensure complete removal of PrV genomic DNA. RNA  
176 integrity was assessed using an Agilent 2100 Bioanalyzer and RNA 6000 nano kits  
177 (Agilent) and the RNA Integrity Number (RIN) (29) was calculated.

#### 178 **Estimation of relative amounts of PrV genomes in trigeminal ganglia**

179 The relative amount of PrV genomes in trigeminal ganglia was estimated by a classical  
180 qPCR approach (30). DNA was extracted from a single whole ganglion per animal and  
181 amplified using primers specific to the GFP gene (primer forward: GCA AAG ACC CCA  
182 ACG AGA AG; primer reverse: TCA CGA ACT CCA GCA GGA CC). For each biological  
183 sample, three technical replicates were run and all qPCR were performed on the same run  
184 to minimize inter-experimental variation. Triplicate reactions (20  $\mu$ L) included 5  $\mu$ L genomic  
185 DNA (corresponding to 100 ng of DNA), 10  $\mu$ L of SYBRGreen PCR master mix and 5  $\mu$ L of  
186 primers (300 nM each). Reactions were incubated in a 96-well optical plate at 95°C for 10  
187 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min using a 7900HT Fast Real-  
188 Time PCR System instrument (Applied Biosystems). To avoid false-positive results, the  
189 DNA of three negative controls was used (samples 22C, 23C and 25C). The PrV genome  
190 copy number was estimated per 100 ng of genomic DNA from a PA-GFP-coilin C2 plasmid  
191 DNA standard curve.

## 192 **RNAseq and Small RNAseq libraries preparation and sequencing**

193 Both RNAseq and Small RNAseq libraries were prepared and barcoded using the TruSeq  
194 RNA sample preparation kits and protocols of Illumina ([www.illumina.com](http://www.illumina.com)).

195 *RNAseq*: libraries were prepared from nine individual samples: three control ganglia, three  
196 ganglia latent for pPrV- $\Delta$ gGG (WT-ganglia) and three ganglia latent for pPrV- $\Delta$ miRNA (M-  
197 ganglia). PolyA-RNA was purified from total RNA using oligo (dT) magnetic beads,  
198 fragmented and reverse transcribed using random primers. Libraries were checked with  
199 the Agilent High Sensitivity DNA Kit and quantified with the qPCR NGS Library  
200 Quantification kit (Agilent). The nine tagged cDNA libraries were pooled, quantitated by  
201 qPCR and sequenced in paired-end mode (100 bp reads) on an Illumina HiSeq2000  
202 instrument (TruSeq PE Cluster v3, TruSeq SBS 200 cycles v3 and TruSeq Multiplex  
203 Primer kit). Quality control analysis of the raw dataset did not indicate any differences

204 among lanes regarding the quality or quantity of the reads generated.

205 *Small RNAseq*: libraries were prepared for three control ganglia, five ganglia latent for  
206 pPrV- $\Delta$ gGG (WT-ganglia) and five ganglia latent for pPrV- $\Delta$ miRNA (M-ganglia). Prior to  
207 library preparation, integrity of the RNAs was assessed using an Agilent 2100 Bioanalyzer  
208 and yields were estimated with a Qubit® Fluorometer. RNAs were fractionated in a 15%  
209 denaturing polyacrylamide gel. Small RNA fragments in the range of 18–30 nt were  
210 excised from the gel and purified. The 5' and 3' termini of the small RNAs were ligated  
211 sequentially with adapters, followed by reverse transcription and PCR amplification. The  
212 amplified cDNA products pooled were sequenced in single-end mode (50 bp reads) using  
213 the TrueSeq SBS kit v3 according to the manufacturer's instruction on a HiSeq1000  
214 Illumina sequencer. Raw reads were analyzed with Casava1.8.2.

215 The raw reads have been deposited at the European Nucleotide Archive (ENA). RNAseq:  
216 accession number PRJEB6754 (<http://www.ebi.ac.uk/ena/data/view/PRJEB6754>); Small  
217 RNAseq: accession number PRJEB6755  
218 (<http://www.ebi.ac.uk/ena/data/view/PRJEB6755>).

### 219 **Deep sequencing and differential expression analysis**

220 *RNAseq*: first, raw 3' ends reads were trimmed for low quality bases. Briefly, the 3' end  
221 bases were sequentially cut off if their Phred quality score was below 10 or until the read  
222 length became less than 40 bp long. Then, trimmed reads were mapped against the *Sus*  
223 *scrofa* reference genome sequence v10.2 (31) using TopHat v2.0.4 (32). A transcript  
224 annotation was downloaded from Ensembl (v.67) ([www.ensembl.org](http://www.ensembl.org)) and supplied to  
225 TopHat option with “-G”. Transcript assembly was performed by providing mapped reads to  
226 Cufflinks v2.1.1 (33), option “-g” was used to report all reference transcripts as well as any  
227 novel genes and isoforms that were assembled. Transcript quantification was performed  
228 using HTSeq-count (from the 'HTSeq' framework, version 0.5.4p3) in default ('union')

229 mode and these counts were used to perform differential expression analysis.

230 Normalization and a GLM likelihood ratio test were performed using the Bioconductor  
231 edgeR package (version 3.2.3) (34) in the R environment (version 3.0.0). Transcripts  
232 showing a Benjamin-Hochberg FDR below 0.05 were considered as differentially  
233 expressed.

234 *Small RNAseq*: first, raw reads were trimmed for adapters and low-quality ends (cutoff  
235 Phred quality score: 20) using cutadapt v.1.3 (35). Scripts from the miRDeep2 (v.2.0.0.5)  
236 software package (36) were then used for the identification and quantification of novel and  
237 known miRNAs from the trimmed reads. Mapping against the pig genome reference  
238 sequence (*Sus scrofa v10.2*) was performed with the script mapper.pl while identification  
239 of known and novel miRNAs was done using miRDeep2.pl script. The known and  
240 predicted miRNAs were then provided to the quantifier.pl script. This module maps the  
241 deep sequencing reads to predefined miRNA precursors. These signatures were then  
242 post-processed using a custom python script to quantify mature miRNAs. To discard  
243 hairpins with a read distribution inconsistent with Drosha and Dicer processing sites (i.e.  
244 reads tiled across the precursor), we expected at least a 3:1 ratio between reads that  
245 matched on any of the stem-loop arms and reads located in the loop. For the remaining  
246 hairpins, reads that mapped inside the loop (more than 3 nucleotides falling in the loop)  
247 were not considered for quantification. When no known mature miRNA matched the same  
248 precursor, putative new mature miRNAs were named based upon the name of the hairpin  
249 on which they were located, or from the name of the known miRNA mapping on the  
250 opposite strand of the precursor. All the reference sequences from mature miRNAs and  
251 their precursors were obtained from miRBase database, v20 ([www.mirbase.org](http://www.mirbase.org)) (37).

252 These counts were used to perform differential expression analysis. Normalization and a  
253 GLM likelihood ratio test were performed using the Bioconductor edgeR package (version  
254 3.2.3) (34) in the R environment (version 3.0.0). The miRNAs showing a Benjamin-



255 Hochberg FDR below 0.05 were considered as differentially expressed.

## 256 **RT-qPCR analyses**

257 *Porcine and viral genes:* to validate the RNAseq data of trigeminal ganglia, 16 genes were  
258 selected to represent most of the predicted PrV miRNA targets (see below) and a wide  
259 abundance range in ganglia (number of RNAseq reads). A second set of genes included  
260 the viral genes LLT, EP0, IE180, US1, US3, US7, US8, UL6, UL28, UL32, UL33, UL43,  
261 UL47, and UL48. With the exception of primers for LLT, all primers for PrV genes have  
262 been reported (38). Primers for LLT and for all porcine genes were designed using  
263 Primer3Plus software (39) and verified for specificity by Blast analysis (Suppl. Table 1).

264 Reverse transcription was performed with the SuperScript III first strand synthesis system  
265 (Invitrogen) using between 800 ng and 1 µg of total RNA, and 50 ng of random hexamers.  
266 The quantity and quality of cDNAs were evaluated using an Agilent 2100 Bioanalyzer and  
267 RNA 6000 pico kits (Agilent). All RT-qPCRs were performed on a 7900HT Fast Real-Time  
268 PCR System instrument (Applied Biosystems) using the SYBRGreen PCR master mix. For  
269 each primer pair, PCR efficiency was evaluated using serial dilutions of cDNA sample. The  
270 potential occurrence of dimers and amplification specificity was assessed by melting curve  
271 analyses. An equivalent of 500 pg of cDNA was used as template for each sample and  
272 three technical replicates were run as previously described (see “Estimation of relative  
273 amounts of PrV genomes in trigeminal ganglia”). A parametric two-tailed Students t-test  
274 was used to assess statistical differences between pairwise comparisons.

275 *PrV miRNAs:* stem loop RT primers, PCR primers and probes were optimized for improved  
276 stability and mismatch discrimination using locked nucleic acid nucleotides (40, 41) (Suppl.  
277 Table 1).

278 Reverse transcription was done using the TaqMan microRNA Reverse Transcription Kit  
279 (Applied Biosystems). In each reaction 10 ng of total RNA from trigeminal ganglia/PK15

280 cells were mixed with 50 nM specific stem-loop RT primer. RT reactions were carried out  
281 at 16°C for 30 min, 42°C for 30 min and 85°C for 5 min. The qPCRs were made using  
282 standard TaqMan PCR protocols on a 7900HT Fast Real-Time PCR System instrument  
283 (Applied Biosystems).

#### 284 **Target gene predictions of PrV miRNAs**

285 The target sites of all PrV miRNAs on differentially expressed genes (Suppl. Table 3) were  
286 predicted by TargetScan 6.0 (42, 43). As few genes had annotated 3'UTRs, we first  
287 manually annotated as many as possible missing genes making use of cross-species  
288 mRNAs where pig specific sequences were unavailable (44). This annotation is available  
289 from the Vega website (<http://vega.sanger.ac.uk>).

290 Predictions could be computed on 34 out of the 54 differentially expressed genes (Suppl.  
291 Table 3). The 3'UTR sequences from EPO (Enredo, Pecan, Ortheus) for 12 eutherian  
292 mammal species multiple alignments were retrieved from Ensembl v.68  
293 ([www.ensembl.org](http://www.ensembl.org)). Genes having target site context score equal to or greater than zero  
294 were filtered out of the analysis. An enrichment analysis was carried out to check if  
295 differentially expressed genes were enriched in miRNA targets compared to the number of  
296 targets predicted on the total number of genes expressed in ganglia using the Fisher's  
297 exact test.

#### 298 **Gene pathway analysis**

299 The Ingenuity Pathways Analysis software IPA ([www.ingenuity.com](http://www.ingenuity.com)) was used to identify  
300 the most relevant biological functions and pathways involving the genes found differentially  
301 expressed in pairwise comparisons between WT, M and control ganglia. Firstly we  
302 uploaded the list of human homologs that corresponded to the pig genes into the  
303 application. The network analysis in the "WT vs. C" and "M vs. C" datasets aimed to  
304 search both direct and indirect interactions (known from the literature) between

305 differentiated genes and all other molecules (genes, gene products or small molecules)  
306 contained in the Ingenuity Knowledge Base (IKB). For a given network the degree of  
307 association is estimated by considering the proportion of eligible genes (genes with at  
308 least one interaction with another full length gene or protein in IKB) and a score is  
309 assigned based on the right-tailed Fisher exact test ( $\log(1/p\text{-value})$ ). The IPA Upstream  
310 Regulator Analysis was used to identify upstream regulators and predict, based on the  
311 literature compiled in the IKB, whether they are activated or inhibited, given the observed  
312 gene expression changes in the “WT vs. C” and “M vs. C” datasets. The activation z-score  
313 predicts the activation state of the upstream regulator, using the gene expression patterns  
314 of the genes downstream of an upstream regulator; an absolute z-score of  $\geq 2$  is  
315 considered significant. Finally, the heatmap comparison analysis tool was used to visualize  
316 clusters of diseases and biofunctions predicted to increase or decrease similarly across  
317 the “WT vs. M” and “M vs. C” datasets. The statistical significance of each biofunction is  
318 expressed as p-values from the Fisher’s exact test and a total absolute z-score across all  
319 the observations is provided.

320

## 321 **Results**

### 322 **Generation and *in vitro* characterization of a PrV miRNA mutant**

323 pPrV- $\Delta$ miRN was generated from the parental pPrV- $\Delta$ gGG (Fig. 1A) (24) by deleting  
324 nucleotides 98100 to 100570 from the right end of the U<sub>L</sub> region of the PrV-Ka genome  
325 (Genbank accession no. JQ809328) (26). The deletion includes nine out of the eleven  
326 described miRNA genes (22, 23), but excludes the two miRNA genes transcribed from the  
327 inverted repeat sequences (prv-miR-LLT10 and prv-miR-LLT11) (Fig. 1B).

328 The deletion is completely located within the intron of the LLT (8), without affecting the  
329 predicted splice donor-, branch-, or acceptor sites. Due to insertion of the bacterial genes

330 (Fig. 1B) the genome size of pPrV- $\Delta$ miRN is reduced by only 1154 bp compared to pPrV-  
331  $\Delta$ gGG, which is unlikely to influence significantly the efficiency of viral DNA replication or  
332 packaging. Consistently, pPrV- $\Delta$ miRN and pPrV- $\Delta$ gGG exhibited almost identical *in vitro*  
333 replication properties with respect to replication kinetics and cell-to-cell spread in RK13  
334 and PK15 cells (Fig. 2).

335 Expression of the genes adjacent the deletion (IE180 and EP0) was profiled by RT-qPCR  
336 in PK15 cells. In cells infected with pPrV- $\Delta$ miRN EP0 was transiently overexpressed  
337 peaking at 8h p.i. (Fig. 3A and D) while IE180 and the spliced LLT product (exon 1 - exon 2  
338 junction of LLT) displayed very similar profiles of expression in cells infected with either  
339 pPrV- $\Delta$ miRN or pPrV- $\Delta$ gGG (Fig. 3B and C). Similar expression profiles were found for  
340 eleven other PrV genes (not shown). Thus, as desired, mutant and wild-type PrV displayed  
341 highly similar *in vitro* properties as an essential prerequisite for the following *in vivo*  
342 studies.

#### 343 **Both pPrV- $\Delta$ miRN (“M”) and pPrV- $\Delta$ gGG (“WT”) establish latency *in vivo***

344 Groups of five animals were infected with pPrV- $\Delta$ gGG (“WT”), pPrV- $\Delta$ miRN (“M”) or mock  
345 infected (“C”). The only clinical symptom detected was intermittent fever until 5 days p.i. All  
346 infected animals recovered, while two non-infected control animals died in the course of  
347 the experiment due to stress reaction.

348 The levels of virus excretion in nasal swabs were heterogeneous. On average the animals  
349 infected by M showed higher excretion levels than those infected by WT with maximum  
350 levels reached earlier (at 2 days p.i.) in two of the M-infected animals. No virus excretion  
351 was detected in nasal swabs from 12 days p.i (Fig. 4A, B). All infected animals developed  
352 a robust immune response with no differences between M and WT (Fig. 4C, D).

353 Animals were sacrificed at day 62 p.i. PrV genomes were detected in the trigeminal

354 ganglia (WT-ganglia and M-ganglia) of all infected animals. Values ranged between 57 and  
355 542 copies per 100 ng of genomic DNA, which is similar to the range found in previous  
356 studies on HSV (45). Some of the highest values were found in M- ganglia (Fig. 5). This  
357 showed that the deletion did not impair the mutant virus in access to and establishing  
358 latency in trigeminal ganglia.

### 359 **Descriptive statistics of Small RNAseq and RNAseq of trigeminal ganglia**

360 *Small RNAseq* - We generated individual libraries and profiled by Small RNAseq the  
361 ganglia derived from all 13 surviving animals. The sequencing depth ranged from 20.7 to  
362 47.9 million reads with a mean depth of 37.9 million reads per sample. After adapter  
363 trimming and filtering out low quality reads, porcine and PrV miRNAs were identified and  
364 mapped on the pig and PrV genomes. This led to the identification of between 5.8 and  
365 20.7 million reads per library mapping to known or novel miRNAs (Table 1).

366 The vast majority of sequences recovered proved, as expected, to be porcine cellular  
367 miRNAs. The most highly expressed miRNAs were ssc-miR-27b-3p and ssc-miR-143-3p,  
368 with average read counts of about 2 and 1 million respectively. Further analysis did not  
369 provide any significant evidence of host miRNAs differentially expressed in the pairwise  
370 comparisons among M, WT and C-ganglia. Differences were observed for ssc-miR-204  
371 expression between WT and C-ganglia, and for ssc-miR-429 expression between M and  
372 WT-ganglia. However, after manual checking of reads, these turned out to be artifacts due  
373 to the abnormally high number of reads in outlier samples, specifically of ssc-miR-204 in  
374 one C-ganglia sample and of ssc-miR-429 in one M-ganglion sample (data not shown).

375 *RNAseq* - We produced individual libraries for a sub-sample of nine animals (3 M, 3 WT,  
376 and 3 C-ganglia). RNAseq profiling generated an average of 65 million reads per library.  
377 Quality check confirmed that over 75% of reads were of good quality. Upon mapping and  
378 transcript assembly, we detected 19,465 pig genes expressed in ganglia. Normalized

379 values are provided in Suppl. Table 2. The most expressed pig genes (average of 700,000  
380 reads per sample) corresponded to the neurofilament medium and light polypeptide genes  
381 (NEFM and NEFL), which are found highly expressed in the cerebral cortex and in the  
382 hippocampus (46, 47). Despite the depth of sequencing, very few reads mapped on the  
383 PrV genome (between 51 and 523 normalized reads). All of them mapped to the LLT gene  
384 locus as expected during latency (Suppl. Table 2).

### 385 **All the known PrV miRNAs are expressed during latency**

386 In the ganglia latent for parental PrV (WT-ganglia), we detected all the mature PrV  
387 miRNAs described so far, which are encoded by 11 miRNA genes clustering in the LLT  
388 intron (22, 23). No new PrV miRNAs were identified (Table 2). Furthermore, we did not  
389 detect the offset-moRNA encoded by the prv-mir-LLT8 gene previously found in dendritic  
390 cells during productive PrV infection, identified as prv-miR-4 by (22) and as moR-8 (23,  
391 48).

392 The PrV miRNAs are still annotated as unique mature sequence in the last version (v21) of  
393 the miRBase database ([www.mirbase.org](http://www.mirbase.org)). However, with few exceptions, all miRNAs  
394 were found expressed by both the 5p and 3p arms of their precursor sequence, and, as  
395 expected, one form was predominant (Table 2). Furthermore, the predominant mature  
396 miRNAs encoded by the prv-mir-LLT7 and prv-mir-LLT8 genes were those of the 3p arm  
397 as previously detected (23, 48) but not yet annotated in miRBase. To clarify the issue, we  
398 revised the nomenclature of all PrV miRNAs by adding the arm of origin information (Table  
399 2).

400 The most abundant viral miRNA was prv-miR-LLT2-5p followed by prv-miR-LLT1-3p (both  
401 deleted in M), and prv-miR-LLT-10-3p (present in both WT and M) (Table 2). The prv-mir-  
402 LLT-10a and prv-miR-LLT-11a genes map to the 3' distal portion of the LLT intron and are  
403 duplicated in the terminal repeat region (TR) of the PrV genome (prv-mir-LLT10b and prv-

404 mir-LLT11b). The mature prv-miR-LLT-10 and prv-miR-LLT-11 showed similar patterns of  
405 expression in M and WT- ganglia, suggesting that the deletion in the mutant virus did not  
406 affect regulatory sequences required for the expression of prv-mir-LLT10a and prv-mir-  
407 LLT11a (Table 2).

408 The RT-qPCRs confirmed the presence of the three most abundant miRNAs (prv-miR-  
409 LLT1-3p, prv-miR-LLT2-5p and prv-miR-LLT-10-3p) (Fig. 6A). For comparison, we checked  
410 the expression of these miRNAs in PK15 cells during productive infection at 12h p.i. Both  
411 prv-miR-LLT1-3p and prv-miR-LLT2-5p were highly expressed while prv-miR-LLT-10-3p  
412 was detected at much lower levels (Fig. 6B). We were unable to assess the expression  
413 levels of other less abundant PrV miRNAs above background values.

#### 414 **Characterization of the pattern of expression of the LAT locus in trigeminal ganglia**

415 RNAseq and Small RNAseq data indicated that only LLT and the viral miRNAs (which, with  
416 the exception of prv-miR-LLT10 and prv-miR-LLT11, are only present in the genome of  
417 WT) were expressed in the porcine ganglia, as it would be expected in established latency.  
418 To better characterize this status in the M-ganglia we performed RT-PCR and RT-qPCR  
419 analyses of different portions of the LAT locus adjacent to the deletion (Fig. 1).

420 In both M and WT-ganglia no expression of IE180 or EP0 could be detected by repeated  
421 tests by primer-specific RT-qPCR. This confirmed that also the second copy of the IE180  
422 gene mapping to the TR region of the PrV genome (1) is inactive during latency. The M  
423 virus expressed lower levels of transcripts comprising the ex1/ex2 junction and exon 2 of  
424 LLT, while the first LLT exon was expressed at similar levels by both viruses (Fig. 7). This  
425 was surprising, given that the splicing of LLT (LLT ex1/ex2 junction) was unaffected *in vitro*  
426 (Fig. 2C) and no expression of genes expressed during the lytic phase was detected in  
427 ganglia.

428 An additional difference was observed in the distribution of RNAseq reads between M and  
429 WT-ganglia at the LAT locus. This revealed that in M-ganglia the portion of the LLT intron ~  
430 1000 bp immediately downstream the deletion is expressed (Fig. 8). We tested by RT-  
431 qPCR if this could indicate the presence in M-ganglia of unspliced transcripts which are  
432 expressed during the PrV lytic phase (12). The results confirmed that the bacterial genes  
433 and the LLT intron region immediately downstream of the bacterial cassette (Fig. 1) were  
434 expressed by the M virus, indicating that the bacterial promoter is active in ganglia.  
435 However, in agreement with the distribution of mapped reads, no expression of the portion  
436 of the LLT intron adjacent to the acceptor site and preceding the peak of reads at the 5' of  
437 LLT exon 2 was detected by RT-qPCR in M and WT-ganglia (not shown). This excluded  
438 the possibility that transcripts covering part of the 3' portion of the intron are expressed in  
439 M-ganglia.

#### 440 **Gene upregulation is prevalent in trigeminal ganglia latent for the mutant PrV**

441 By differential expression analysis of the nine samples profiled by RNAseq, we identified  
442 54 genes (plus two pseudogenes and two miRNA precursors predicted in the cow  
443 genome), each significantly differentially expressed (DE) in at least one of three pair-wise  
444 comparisons among WT, M and C- ganglia (WT vs. C, M vs. C and M vs. WT). Values of  
445 differential expression (DE and p-values of significance) are provided in Suppl. Table 3.

446 M-ganglia and WT-ganglia differed considerably in their patterns of gene expression. DE  
447 genes reaching significance were more abundant in WT- (34) than in M-ganglia (22), and  
448 only eight genes were common to the M vs. C and WT vs. C comparison. An additional six  
449 genes were significantly DE only in the direct comparison between WT vs. M.

450 Remarkably, while in the WT-ganglia we found a prevalence of significantly downregulated  
451 genes (20 downregulated vs. 14 upregulated), the opposite trend was found in M-ganglia  
452 (19 upregulated vs. only 3 downregulated) (Suppl. table 3). Only BTNL9 (butyrophilin-like



453 9), MTNR1B (melatonin receptor 1B), NR1D2 (nuclear receptor subfamily 1, group D,  
454 member 2, which is a transcriptional repressor with roles in circadian rhythms and  
455 carbohydrate and lipid metabolism) and MAPK4 (Mitogen-activated protein kinase 4) were  
456 more significantly downregulated in M-ganglia in comparison to WT-ganglia or controls.

457 The eight genes shared by the M vs. C and WT vs. C comparisons included only highly  
458 upregulated host immune genes: CXCL13 (a chemokine ligand); five immunoglobulins  
459 (IGJ, Igk-V, IGKV-6, one IGLC member and IGLL5); TNFRSF10B (member 10B of the  
460 TNF-receptor superfamily, the most upregulated gene found in both M and WT-ganglia (>  
461 4 logFC); and a protein annotated as novel in the pig genome similar to SLC2A7, which is  
462 a glucose transporter (Suppl. Table 3).

463 The RT-qPCR of 16 genes represented by either a high or low number of RNAseq reads  
464 was carried out in the whole set of WT, M and C-ganglia samples. The results showed  
465 excellent concordance between RNAseq and RT-qPCR. Furthermore, the profile of these  
466 few additional animals provided significance to previously suggestive values (Table 3,  
467 Suppl. Table 3). In particular, PLA2G2D (Phospholipase A2, group IID), CD8A (T-cell  
468 surface glycoprotein CD8 alpha chain) and CXCL9 became significant also in the M vs.  
469 WT contrast, strengthening the pattern of gene upregulation found in M-ganglia.  
470 Furthermore, RT-qPCR confirmed that VIP (vasointestinal peptide) was detectable only in  
471 the three M-ganglia samples carrying the highest numbers of copies of PrV genomes (Fig.  
472 5).

473 Fifteen DE genes harbored one or multiple targets for one or more of PrV's miRNAs  
474 (Suppl. Table 3). However, the generalized pattern of gene downregulation in WT-ganglia  
475 and of gene upregulation in M-ganglia hid any putative modulatory effect of PrV miRNAs.  
476 Furthermore, we did not detect any relative enrichment of putative targets for the most  
477 expressed PrV miRNAs compared to the whole set of porcine genes expressed in ganglia

478 (not shown).

### 479 **The LAT deletion affects the host pro-inflammatory response**

480 We used the Ingenuity Pathway Analysis (IPA) software ([www.ingenuity.com](http://www.ingenuity.com)) to analyze  
481 the expression patterns of latently infected ganglia. All the genes which were DE in at least  
482 one of the three comparisons (Suppl. Table 3) were included in the analysis, with the  
483 exception of the two putative miRNA precursors so far annotated only in the cow genome  
484 (bta-mir-2887 and bta-miR-2904) and C3 (missing in the IPA reference database). By this  
485 analysis we could assign a total of 44 these DE genes to top gene networks and/or  
486 biofunctions.

487 The top network identified by IPA in both M- and WT-ganglia was “Cell-mediated Immune  
488 Response, Cellular Movement, Hematological System Development and Function” (17  
489 genes; score 39), followed by “Hereditary Disorder, Neurological Disease, Psychological  
490 Disorders” (15 genes; score 34) (Suppl. Table 4). Other networks were identified by less  
491 than six genes in either the WT vs. C or M vs. C comparisons.

492 IPA identified INFG and two inflammatory cytokines (TNF and IL6) as most significant top  
493 upstream regulators. The state of activation of these regulators was globally coherent with  
494 the pattern of expression of 20 DE genes in the WT vs. C and M vs. C comparisons (Fig.  
495 9). As expected, the large majority (15) of these genes belonged to the cell-mediated  
496 immune response network (Suppl. Table 4). This network added evidence for the pattern of  
497 expression of VIP being inconsistent with the activation of TNF while the pattern of  
498 CYP2E1 (cytochrome P450, family 2, subfamily E, polypeptide 1) is inconsistent with all  
499 the three regulators (Fig.9).

500 In order to compare the WT and M-ganglia for their respective status of activation of  
501 specific diseases and biofunctions, we generated a comparative heatmap of M vs. WT-

502 ganglia reporting the IPA z-scores of activation besides the p-values of biofunctions'  
503 significance (Table 3). The differences in the trend of activation/inhibition between WT- and  
504 M-ganglia are largely determined by few genes participating to several biofunctions. The  
505 “migration of dendritic cells” was, together with the more general ones related to tissue and  
506 cell homeostasis, the most significant biofunction of latent ganglia (p-value:  $6.77E^{-06}$ ).  
507 Differently than in WT-ganglia, in M-ganglia this biofunction had a trend of inhibition. This  
508 difference was due to the combined effect of three genes: VIP (only expressed in M-  
509 ganglia); AGT (angiotensinogen - serpin peptidase inhibitor, clade A, member 8; less  
510 downregulated in M-ganglia) and ICOS (inducible T-cell co-stimulator; more upregulated in  
511 M-ganglia). A similar effect was found for other biofunctions (“activation of leukocytes”,  
512 “activation of T lymphocytes”, and “inflammatory response”). Conversely, “expansion of T  
513 lymphocytes” and “stimulation of cells” had a trend of activation in M-ganglia due to the  
514 combined effect of VIP and BTNL9 (the latter more downregulated in M-ganglia).  
515 Interestingly, M-ganglia showed also a less efficient inhibition of “synthesis of fatty acid”  
516 and “concentration of fatty acid” (Table 3).

517

## 518 **Discussion**

519 We show here that deletion of nine of the eleven known PrV miRNA genes, contained in a  
520 cluster within the LLT intron sequence, does not impair establishment of latency in  
521 trigeminal ganglia. The PrV genome was detected in the trigeminal ganglia of all infected  
522 animals beyond the termination of clinical symptoms and viral excretion (Fig. 4, Fig. 5).  
523 Moreover, the mutant virus displayed almost identical properties with the parental pPrV-  
524  $\Delta$ gGG, a BAC clone derived from PrV-Ka, during propagation *in vitro* (Fig. 1, Fig. 2, Fig. 3).  
525 The value of our experimental approach lies in the use of a natural virus-host system to  
526 analyze the importance of miRNA-containing regions on herpesvirus latency. Most of the

527 current knowledge on latency has been obtained from studies of HSV-1 and HSV-2 in  
528 rodent models. In these settings, all LAT mutants that ablate LAT expression and, thus, the  
529 expression of multiple miRNAs, can establish and maintain latency (18, 41-43). It has been  
530 reported earlier that PrV mutants unable to express LAT and EP0 were also able to reach  
531 and persist in porcine trigeminal ganglia after intranasal infection (49). This makes it  
532 unlikely that removal of the entire cluster of eleven PrV miRNA genes would make a  
533 difference for the ability of PrV to establish latency.

### 534 **Transcriptional patterns of the PrV genome during latency**

535 With the exception of the deleted miRNAs, the viral transcriptional profiles of ganglia latent  
536 for the mutant “M” PrV displayed only subtle differences compared to the parental “WT”  
537 virus. The finding that the levels of ex1/ex2 junction and exon 2 of LLT were decreased in  
538 M-ganglia (Fig. 7) is difficult to explain in absence of any evidence of viral reactivation. In  
539 HSV several results point to products of the LAT locus functioning in repression of lytic  
540 gene expression, which would favor establishment and maintenance of latency, and LAT  
541 has been proposed to silence viral gene expression as a long non-coding RNA (50-52).  
542 However, in HSV the number of neurons harboring virus is decreased after infection by  
543  $\Delta$ LAT mutants, as reviewed by (53), while ganglia latent for the mutant PrV (M-ganglia)  
544 carried similar amounts of latent PrV genomes compared to WT-ganglia (Fig. 5).  
545 Decreased levels of LLT in ganglia latent for the nine miRNA-deleted virus are also  
546 inconsistent with the predicted ability of multiple PrV miRNAs to target LLT, as well as  
547 IE180 and EP0 (23). Finally, given the limited knowledge of the PrV LAT locus, we cannot  
548 totally exclude that the 2.5 kb deletion removed regulatory sequences which may affect  
549 LLT expression in neurons (54).

550 In addition to LLT, all the PrV miRNAs previously described from productively infected cells  
551 (22, 23) were detected in latent ganglia (Table 2, Fig. 6). The two most abundant miRNAs

552 in WT-ganglia prv-miR-LLT1-3p, prv-miR-LLT2-5p, which are absent from the mutant PrV  
553 genome, are highly expressed during productive infection in PK15 cells (Table 2, Fig. 6).  
554 Prv-miR-LLT1 is also the most highly expressed PrV miRNA in dendritic cells (22) and is  
555 the only one detected in trigeminal ganglia of pigs during acute infection, albeit at reduced  
556 sequencing depth (48). It is interesting that prv-miR-LLT10-3p, which is not included in the  
557 deletion, was expressed by both M and WT at similar levels as prv-miR-LLT1-3p and prv-  
558 miR-LLT2-5p, which contrasts with the low expression of this miRNA during productive  
559 infection (Table 2, Fig. 6). It should be noted that the gene coding for this miRNA is  
560 duplicated, and maps at the 3' end of both copies of IE180 (1). The fact that IE180  
561 expression was not detected suggests that the mature prv-miR-LLT10 is expressed only  
562 by the miRNA gene copy adjacent to the LAT locus.

563 These findings suggest that, as for HSV (55), different PrV miRNAs may be expressed  
564 preferentially during productive infection in cell culture and during latent infection in  
565 sensory ganglia. In this context, it is interesting that the only difference found was a  
566 transient upregulation of EP0 at 8h pi (Fig.3A and D) in PK15 cells infected by the mutant  
567 virus. Otherwise, the absence of miRNAs did not affect the replication properties of PrV  
568 (Fig. 2).

### 569 **Transcriptional patterns of the host genome during latency**

570 Our findings add to what had been proposed for HSV, i.e. that host parameters such as  
571 innate immunity (56), the repressive effects of immune cells in ganglia (57), or the  
572 neuronal environment (58) promote the establishment and maintenance of latency (52).  
573 Host genes which are differentially expressed during PrV latency are involved in  
574 biofunctions related to expansion, activation and cell death of T lymphocytes and of  
575 dendritic cell migration. This parallels data from HSV latency, where the LAT locus has  
576 been shown to function as an “immune evasion gene” by promoting functional exhaustion

577 of virus-specific CD8<sup>+</sup> T cells in latently infected trigeminal ganglia and by inhibiting the  
578 phenotypic and functional maturation of dendritic cells (59, 60).

579 Indeed, the most prominent differences between ganglia latent for the miRNA-deleted (M)  
580 and parental (WT) PrV were found in host response, and, interestingly, without any  
581 evidence for differential expression of host miRNAs. Both viruses triggered a robust pro-  
582 inflammatory immune response (Suppl. Table 4, Fig. 9) but a pronounced pattern of gene  
583 upregulation was found in ganglia latent for the mutant virus (Suppl. Table 3, Table 3). The  
584 impairment of the host pro-inflammatory response is reflected by differential expression of  
585 a limited number of genes acting in several pathways (Table 4). VIP acts as an inhibitor in  
586 many biological functions. Its absence induces better Th1 polarization and antiviral  
587 immunity in mice (61) and VIP-knockout mice have enhanced cellular immune responses  
588 and increased survival following murine cytomegalovirus infection (62). Various reports  
589 indicate CYP2E1 (as a gene downregulated by various stimuli, including inflammation (63)).  
590 Thus, in M-ganglia VIP would be a factor of less efficient cell mediated host response, and  
591 the upregulation of CYP2E1 would be a global indicator of reduced inflammatory response  
592 (Table 3, Figure 9). Conversely, reduced levels of BTNL9 suggest a reduced ability of the  
593 mutant virus to control T cell activation (Table 3). The butyrophilin-like family encodes  
594 transmembrane glycoproteins with roles in immune co-regulation and antigen  
595 presentation, and some of them are functionally implicated in T cell inhibition and in the  
596 modulation of epithelial cell-T cell interactions (64-66).

597 The pattern of gene upregulation found in the ganglia latent for the mutant virus is  
598 suggestive of a role for PrV miRNAs in regulating the host genome during latency.  
599 However, presumably only a fraction of the observed effects can be attributed to PrV  
600 miRNAs. Other regulatory sequences controlling the latent virus genome at the epigenetic  
601 level (54, 67) may map to the 2.5 kb region deleted from the PrV LAT locus and alter host  
602 transcription and immune responses. Additional functional studies are required to

603 investigate the relative contribution of these different factors during PrV latency.

604

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617

## 618 **Figure legends**

619 Figure 1

620 (A) Physical map of the PrV-Ka genome containing unique ( $U_L$ ,  $U_S$ ) and inverted repeat  
621 ( $I_R$ ,  $I_L$ ) sequences. BamHI restriction sites and fragments, as well as the insertion of a  
622 bacterial vector and of an EGFP reporter gene cassette at the gG gene locus in pPrV-  
623  $\Delta gGG$  (Fuchs et al., 2012) are indicated. (B) An enlarged section shows the boundary  
624 between  $U_L$  and  $I_R$  with the open reading frames of the regulatory proteins EP0 and IE180.  
625 Viral mRNAs and the spliced large latency transcript (LLT) are indicated by dotted arrows.  
626 Identified miRNAs (Wu et al., 2012) are shown as red arrowheads numbered from 1 to 11

627 (corresponding to miRNA genes: from prv-mir-LLT1 to prv-mir-LLT11). In pPrV- $\Delta$ miRN the  
628 majority of the miRNA genes was deleted and replaced by selection markers (RpsL, KanR)  
629 used for BAC mutagenesis in *E. coli*.

#### 630 Figure 2

631 Replication of pPrV- $\Delta$ gGG and pPrV- $\Delta$ miRN in PK15 (A) and RK13 (B) cells. Progeny virus  
632 titers were determined between 4 and 24h after infection at multiplicity of infection (MOI) of  
633 10 (PK15) or 5 (RK13). Titters represent mean values of three independent experiments  
634 with standard deviation bars.

#### 635 Figure 3

636 RT-qPCR expression kinetics of LLT during PrV infection *in vitro*. PK15 cells were infected  
637 with pPrV- $\Delta$ miRN (light gray) and pPrV- $\Delta$ gGG (dark gray) at a MOI of 10. Values are  
638 provided as mean Ct values and are the average of three biological replicates (higher Ct  
639 values mean decreased gene expression levels). The qPCRs were normalized to input  
640 amount of total RNA.

#### 641 Figure 4

642 Establishment of latency *in vivo*. Pigs were infected with either pPrV- $\Delta$ gGG ("WT" 54-58),  
643 pPrV- $\Delta$ miRN ("M" 49-53) or mock infected ("C" 43-48). A, B): DNAs from nasal swabs of  
644 animals infected by WT PrV (A) or M PrV (B) were analyzed by RT-qPCR of the PRV gB  
645 gene. C, D): The host antibody response was analyzed by ELISA using PrV gB as antigen.  
646 The threshold value of the assay (0.7) is indicated as a red line.

#### 647 Figure 5

648 Relative amounts of PrV genomes in latent trigeminal ganglia. A): The PrV genome copy  
649 value per 100 ng of genomic DNA was quantified by qPCR using a GFP amplicon. B): PA-  
650 GFP-coilin C2 plasmid DNA standard curve. The x-axis represents the input copies of



651 plasmid DNA and the y-axis the mean cycle of threshold (Ct mean).

652 Figure 6

653 RT-qPCR profiles of prv-miR-LLT1, prv-miR-LLT2 and prv-miR-LLT10 in (A) trigeminal

654 ganglia latent for the WT or M PrV and (B) in PK15 cells at 12h p.i. with the WT PrV.

655 Values are normalized against background and indicated as  $2^{-\Delta Ct}$  ( $\pm$  standard deviation).

656 Figure 7

657 Pattern of transcription of three regions of LLT (exon 1, ex1/ex2 junction and exon 2) in

658 trigeminal ganglia latent for the WT or M PrV. RT-qPCR values were calibrated vs. the

659 relative amount of PrV genomes. Values are the  $2^{-\Delta Ct}$  ( $\pm$  standard deviation) calculated

660 from three technical replicates.

661 Figure 8

662 Visualization of the distribution of RNAseq reads obtained by RNAseq profiling of

663 trigeminal ganglia latent for the mutant (M) or parental (WT) PrV on the PrV genome.

664 Figure 9

665 IL6, IFNG and TNF were identified by IPA as most significant upstream regulators (z

666 scores > 2) to explain the pattern of transcription of 20 DE genes, of which 15 belong to

667 the top IPA network “Cell-mediated Immune Response, Cellular Movement,

668 Hematological System Development and Function” (17 DE genes). Left: WT vs. C; right:

669 M vs. C. Numbers are the logFC values of each comparison.

670 Red: upregulated; green: downregulated; orange: leads to activation; blue: leads to

671 inhibition; yellow: finding inconsistent with state of downstream molecules; grey: effect not

672 predicted.

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885 **Table 1.** Descriptive statistics of Small RNA profiling of porcine trigeminal ganglia latent for  
 886 either the WT (parental) or M (mutant) PrV. Thirteen individual small RNAseq libraries  
 887 were constructed from five WT-infected animals (54 WT, 55 WT, 56 WT, 57 WT, 58 WT),  
 888 five M-infected animals (49M, 50 M, 51 M, 52 M, 53 M) and three mock-infected animals  
 889 (22 C, 23 C, 25C). Values are indicated as millions of normalized reads. The average  
 890 counts are provided at the bottom of each column.

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<b>Samples</b>	<b>Reads mapping on miRNAs</b>	<b>Reads &lt;16nt and &gt;29nt</b>	<b>Total reads</b>
<b>22 C</b>	7.8	16.9	42.9
<b>23 C</b>	5.8	8.7	46.8
<b>25 C</b>	7.2	12.9	27.3
<b>49 M</b>	6.9	14.8	55.3
<b>50 M</b>	20.7	34.7	47.9
<b>51 M</b>	11.3	17.7	43.8
<b>52 M</b>	16	15.9	46.1
<b>53 M</b>	16.3	19.8	39.2
<b>54 WT</b>	10	8	20.7
<b>55 WT</b>	5.7	16.5	27.4
<b>56 WT</b>	25.6	32.8	46.5
<b>57 WT</b>	15	19	20.7
<b>58 WT</b>	13.5	15.9	28.4
	<b>12.4 ± 6.1</b>	<b>18.0 ± 7.8</b>	<b>37.9 ± 11.5</b>

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**Table 2.** Expression levels of PrV miRNAs in trigeminal ganglia latent for the WT or M PrV. In bold the miRNAs deleted in the M genome. Both prv-mir-LLT10 and prv-mir-LLT11 are duplicated in PrV genome as identical genes (prv-mir-LLT10a and prv-mir-LLT10b; prv-mir-LLT11a and prv-mir-LLT11b). Values are provided as counts per million of reads (cpm).

microRNA name	miRBase ID	miRBase Accession number	mature miRNA sequence (5'-3')	54 WT	55 WT	56 WT	57 WT	58 WT	49 M	50 M	51 M	52 M	53 M
prv-mir-LLT1-5p			GACGGCTCCTGGGGCTGAAAGC	0.18	0.60	1.84	0.27	0.15	-	-	-	-	-
<b>prv-mir-LLT1-3p</b>	<b>prv-miR-LLT1</b>	<b>MIMAT0025304</b>	UCUCACCCCUGGGUCCGUCGC	25.11	43.27	76.90	5.54	17.18	-	-	-	-	-
<b>prv-mir-LLT2-5p</b>	<b>prv-miR-LLT2</b>	<b>MIMAT0025305</b>	CUCAUCCCGUCAGACCUGCG	55.17	344.02	153.63	14.34	35.02	-	-	-	-	-
<b>prv-mir-LLT2-3p</b>			CGCGGGGCAACGGTGGTGAG	0.35	-	0.31	0.13	0.07	-	-	-	-	-
<b>prv-mir-LLT3-5p</b>			GAGCCGGGGGGTTCGAGTG	-	-	-	-	-	-	-	-	-	-
<b>prv-mir-LLT3-3p</b>	<b>prv-miR-LLT3</b>	<b>MIMAT0025306</b>	CGCACACGCCCCUCUCGCGCAC	0.18	0.70	1.80	-	0.37	-	-	-	-	-
<b>prv-mir-LLT4-5p</b>	<b>prv-miR-LLT4</b>	<b>MIMAT0025307</b>	AGAGUAUCAGCGUGGCUUUUUU	4.42	4.59	23.07	1.40	2.59	-	-	-	-	-
<b>prv-mir-LLT4-3p</b>			AAAAGGCACGCTGATGCGTCC	-	-	0.12	-	-	-	-	-	-	-
<b>prv-mir-LLT5-5p</b>				-	-	-	-	-	-	-	-	-	-
<b>prv-mir-LLT5-3p</b>	<b>prv-miR-LLT5</b>	<b>MIMAT0025308</b>	UGAGUGGAUGGAUGGAGGCGAG	-	0.50	1.21	0.20	-	-	-	-	-	-
<b>prv-mir-LLT6-5p</b>	<b>prv-miR-LLT6</b>	<b>MIMAT0025309</b>	CGUACCGACCCGCCUACCAGG	-	3.39	1.02	-	-	-	-	-	-	-
<b>prv-mir-LLT6-3p</b>			CTTGGCAGCGGGTGGGTACC	-	0.80	0.70	0.07	0.22	-	-	-	-	-
<b>prv-mir-LLT7-5p</b>	<b>prv-miR-LLT7</b>	<b>MIMAT0025310</b>	CCGGGGGUUGAUGGGGAU	-	-	-	-	-	-	-	-	-	-
<b>prv-mir-LLT7-3p</b>			ACCACCGTCCCCCTGTCCCT	1.42	4.29	5.70	2.27	2.00	-	-	-	-	-
<b>prv-mir-LLT8-5p</b>	<b>prv-miR-LLT8</b>	<b>MIMAT0025311</b>	GUGGGGGCGAAGAUUGGGUU	-	-	1.84	0.07	-	-	-	-	-	-
<b>prv-mir-LLT8-3p</b>			CAACCCTTCTGGAGCCCTACC	10.79	8.57	30.25	2.27	5.48	-	-	-	-	-
<b>prv-mir-LLT9-5p</b>	<b>prv-miR-LLT9</b>	<b>MIMAT0025312</b>	AUCGAGGAGAUGUGGAGGGG	-	0.20	0.59	-	0.07	-	-	-	-	-
<b>prv-mir-LLT9-3p</b>			CCCTCCCCCGCATCTCTTCTC	-	-	0.43	0.13	-	-	-	-	-	-
<b>prv-mir-LLT10b-5p</b>				-	-	-	-	-	0.15	-	-	-	-
<b>prv-mir-LLT10b-3p</b>	<b>prv-miR-LLT10a</b> <b>prv-miR-LLT10b</b>	<b>MIMAT0025313</b> <b>MIMAT0025314</b>	CCGAGCCUGCCCCUCCGUCGCA	24.05	51.74	74.05	4.47	10.51	18.93	4.68	11.13	17.36	75.16
<b>prv-mir-LLT11b-5p</b>	<b>prv-miR-LLT11a</b> <b>prv-miR-LLT11b</b>	<b>MIMAT0025315</b> <b>MIMAT0025316</b>	AGGCUGGGAGUGGGGACGGAAGA	0.18	-	1.02	0.13	-	-	-	-	0.08	0.37
<b>prv-mir-LLT11b-3p</b>				-	0.80	1.17	0.07	0.07	-	0.05	-	-	0.25

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898 **Table 3.** Comparison of RNAseq and RT-qPCR data of 16 genes differentially expressed in trigeminal ganglia latent for the WT or M PrV. Values  
899 are reported as fold change (logFC) for each of the three pairwise comparisons (WT vs. C, M vs. C, and M vs. WT). VIP was only detected in  
900 three M-ganglia samples.

Accession number	Gene symbol	Gene name	WT vs. C		M vs. C		M vs. WT	
			RNAseq	RT-qPCR	RNAseq	RT-qPCR	RNAseq	RT-qPCR
ENSSSCG00000028488	LTC4S	Leukotriene (LT) C(4) synthase	-1.38 *	-0.23	-0.72	-0.76	0.66	0.52
ENSSSCG00000013022	PYGM	Phosphorylase	-1.46 *	-0.88	-0.33	0.32	1.13	1.20 **
ENSSSCG00000010506	Opalin	Opalin (specifically expressed in brain)	-3.11 *	-3.06	-0.67	-0.40	2.44	2.66 *
ENSSSCG00000025434	CGA	Glycoprotein hormones alpha chain	-3.13 *	-2.27 *	-0.38	-0.72	2.75	1.55 *
ENSSSCG00000016664	NPSR1	Member of G-protein coupled receptor 1 family	-2.66 *	-0.27	-2.26	0.58	0.40	0.85
ENSSSCG00000003345	TMEM88B	Transmembrane protein 88B	-1.98 *	-1.29 *	-0.84	0.01	1.14	1.31 **
ENSSSCG00000000133	TST	Sulfurtransferase	-0.88 *	-0.74	-0.16	-0.02	0.73	0.72 *
ENSSSCG00000008648	RSAD2	Viperin antiviral protein	1.36 **	1.29	0.62	1.09	-0.73	-0.20
ENSSSCG00000003497	PLA2G2D	Phospholipase A2. group IID	1.30	0.70 *	2.25 **	1.31 *	0.95	0.61
ENSSSCG00000008217	CD8A	T-cell surface glycoprotein CD8 alpha chain	0.50	-0.31	1.37 *	1.10 *	0.87	1.41 **
ENSSSCG00000023489	CXCL9	Chemokine (C-X-C motif) ligand 9	0.81	1.02	1.92 ***	2.28 *	1.12	1.26 *
ENSSSCG00000010780	CYP2E1	Cytochrome P450 2E1	-2.28	-1.41	0.78	0.81	3.06 *	2.23 **
ENSSSCG00000025614	PRICKLE4	LIM protein family member	-1.14	-0.15	1.56	0.56 *	2.71 *	0.71 *
ENSSSCG00000004492	SLC14A1	Membrane transporter of urea in erythrocytes	-1.96 ***	-1.32 *	-0.33	0.34	1.63 **	1.66 **
ENSSSCG00000009672	SCARA5	A ferritin receptor mediating non-transferrin iron delivery	-0.77 *	-0.73	0.27	0.16	1.05 ***	0.89 *
ENSSSCG00000004078	VIP	Vasointestinal neuropeptide	-	-	-	-	5.85 **	6.66 *

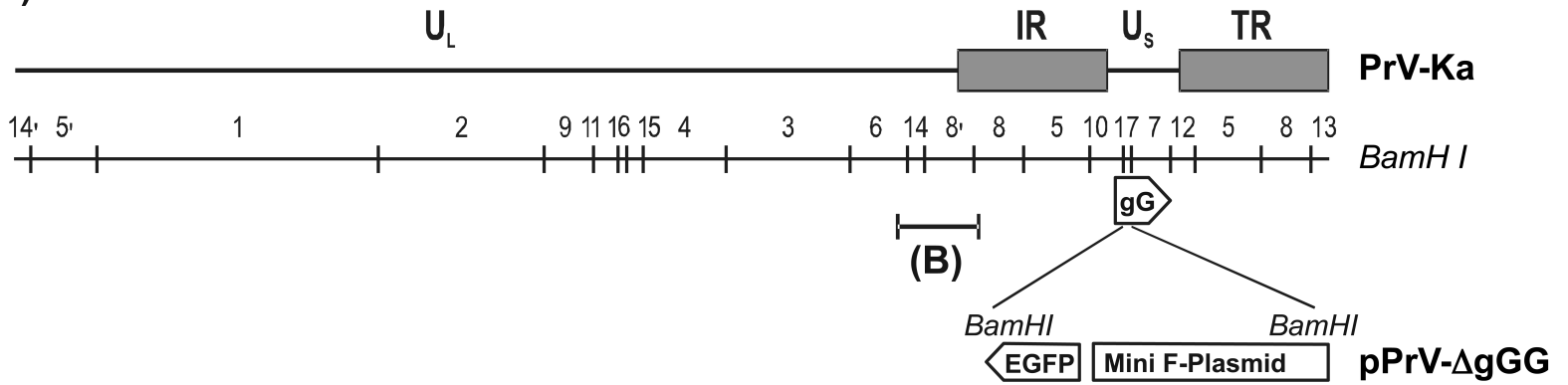
901 \*: p≤0.05; \*\*: p ≤0.01; \*\*\*: p ≤ 0.001.

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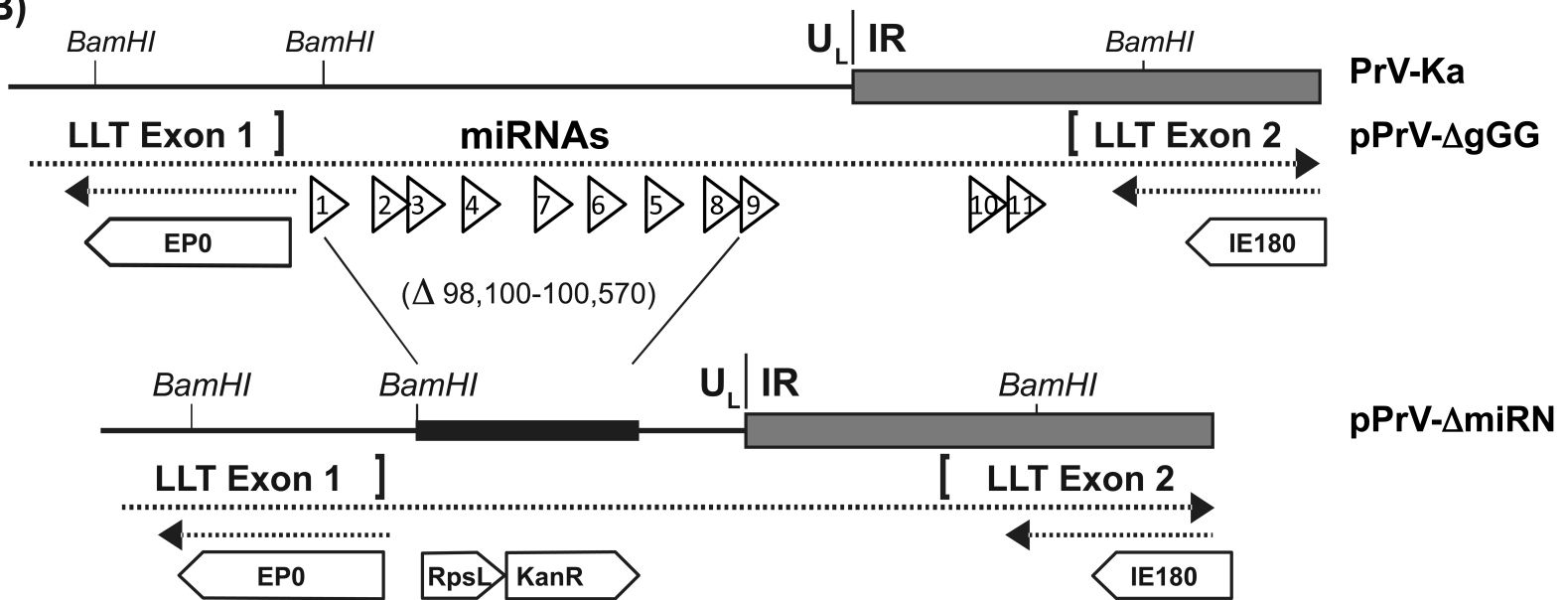
904 **Table 4.** Diseases and biofunctions in trigeminal ganglia latent for M or WT PrV. The “M vs. C” and “WT vs. C” columns report the IPA z-scores of  
905 activation (positive values) or inhibition (negative values) in the two comparisons. The most different ones (in *italic*) are reported in bold on top  
906 (more inhibited/less activated in M) and bottom (more activated/less inhibited in M). The most significant p-values of each biofunction are in bold.

Diseases and Bio Functions	M vs C	WT vs C	p-Value	Genes
<b>inflammation of organ</b>	-1.17	1.05	1.38E-03	AGT,CD8A,CXCL9,CXCL13,CYP2E1,GPD1,ICOS,SCARA5,TNFRSF10B,VIP
<b>inflammatory response</b>	0.26	1.61	3.15E-03	AGT,CXCL13,CXCL9,ITGA2,PLA2G2D,SCARA5,VIP
<b>cell death of T lymphocytes</b>	-0.42	0.91	9.16E-03	GZMA,ICOS,LAG3,VIP
<b>migration of dendritic cells</b>	-0.69	0.44	<b>6.77E-06</b>	AGT,CXCL13,CXCL9,ICOS,VIP
<b>recruitment of cells</b>	-0.10	0.93	1.79E-03	AGT,CD8A,CXCL9,CXCL13,VIP
<b>activation of T lymphocytes</b>	0.52	1.56	2.45E-03	CD8A,GZMA,ICOS,LAG3,VIP
recruitment of lymphocytes	0.25	1.10	<b>4.88E-05</b>	AGT, CD8A, CXCL9, VIP
recruitment of granulocytes	-1.10	-0.25	1.57E-03	AGT, CD8A, CXCL9, VIP
cell movement of leukocytes	-0.46	0.29	1.07E-03	AGT,CD8A,CXCL9,CXCL13,ICOS,LAG3,LTC4S,VIP
survival of organism	-2.06	-1.34	<b>5.00E-04</b>	AGT, CD8A, CXCL9, GZMA, LAG3, RSAD2, SLC14A1, VIP
infiltration by mononuclear leukocytes	-0.62	0.06	<b>6.45E-05</b>	AGT,CXCL9,ICOS,LAG3,VIP
leukocyte migration	0.17	0.80	<b>6.04E-04</b>	AGT,CD8A,CXCL9,CXCL13,ICOS,ITGA2,LAG3,LTC4S,VIP
infiltration of leukocytes	-0.94	-0.32	<b>4.68E-04</b>	AGT,CXCL9,ICOS,LAG3,LTC4S,VIP
cell movement of T lymphocytes	-0.54	0.05	<b>1.43E-05</b>	AGT,CXCL9,CXCL13,ICOS,LAG3,VIP
Lymphocyte migration	0.25	0.77	<b>2.06E-05</b>	AGT,CD8A,CXCL9,CXCL13,ICOS,LAG3,VIP
activation of cells	0.65	1.15	<b>3.02E-04</b>	AGT,CD8A,CXCL9,GABRA1,GZMA,ICOS,LAG3,PLA2G2D,TNFRSF10B,VIP
activation of leukocytes	0.42	0.91	<b>6.25E-05</b>	AGT,CD8A,CXCL9,GZMA,ICOS,LAG3,PLA2G2D,TNFRSF10B,VIP
quantity of IgG	2.19	2.19	4.04E-03	CXCL9, ICOS, IGJ, IGLL1/IGLL5, RSAD2
binding of blood cells	1.87	1.87	2.44E-03	CXCL9, CXCL13, ICOS, ITGA2
binding of cells	1.60	1.60	<b>2.87E-04</b>	AGT,CXCL9,CXCL13,ICOS,ITGA2,SCARA5,VIP
differentiation of blood cells	0.42	0.42	4.51E-03	AGT,CD8A,ICOS,IGLL1/IGLL5,RSAD2,TNFRSF10B,VIP
accumulation of leukocytes	-0.46	-0.46	<b>4.47E-04</b>	AGT,CXCL9,ICOS,ITGA2,LTC4S
quantity of helper T lymphocytes	-0.57	-0.57	1.35E-03	CD8A, ICOS, LAG3, PLA2G2D
activation of phagocytes	-0.69	-0.69	8.01E-03	AGT,GZMA,PLA2G2D,TNFRSF10B
quantity of antigen presenting cells	-1.07	-1.07	4.34E-03	AGT,CXCL13,LTC4S,PLA2G2D
transport of molecule	-1.22	-1.39	2.94E-03	AGT,CD8A,CGA,CYP2E1,EPHX1,GABRA1,ICOS,MX2,RSAD2,SLC14A1,VIP
secretion of molecule	-1.22	-1.41	<b>4.84E-04</b>	AGT,CD8A,CGA,CYP2E1,ICOS,RSAD2,VIP
flux of Ca <sup>2+</sup>	0.91	0.54	<b>6.80E-04</b>	AGT,CD8A,CXCL13,ICOS,VIP
cellular homeostasis	-0.29	-0.66	<b>4.68E-06</b>	AGT,CD8A,CXCL13,CYP2E1,GABRA1,GZMA,ICOS,LAG3,MTNR1B,PYGM,RSAD2,SCARA5,SLC14A1,TNFRSF10B,VIP
production of reactive oxygen species	-0.09	-0.60	2.35E-03	AGT,CXCL9,CYP2E1,GZMA,VIP
ion homeostasis of cells	0.25	-0.25	<b>9.97E-06</b>	AGT,CD8A,CXCL13,GABRA1,ICOS,PYGM,SCARA5,SLC14A1,VIP
quantity of cells	0.42	-0.18	<b>7.95E-04</b>	AGT,CD8A,CGA,CXCL13,GABRA1,ICOS,IGJ,IGLL1/IGLL5,LAG3,LTC4S,PLA2G2D,SLC14A1,VIP
quantity of blood cells	0.78	0.14	<b>1.32E-04</b>	AGT,CD8A,CXCL13,ICOS,IGJ,IGLL1/IGLL5,LAG3,LTC4S,PLA2G2D,SLC14A1,VIP
mobilization of Ca <sup>2+</sup>	0.18	-0.46	<b>2.74E-04</b>	AGT,CD8A,CXCL9,CXCL13,NPSR1,VIP
quantity of leukocytes	0.58	-0.07	<b>2.09E-04</b>	AGT,CD8A,CXCL13,ICOS,IGJ,IGLL1/IGLL5,LAG3,LTC4S,PLA2G2D,VIP
quantity of lymphocytes	0.98	0.26	<b>1.31E-04</b>	AGT,CD8A,CXCL13,ICOS,IGJ,IGLL1/IGLL5,LAG3,PLA2G2D,VIP
quantity of T lymphocytes	0.02	-0.85	3.46E-03	AGT,CD8A,ICOS,LAG3,PLA2G2D,VIP
proliferation of lymphocytes	1.97	1.03	3.33E-03	BTNL9,CD8A,ICOS,IGLL1/IGLL5,LAG3,TNFRSF10B,VIP
quantity of Ca <sup>2+</sup>	0.99	0.01	4.55E-03	AGT,CD8A,CXCL9,CXCL13,VIP
<b>synthesis of fatty acid</b>	-0.14	-1.14	7.55E-03	AGT,CYP2E1,LTC4S,PLA2G2D
<b>stimulation of cells</b>	0.88	-0.13	<b>8.85E-04</b>	AGT,BTNL9,CD8A,ICOS,VIP
<b>expansion of T lymphocytes</b>	1.70	0.27	<b>3.88E-04</b>	BTNL9,ICOS,LAG3,VIP
<b>concentration of fatty acid</b>	-0.28	-1.94	5.64E-03	AGT,CYP2E1,LTC4S,VIP

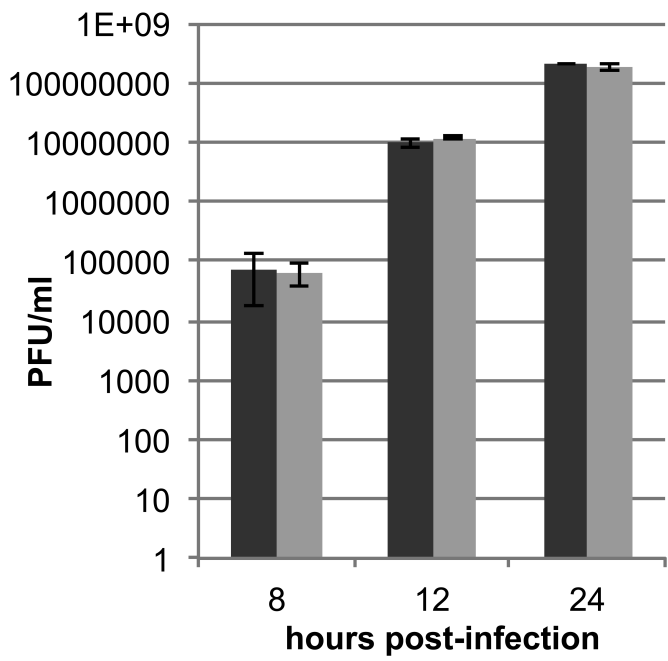
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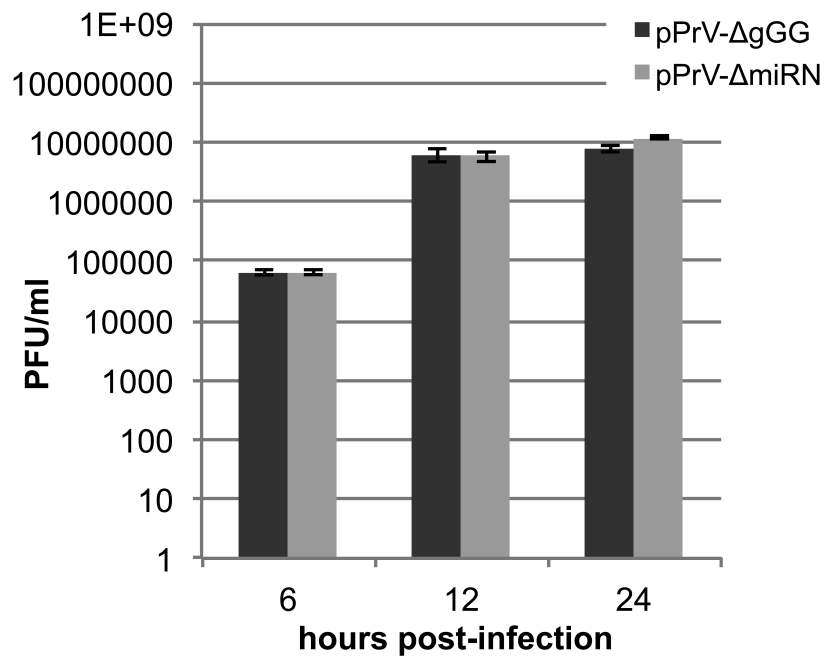
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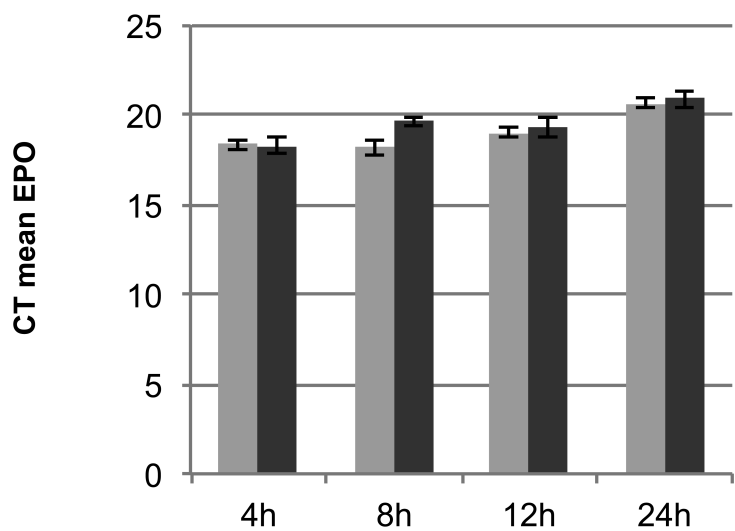
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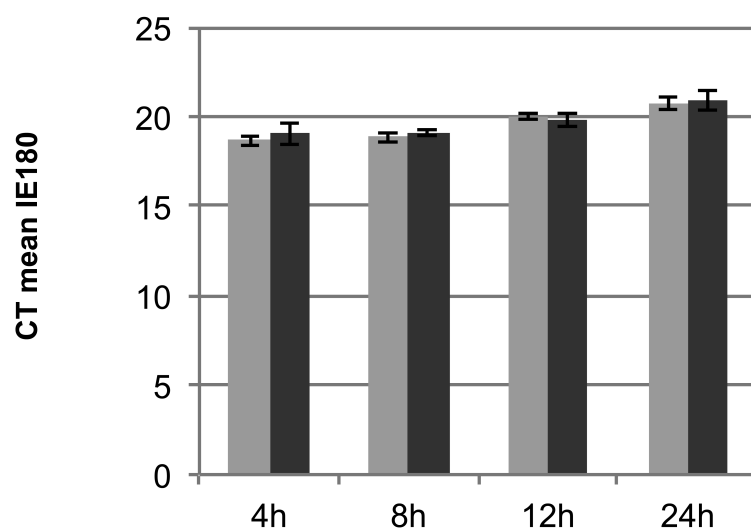
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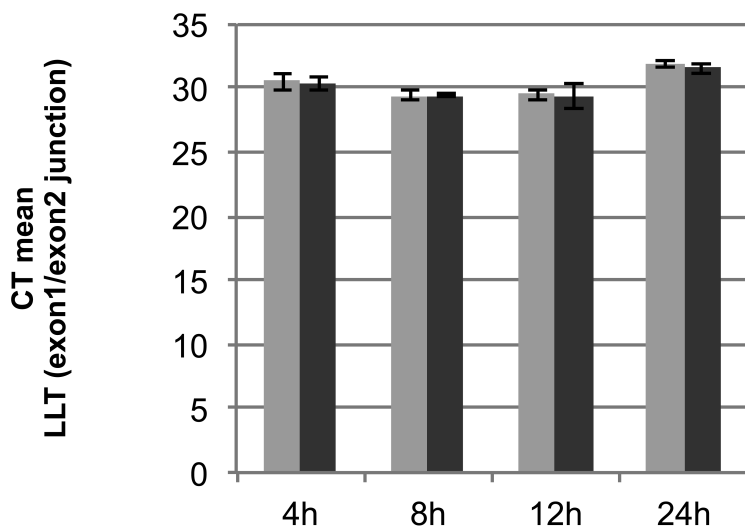
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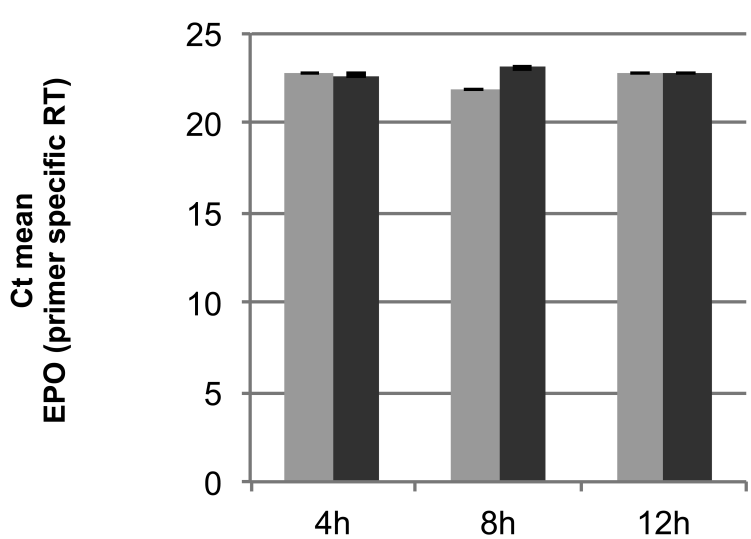
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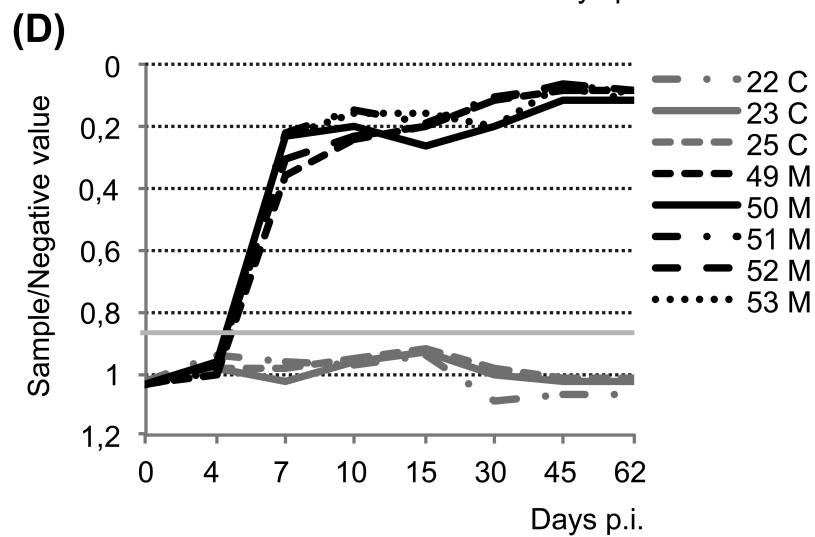
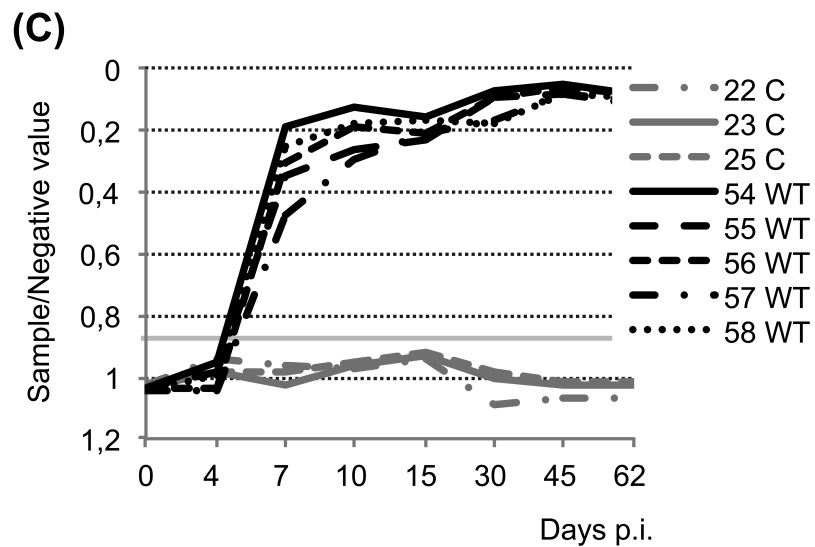
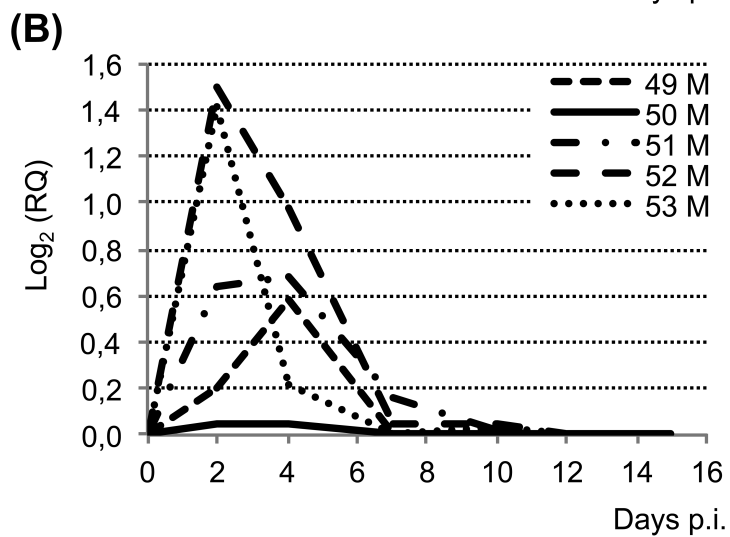
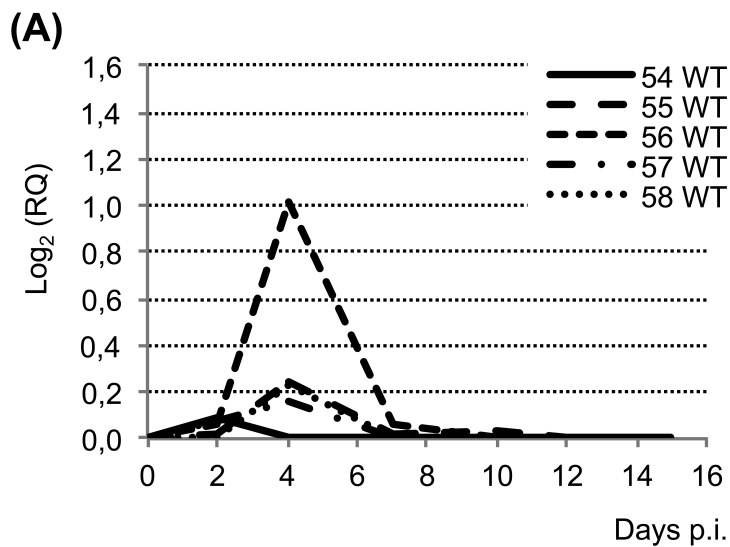


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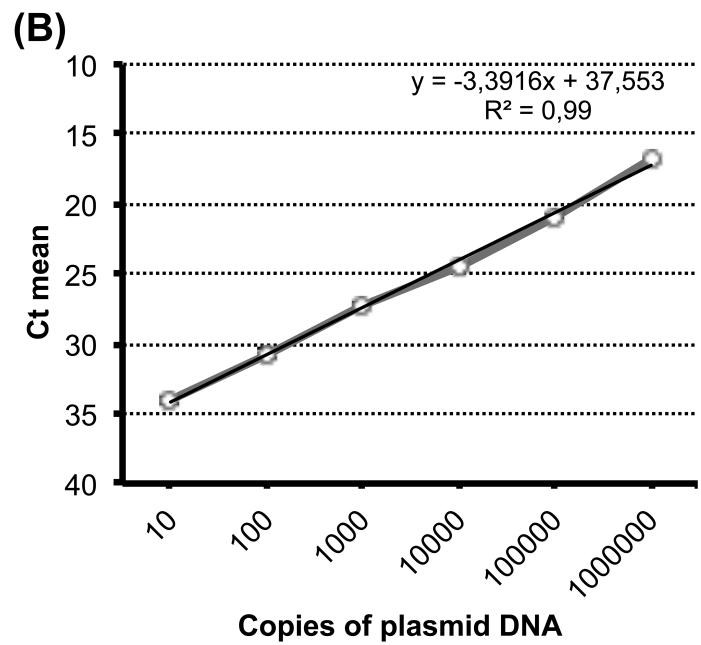
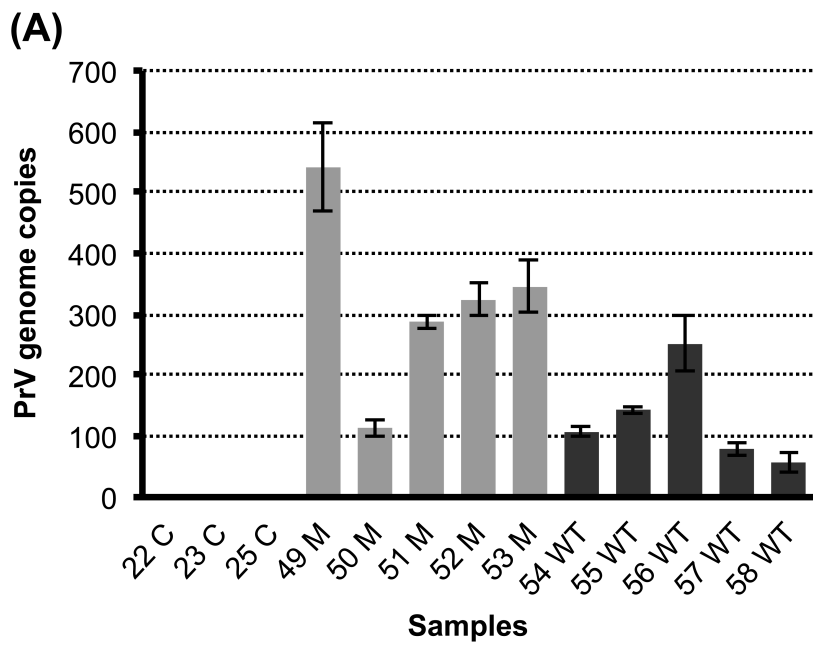


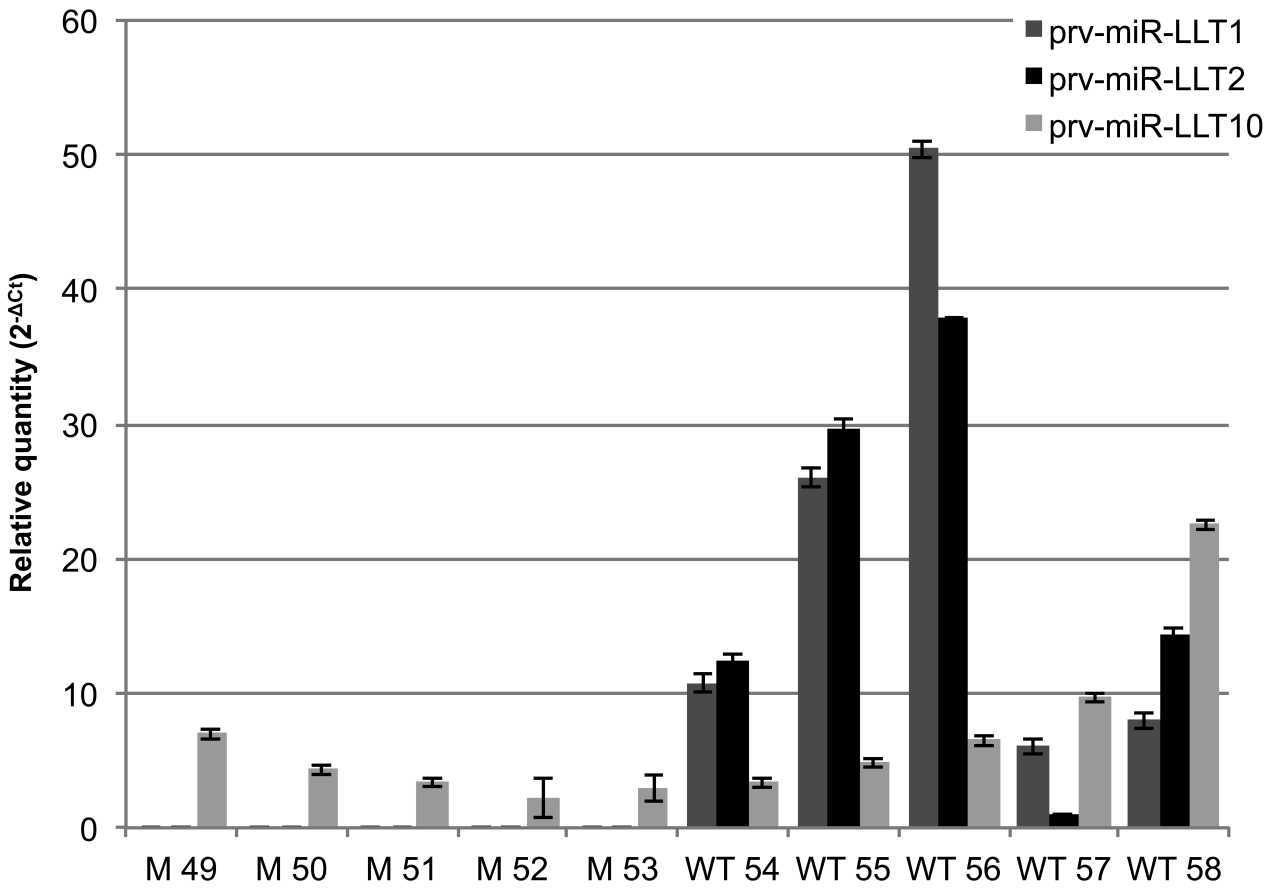
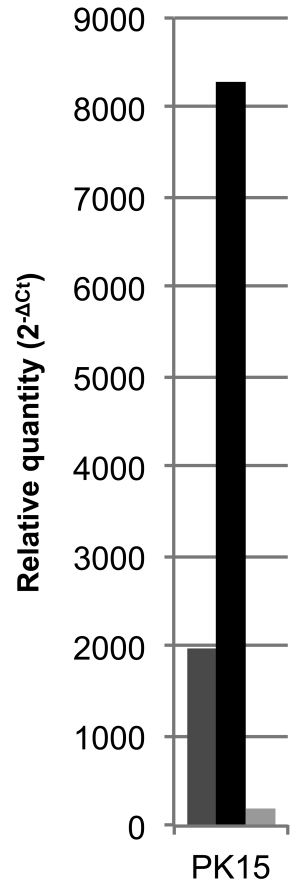
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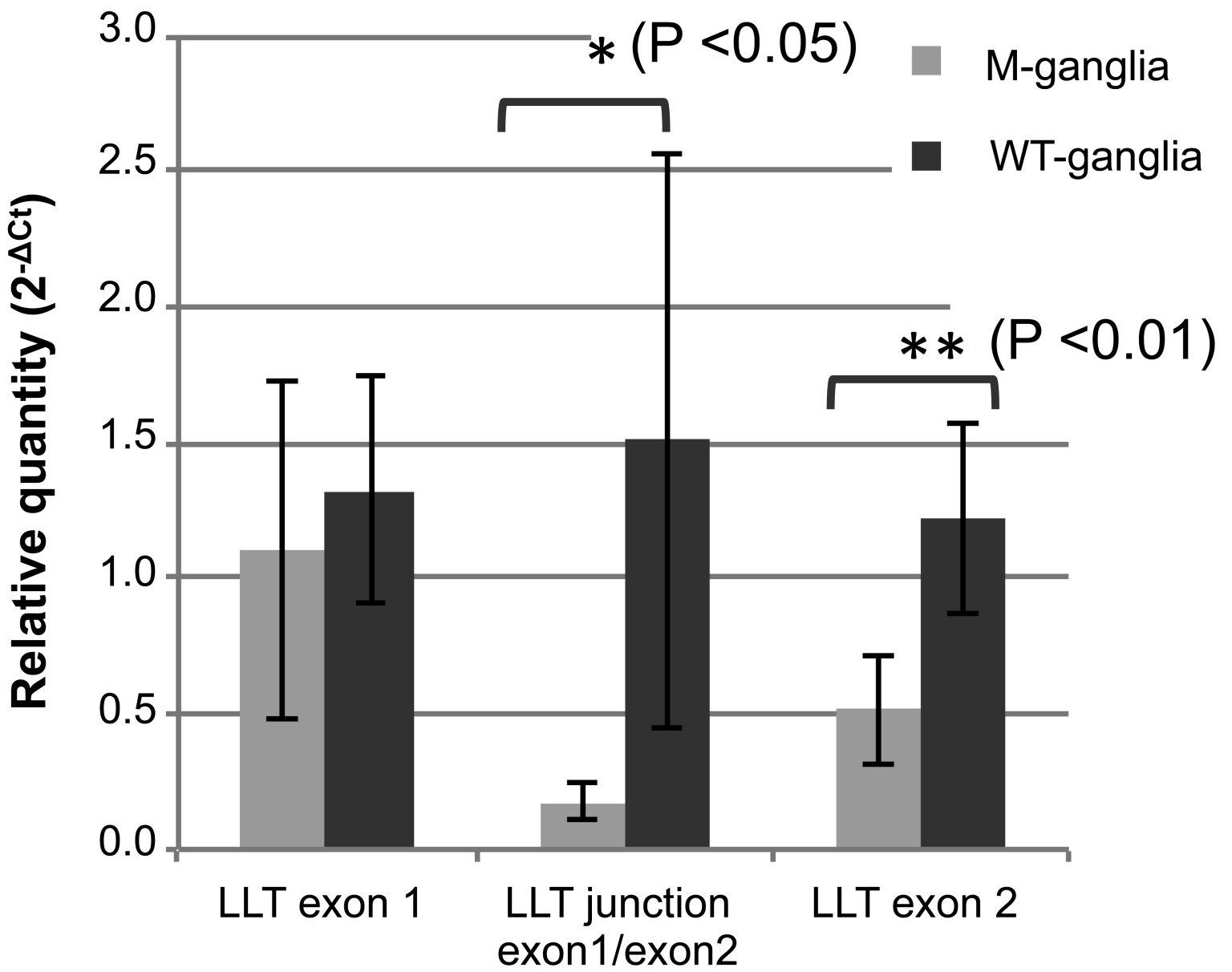








**(A)****(B)**



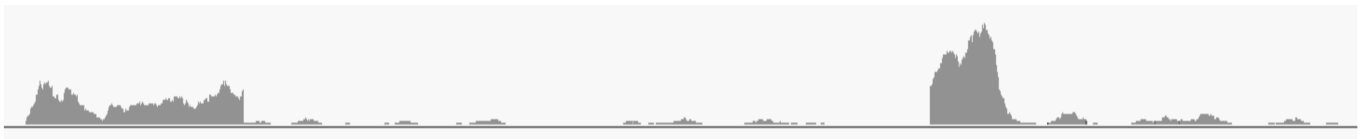
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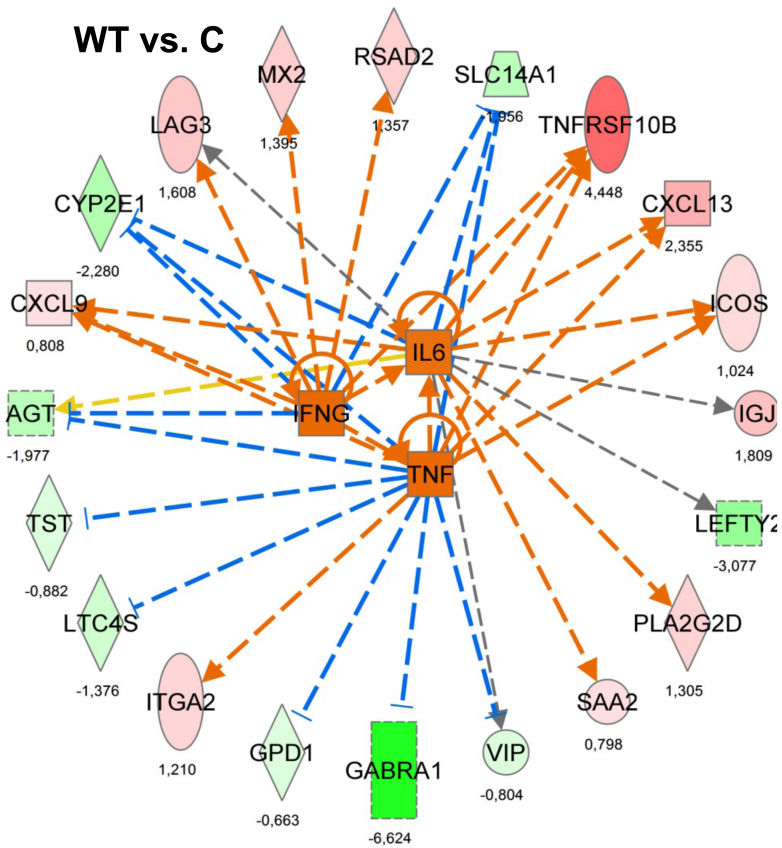
M



WT



### WT vs. C



### M vs. C

