[redacted]

From: Heidi Drumme [redacted]
Sent: Tuesday, 3 September 2019 4:11 PM
To: Joshua Hayward
Cc: mary.tachedjian; Kohl, Claudia; Adam Johnson; Dearnley, Megan (AAHL, Geelong AAHL); Brianna Jesaveluk; Christine Langer; philip-daniel.solymos [redacted] Hille, Georg; Andreas; Cecilia Sanchez; Adam Werner; dimitri.kontos [redacted] garycrameri [redacted] Andy Poumbourios; Glenn.Marsh; Michelle Baker; Edward Holmes; Wang Linfa; <Ina.Smith [redacted] Gilda Tachedjian
Subject: Re: HPG Bat Retrovirus Manuscript
Attachments: HPG paper Manuscript Final v2 HD.docx; HPG paper - SI Appendix Final v2 _HD.docx

Attachments Removed

Hi Joshua and all authors,
Great job from everyone to pull all this work together. Here are a few more comments on the latest version.
Cheers and good luck
Heidi

[redacted]
Duplicate Email - Removed

s22

From: Joshua Hayward s22
Sent: Tuesday, 3 September 2019 7:11 PM
To: Heidi Drummer
Cc: mary.tachedjian; Kohl, Claudia; Adam Johnson; Dearnley, Megan (AAHL, Geelong AAHL); Brianna Jesaveluk; Christine Langer; philip-daniel.solymosi s22 Hille, Georg; Andreas; Cecilia Sanchez; Adam Werner; dimitri.kontos s22 garycrameri s22 Andy Poumbourios; Glenn.Marsh; Michelle Baker; Edward Holmes; Wang Linfa; <lna.Smith s22 Gilda Tachedjian
Subject: Re: HPG Bat Retrovirus Manuscript

Hi Heidi,
Thank you for that!
Cheers,
Josh

Joshua Hayward PhD

Research Officer
Retroviral Biology and Antivirals Research Laboratory

Burnet Institute
Disease Elimination and Maternal & Child Health Programs

s22

GPO Box 2284, Melbourne, Victoria, Australia 3001

s22

Duplicate Email - Removed

[redacted]

From: Joshua Hayward [redacted]
Sent: Thursday, 5 September 2019 12:46 PM
To: mary.tachedjian; Kohl, Claudia; Adam Johnson; Dearnley, Megan (AAHL, Geelong AAHL); Brianna Jesaveluk; Christine Langer; Solymosi, Philip; Hille, Georg; Andreas; Cecilia Sanchez; Adam Werner; dimitri.kontos [redacted] garycrameri [redacted] Heidi Drummer; Andy Poumbourios; Glenn.Marsh; Michelle Baker; Edward Holmes; Wang Linfa; <Ina.Smith [redacted]; Gilda Tachedjian
Subject: HPG Paper - Fwd: PNAS MS#2019-15400 Submitted
Attachments: HPG paper - Manuscript and SI v1.pdf

Dear all,
I am happy to report that the HPG manuscript has been submitted! The submitted single-document manuscript/SI is attached.
Best regards,
Josh

Joshua Hayward PhD

Research Officer
Retroviral Biology and Antivirals Research Laboratory

Burnet Institute
Disease Elimination and Maternal & Child Health Programs

[redacted]
GPO Box 2284, Melbourne, Victoria, Australia 3001
[redacted]

---------- Forwarded message ---------
From: **Gilda Tachedjian** [redacted]
Date: Thu, 5 Sep 2019 at 12:30
Subject: Fwd: PNAS MS#2019-15400 Submitted
To: Joshua Hayward [redacted]

Can you please forward this email to all authors along with the submitted manuscript attached for their records

Begin forwarded message:

From: journalstaff@pnascentral.org
Subject: PNAS MS#2019-15400 Submitted
Date: 5 September 2019 at 12:25:45 pm AEST
To: gildat [redacted]
Cc: gtachedjian [redacted]
Reply-To: pnas@nas.edu

September 4, 2019

Dear Dr. Tachedjian,

Thank you for your submission to PNAS. Your Direct Submission was received and will be processed within 24–48 hours. You will receive an email once the staff have confirmed everything is in order or letting you know if any changes are necessary.

Thank you for submitting to PNAS.

Sincerely yours,

s22

SI Appendix

Contents

Supplementary Methods

Ethics

Fieldwork in all locations and on all occasions was approved under the following permits: in Queensland, the (then) Department of Employment, Economic Development and Innovation Animal Ethics Committee (AEC) Permit SA 2011/12/375 and 1710 the Environmental Protection Agency/Department of Environment and Resource Management Scientific Purposes Permits WISP14939514, WISP05810609, and WISP14100614; in New South Wales, The University of Sydney AEC Permit 04/3 2011/1/5498, the Elizabeth Macarthur Agricultural Institute AEC Permit M11/15, the Office of Environment and Heritage AEC Permit 120206/02, and the Office of Environment and Heritage Scientific Licenses SL100086 and SL 100537.

Sample collection along east coast of Australia and Daintree Rainforest

To assess Australian bats for the presence of unidentified viruses, samples including scat, blood, urine, and oral swabs were collected from multiple species of bats, including 373 bats across Hervey Bay, Boonah, Byron Bay, Alstonville, Redcliffe, and Nambucca Heads, and 106 bats in the Daintree rainforest, between 2007 and 2014. Bats were macroscopically identified. To confirm the species of origin of each sample, nucleic acids were extracted as described below in supplementary methods section "RT-qPCR for presence of Gammaretroviral nucleic acids in bat samples", and a cytochrome B gene TaqMan PCR assay for species determination was performed, as described in (1).

Metagenomic analysis of viral nucleic acid in bat samples

For samples collected in the Daintree rainforest, total RNA was extracted with the QIAamp viral RNA Mini Kit (QIAGEN) and DNA was digested using the TURBO DNA-free kit (Thermo Fisher Scientific), all according to the manufacturer's protocol. Other samples were processed as follows: Briefly, PBS homogenized bat feces was enriched for viral particles using a discontinuous sucrose gradient (2, 3). Total RNA was extracted with the QIAamp viral RNA Mini Kit (QIAGEN) except carrier RNA (poly-A) was omitted from Buffer AVL and genomic DNA was removed with DNase I digestion prior to RNA extraction as previously described (2). Random RT-PCR amplification and double-stranded cDNA was prepared as previously described (2) except K8N random primers were replaced with (5' GTTTCCCAGTAGGTCTCNNN NNNNN-3') for cDNA synthesis and 5'-A*G*C*A*C TGTAGGTTTCCCAGTAGGTCTC-3' for double-stranded cDNA amplification (4). Sequencing

libraries were generated using Illumina Nextera-XT library construction, sequencing was performed on the Illumina MiSeq platform, and bioinformatics including FASTQ paired-end read quality control and *de novo* assembly was performed as described previously (3). KoRV-related viral contigs were identified by BLASTn and BLASTx analysis using the assembled contigs as query sequences against the NCBI nucleotide collection database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) using default parameters.

Isolation and assembly of the HPG genome sequence

Partial HPG sequences were initially identified in the metagenomic analysis of a *P. alecto* scat sample obtained in Hervey bay, in 2011, using the method described above in "Metagenomic analysis of viral nucleic acid in bat samples", employing random RT PCR amplification. To generate the complete HPG genome sequence we used a modified single-cell whole transcriptome amplification (WTA) procedure for detecting ultra-low-copy viral RNA, and *de novo* sequence assembly pipeline.

Total RNA was purified from the same fecal sample and using the same procedure as described in the initial metagenomics study with the exception that DNase I digestion was performed after extraction of total RNA with the QIAamp viral RNA mini kit (QIAGEN) and final purified total RNA was eluted in a total volume of 20 µL. Concentration of both DNA and total RNA was determined with the Qubit HS DNA and HS RNA assays (Invitrogen) read on the Qubit 3.0 fluorimeter (Invitrogen) and was below the level of detection for both assays, < 0.5 ng/mL and < 20 ng/mL, respectively.

Eight microliters of purified total RNA was converted to cDNA, ligated, and then isothermally amplified using the REPLI-g WTA Single Cell kit (QIAGEN), according to the manufacturer's protocol, except the amplicons were purified using the Genomic DNA Clean and Concentrator-10 kit (Zymo Research, Irvine, USA). Briefly, 60 µL of amplified cDNA was diluted in a total volume of 100 µL with 40 µL of 10 mM Tris pH 8.5 (QIAGEN), to which was added 200 µL of DNA binding buffer and processed according to the manufacturer's protocol. Purified cDNA was eluted sequentially with 20 µL and 15 µL of 70°C pre-heated 10 mM Tris pH 8.5 (QIAGEN) buffer for 2 min prior to elution. The total amount of amplified product was 3.15 µg (89.95 ng/µL), as determined with the Qubit dsDNA BR Assay Kit (Invitrogen).

Dual indexed libraries were prepared according to the Illumina Nextera XT DNA Library Prep Kit (Illumina). Library concentration was determined with the Qubit HS dsDNA assay (Invitrogen). Library quality and distribution was determined by loading 3 ng of sample on an Agilent Technology 2100 Bioanalyzer using the Agilent High Sensitivity DNA assay.

Libraries were normalized, denatured then diluted to a final concentration of 10 pM with HT1 buffer (Illumina) and spiked with 1% PhiX control library (Illumina). Libraries were sequenced on the Illumina MiSeq platform, using the MiSeq Reagent v2 kit (300 cycles), generating 150 bp paired end reads.

Illumina FASTQ paired-end reads (8,162,956) were imported into CLC Genomics Workbench v10.1.1 using default Illumina import parameters, and then trimmed for size, quality, and ambiguous bases using default parameters except for the following: Quality Limit = 0.01, Ambiguous limit = 2 and Minimum number of nucleotides in reads = 30. Host reads (*Pteropus alecto* draft genome assembly GenBank assembly accession GCA_000325575.1 and mitochondrion Genbank accession NC_023122) were removed by read mapping using default settings on the CLC Genomics Grid Worker v7.0.1 except "Length" and "Similarity" fractions were both set to 0.9.

Host subtracted, trimmed, FASTQ paired end reads (6,836,522) were imported into Geneious v10.2.2 with "Read Technology" set to Illumina and the default paired end insert size selected (500 nt). Reads were error corrected and normalised with the Kmer-based tool, BBNorm v37.25 (https://sourceforge.net/projects/bbmap/), using default settings except "Minimum Depth" normalization was increased from 6 to 40. Normalized paired-end reads (295,939) were *de novo* assembled using default settings for SPAdes v3.10.0 (5, 6) selecting the "Multi Cell" Data Source option with error correction. The *de novo* assembled contig (8,040 bp) was verified by mapping trimmed reads to obtain the final genome sequence (8,030 bp).

Identification and assembly of KoRV related viruses in publicly available databases

To identify KoRV-related gammaretroviruses in public databases, data from the Sequence Read Archive (SRA) derived from bat RNA and DNA were subjected to SRA-BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&BLAST_SPEC=SRA&LINK_LOC=blasttab). The genome sequences of the assembled Hervey pteropid gammaretrovirus (HPG) was used as the query sequence. The algorithm parameters set were to: Program = blastn, Max target sequences = 1000, Expect threshold < $1x10^{-10}$, word size = 11, match score = 2, mismatch cost = -3, gap costs = existence 5 extension 2, no filtering or masking. SRA that contained reads aligning to the query sequences were from the Chinese microbats *Rhinolophus hipposideros* (Genbank: SRX1059482 & SRX1059481) and *Hipposideros larvartus* (Genbank: SRX1059446). Sequencing reads aligning to the query sequences were downloaded and assembled into the partially complete genomes of RhGRV and HlGRV as follows: Reads were downloaded and assembled using the CLC Genomics Workbench 11.0 (QIAGEN, Aarhus, Denmark)

"Assemble Sequences" tool into a contiguous consensus sequence using the following parameters: Minimum aligned read length = 20, alignment stringency = high, conflicts = Vote (A, C, G, T). Assembled contigs were subsequently used as a new query in an otherwise identical BLASTn search against the same SRA. This process was iteratively repeated until all contigs could be extended out until they overlapped with each other or reached a region of zero read coverage. The extended and overlapping contigs were assembled by alignment against the reference/query HPG genome sequence in CLC Genomics Workbench.

Annotation of Retroviral Genomes

Bat retroviral genome sequences were annotated using CLC Genomics Workbench by alignment using MUSCLE, and comparison against the genomes of KoRV-A (Genbank: AF151794) and M-MLV (Genbank: NC001501).

HPG specific analysis of Pteropid genomes

To determine whether HPG could be identified as an endogenous retrovirus within the genomes of Pteropid bats, we performed *in vitro* and *in silico* analyses. For the *in silico* analysis, we performed a BLAST analysis using CLC Genomics Workbench, of the genomes of *Pteropus alecto* (Genbank: PRJNA232518) and *P. vampyrus* (Genbank: PRJNA275879) using the HPG genome as the query sequence, with the algorithm parameters: Expect threshold = $1x10^{-10}$; word size = 11; Low complexity regions filtered.

For the *in vitro* analysis, two sources of *P. alecto* genomic DNA were analyzed by PCR for the presence of HPG. The first source of *P. alecto* genomic DNA was extracted from pooled heart and muscle tissue of a male bat captured in Brisbane QLD, November 2008. DNA was extracted using the QIAGEN Gentra Puregene Tissue Kit (QIAGEN) and further purified with the MO BIO Powerclean DNA clean up kit (MO BIO, Carlsbad, USA) and then AMPure XP beads (Beckman Coulter, Brea, USA), all of which were performed according to the manufacturer's protocol. The second source of *P. alecto* genomic DNA was extracted from a primary kidney (PaKi) cell line (7) using the QIAamp DNA Mini kit (QIAGEN), following the manufacturer's protocol.

HPG positive controls were derived from two regions within HPG [1.34 & 1.55 kb in length (Supplementary Figure 3), which were identified in the NGS metagenomics analysis described above in "Metagenomic analysis of viral nucleic acid in bat samples". The two sequences were amplified by PCR with the QIAGEN HotStar HiFidelity polymerase

according to the manufacture's protocol from random RT PCR amplified bat scat sample used for the initial metagenomics NGS using primers designed to amplify each sequence (Supplementary Table 5). The two amplicons were cloned into the pCR4-TOPO (Invitrogen) vector using the Zero Blunt TOPO PCR cloning kit (Invitrogen) according to the manufacturer's protocol. Primer sensitivity tests were conducted to determine the template copy number required for a band to be present in this analysis using the 1.3 & 1.5 kb sequence primers (Supplementary Table 5) based on plasmid controls. Primers specific for the 1.3 & 1.5 kb HPG sequences were found to be sensitive to $1.4x10^8$ and $9.0x10^3$ copies, respectively.

Phylogenetic analysis

To determine the evolutionary relationships among KoRV-related gammaretroviruses, we performed phylogenetic analyses using aligned complete genome nucleotide sequences (Supplementary Table 2). Accordingly, a multiple sequence alignment of 19 complete genomes was performed using a combination of MAFFT (8) and MUSCLE algorithms (9). Following alignment, regions of ambiguous and uncertain alignment were removed using Gblocks (10). This resulted in final alignment of 6,925 nt that was used to infer evolutionary relationships. A phylogenetic tree of these data was estimated using the maximum likelihood (ML) method available in the PhyML program (11), assuming a GTR model of nucleotide substitution with a proportion of invariant sites (I) and a gamma distribution of among-site rate variation (Γ). To determine the robustness of each node, a bootstrap resampling analysis (1,000 replications) was performed using the same nucleotide substitution model. The *Mus caroli* ERV, McERV (Supplementary Table 2), sequence was used as an outgroup to root the tree.

Generation of HPG proviral sequence and synthesis of HPG proviral expression construct

To generate a synthetic HPG provirus *in silico*, with the 5' and 3' long terminal repeats (LTRs) necessary for retroviral gene expression, the HPG genome sequence was modified by copying the unique 5' (U5) region and inserting it immediately following the 3' terminal repeat (R) region, and copying the unique 3' (U3) region and inserting it immediately prior to the 5' terminal R region. The HPG proviral sequence was chemically synthesized (GenScript, Nanjing, China) and inserted within the pCC1BAC cloning plasmid (GenBank: EU140750) at the *Eco*RI 333 site, generating the pCC1-HPG proviral expression construct.

SI Appendix

Cell cultures

Human embryonic kidney (HEK) 293T cells (kindly provided by Richard Axel, Columbia University), human epithelial cervical adenocarcinoma (HeLa) cells (NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH), NIH Swiss mouse embryo (NIH/3T3) fibroblast cells (American Type Culture Collection), a *P. alecto* kidney (PaKi) cell line (7), and HEK cells that express M-MLV Gag and Pol polyproteins (GP2-293 cells; Takara Bio) were utilized. 293T and HeLa cells were authenticated using the Promega GenePrint 10 system performed by the Australian Genome Research Facility (AGRF). The short tandem repeat (STR) profile was used to search the ATCC STR database https://www.atcc.org/en/STR_Database.aspx and the DSMZ-German Collection of Microorganisms and Cell cultures database https://www.dsmz.de/services/human-and-animal_cell-lines/online-str-analysis. All cell cultures were maintained at 37°C, 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific) supplemented with heat-inactivated fetal bovine serum (100mL/l; Invitrogen), glutamine (292 mg/mL; Invitrogen), and the antibiotics penicillin (100 units/mL; Invitrogen) and streptomycin (100 units/mL; Invitrogen), with the exception of the PaKi cells for which DMEM was substituted with DMEM/F-12 1:1 medium (Thermo Fisher Scientific).

Transfection of 293T cells for generation of HPG & M MLV viral particles

HPG and M-MLV viral particles were generated by transfection of 293T cells with the pCC1-HPG and pNCS (Addgene: 17362) plasmids, respectively. 293T cells were transfected at 50% confluency with 20 μg or pCC1-HPG or 10 μg of pNCS, using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's instructions. Untransfected cells and cells transfected with 20 μg of either the empty plasmid pCR2.1 (Thermo Fisher Scientific) or the empty plasmid pcDNA3.1 (Invitrogen), were used as controls. Transfected cells were incubated at 37°C, 5% CO_2 for 48 h, and then virion-containing supernatants were collected and clarified by centrifugation at 200 x g for 5 min. Virus production was determined by quantifying virion-associated RT activity, as previously described (12).

Electron Microscopy and viral particle morphology

HPG, M-MLV, and pcDNA3.1 transfected cell cultures were generated as described above, in "Transfection of 293T cells for generation of HPG & M-MLV viral particles", using Lipofectamine 2000 (Invitrogen). Untransfected cells were used as a control. For thin

section electron microscopy (EM), cells were pelleted and immersed in 2.5% glutaraldehyde in 0.1 M Sorenson's phosphate buffer pH 7.2, (300 mOsmol/kg) for 1 h at room temperature. After washing with Sorensen's phosphate buffer the cells were fixed with 1% osmium tetroxide for 1 h and dehydrated in a graded ethanol series at room temperature. Samples were embedded in Spurr's resin (ProSciTech, Australia) according to the manufacturer's protocol. Ultrathin sections were obtained using a Leica ultracut UCT Microtome and stained with saturated uranyl acetate in 50% ethanol and lead citrate. All prepared grids were examined using a Philips CM120 or JEOL JEM-1400 transmission electron microscope at 120kV. Electron micrographs representing negative control untransfected and mock transfected cells did not reveal the presence of viral-like particles (Supplementary Figure 8).

Reverse transcriptase divalent cation preference

To evaluate the divalent cation preference of HPG reverse transcriptase (RT), we performed a virion associated RT assay using the gammaretroviral RT co-factor, manganese (Mn^{2+}), and the lentiviral RT co-factor, magnesium (Mg^{2+}). We compared HPG RT activity to HIV-1 RT activity in the presence of each co-factor. 293T cells were co-transfected with different quantities of HPG (pCC1-HPG; 0.04 - 0.22 pmol), M-MLV (pNCS; 0.04 - 0.22 pmol), or HIV-1 (pNL4-3; 0.01 0.04 pmol), with the total mass of transfected plasmid DNA equalized by the addition of the empty expression vector pCR2.1 (Thermo Fisher Scientific). Cells transfected only with pCR2.1 (0.04 0.22 pmol) were used as controls. Transfections were performed using Lipofectamine 2000 according to the manufacturer's protocol. Transfected cell cultures were incubated at 37°C, 5% CO_2 for 48 h, and then virion-containing supernatants were collected and clarified by centrifugation at 200 x g for 5 min. To assess magnesium co-factor usage by the viral reverse transcriptase, we performed a virion-associated RT activity, as previously described (12). To assess manganese co-factor usage, the virion-associate RT activity assay was modified by the replacement of magnesium with 0.1M manganese.

Replication kinetics assay

To determine the cell tropism of HPG compared to ecotropic M-MLV, HPG and M-MLV transfected cell cultures were generated as described in the previous section "Transfection of 293T cells for generation of HPG & M-MLV viral particles". Virion-containing supernatants were normalized by virion-associated RT activity, using manganese as the cofactor, as described in (12) except with the above-mentioned modifications. Virion containing supernatants were used to infect human HeLa, mouse

3T3, and bat PaKi cells. Untransfected 293T cell culture supernatant was used as a control. Cells were seeded in a 96-well plate (Sarstedt, Nümbrecht, Germany) at a density of 15,000 cells/well. When cells reached ~50% confluency, the media was replaced with 225 µL of normalized HPG or M-MLV virion containing supernatant with the addition of DEAE-Dextran (Sigma-Aldrich) at a final concentration of 10 µg/mL. Cells were incubated for 6 h, and then the supernatant was removed, cells were washed twice with phosphate buffered saline (PBS), and 250 µL of DMEM medium was added. To assess cell culture supernatants for the release of viral particles, 20 µL samples were collected from the supernatant of each well at 24 h intervals for 5 days. To measure the presence of HPG or M-MLV virions in the cell culture supernatant samples, we performed a virion-associated RT activity assay.

Generation of gammaretroviral *env* gene expression constructs for pseudotyping

Cloning constructs pUC57-GALV-env and pUC57-KoRV-A env encoding the Envelope proteins of GALV (Genbank: KT724048) and KoRV-A (Genbank: NC039228), respectively, were chemically synthesized (GenScript). The Envelope sequences were enzymatically cut from the cloning plasmids using *Bam*HI and *Xho*I enzymes (New England Biolabs) and ligated into the mammalian expression vector pcDNA3.1 (Invitrogen), using T4 DNA ligase (New England Biolabs) following the manufacturer's protocols, generating the expression vectors pcD-GALV-env & pcD-KoRV-A-env. To generate an expression plasmid for the HPG Envelope protein, the HPG *env* gene was amplified from the pCC1-HPG plasmid using primers (HPG-env-F and HPG-env-R; Supplementary Table 5) designed to anneal upstream of the cytoplasmic accumulation element (13) and downstream of *env* stop codon. To facilitate directional cloning, *Eco*RI and *Xba*I restriction sequences were incorporated into the forward and reverse primers, respectively. The HPG *env* gene was amplified using the Phusion High-Fidelity PCR Kit (New England Biolabs) according to the manufacturer's instructions and using 50 ng of pCCI-HBPG template and 0.5 µM of each forward and reverse primer in a 20 µL reaction. The HPG *env* amplicon was ligated into the pcDNA3.1 vector using T4 DNA ligase (New England Biolabs) following the manufacturer's protocols, using the restriction enzymes *Eco*RI and *Xba*I (New England Biolabs), generating the expression plasmid pcD-HPG-env. The sequences of all expression plasmids were confirmed by Sanger sequencing. Expression plasmids for other Envelope proteins including VSV-G (pVSV-G), ecotropic MLV (pEco), 4070A amphotropic MLV (pAmpho), and 10A1 amphotropic MLV ('dualtropic' MLV, p10A1) were obtained from the Retro-X Universal Packaging System (Takara Bio).

SI Appendix

Generation of pseudotyped retroviruses and assay of host cell tropism

To determine the tropism of HPG in comparison to M-MLV, pseudotyped viral particles were produced using the Retro-X Universal Packaging System (Takara Bio). To generate viral particles pseudotyped with each gammaretroviral Envelope protein, GP2-293 cells that express M MLV Gag and Pol were transfected with the reporter vector, pQCLIN, and the Envelope expression vector, pVSV-G, pEco, pAmpho, p10A1, pcD-HPG-env, pcd-KoRV-A env, or pcD-GALV-env, to generate viral particles pseudotyped with Env derived from VSV-G, Ecotropic MLV, Amphotropic MLV, HPG, KoRV_A, and GALV, respectively. T75 tissue culture flasks (Nunc/Thermo Fisher Scientific) were seeded with $2.1x10^6$ GP2-293 cells. Cells were incubated for 16 h and then transfected with 5 µg of pQCLIN and 10 µg of the Env expression plasmid using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's protocol. Transfected cells were incubated for 48 h, and then viral particles were collected from clarified supernatants and concentrated using the Lenti-X Concentrator (Takara Bio).

Human HeLa and mouse 3T3 cells were seeded in 96-well plates (Nunc) at a density of 20,000 cells/well, and incubated for 16 h. Following incubation, equal volumes of pseudotyped viral particles containing DEAE-Dextran (Sigma Aldrich) at a final concentration of 10 µg/mL was added to the cells. Cells were incubated for 48 h and then viral cell entry was determined by the presence of blue cell-forming units as previously described (12).

RT qPCR for detecting the presence of gammaretroviral nucleic acids in bat samples

Nucleic acids from 50 µL PBS resuspended bat scat samples were extracted on a KingFisher Flex Purification System (Thermo Fisher Scientific) using the Applied Biosystems MagMAX-96 Viral RNA Isolation Kit (Thermo Fisher Scientific) according to manufacturer's protocol except final purified nucleic acids were eluted in 50 µL nuclease free water instead of 90 µL elution buffer. No DNase I digestion was performed. 'Broad' primers (HPG-rel-F and HPG-rel-R) were designed to bind to HPG, FFRV1, MmGRV, and SaGRV, in the region upstream of the *gag* gene. Primers specific for HPG (HPG gag F and HPG-gag-R) were designed to bind to within the *gag* gene. Sequences for all primers are provided in (Supplementary Table 5). The presence of viral nucleic acids was determined by reverse transcriptase quantitative PCR (RT-qPCR) using the Power SYBR Green RNA to-CT 1-Step Kit (Thermo Fisher Scientific), with a cycle threshold of 36. Reaction mixtures contained 4.5 µL of purified RNA, 200 nM of each primer, and RT enzyme mix. Reactions were performed in either 384- or 96-well plates on the QuantStudio 7 Flex qPCR machine (Thermo Fisher Scientific). To determine if nucleic acid amplification was from RNA or

DNA, an identical reaction was performed where the RT enzyme mix was excluded, to prevent amplification from RNA. Cycling conditions were as follows: 1x cycle of 48°C for 30 min, then 95°C for 10 min, and 40x cycles of 95°C for 15 s then 60°C for 1 min.

Generation of HPG Envelope protein for serological assays

A codon-optimized (*Homo sapiens*) synthetic gene, encoding the predicted HPG retrovirus Env ectodomain (Glu^{38}-Ser^{603}), was chemically synthesized (GeneArt, Regensburg, Germany). The synthetic gene incorporated an in-frame 5' *Nhe*I site, a C-terminal His_6 tag followed by a termination codon and 3' *Xba*I site. In addition, the putative SU-TM cleavage site, Arg^{473}LeuLysArg, was ablated by substitution with Ser^{473}LeuGlnSer. The synthetic gene was ligated downstream of the tissue plasminogen activator leader sequence in the pcDNA3-based vector, pcE2^{661}myc (14) to give pcHPG-Env^{603}. For expression, 293-F cells were transfected with pcHPG-Env^{603} using 293fectin (Thermo Fisher Scientific). At 24 h post-transfection, 0.5% (w/v) lupin peptone and 0.02% (w/v) pluronic F-68 were added to cells. Proteins were harvested following 3-5 days of incubation by centrifugation at 1,500 x *g* for 5 min, followed by filtration through a 0.45 μM filter before storage at 4°C. The Envelope protein was purified using Talon metal affinity resin (Takara) and 250 mM imidazole/PBS as the elution buffer. The protein was exchanged into PBS and concentrated using an Amicon centrifugal filter device (Merck Millipore, Burlington, USA). SDS-PAGE in the presence and absence of β-mercaptoethanol revealed a single diffuse band with a molecular weight range of ~80-90 kDa (Supplementary Figure 8), consistent with the molecular weight predicted from the amino acid sequence (62,805 Da) with 6 N-linked glycans (~ 18 kDa).

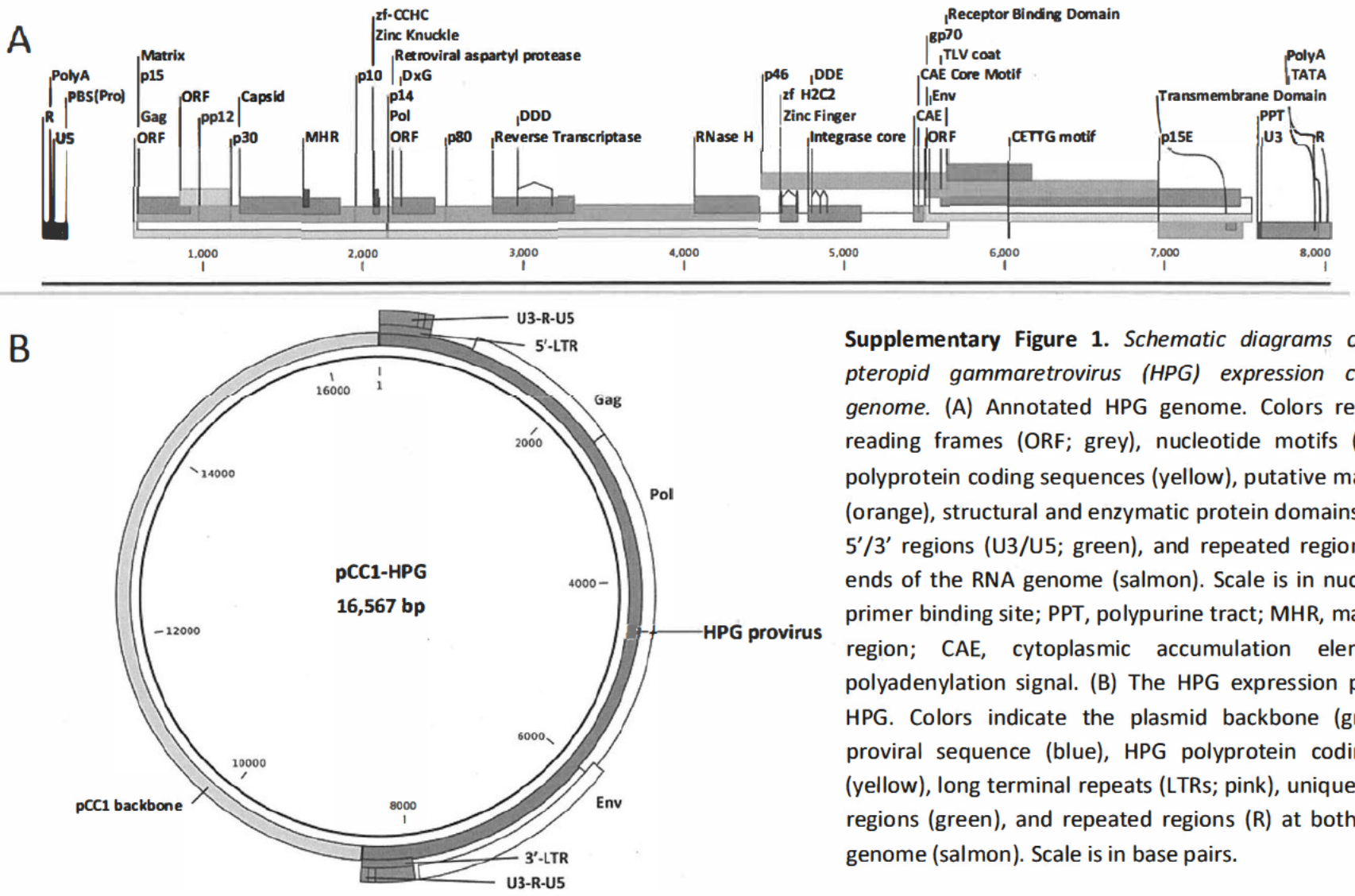
Generation of anti-HPG Envelope sera

Rabbit polyclonal anti-HPG-Envelope sera was generated by the Antibody Services at the Walter and Eliza Hall Institute Biotechnology Centre (Melbourne, Australia), using the HPG Envelope protein described above in "Generation of HPG Envelope protein for serological assays" as the antigen. Rabbits were immunized with 200 μg of the HPG Envelope protein three times with a 4-week interval between immunizations. Sera was collected over the course of 68 days and included the collection of pre-bleed sera as a control.

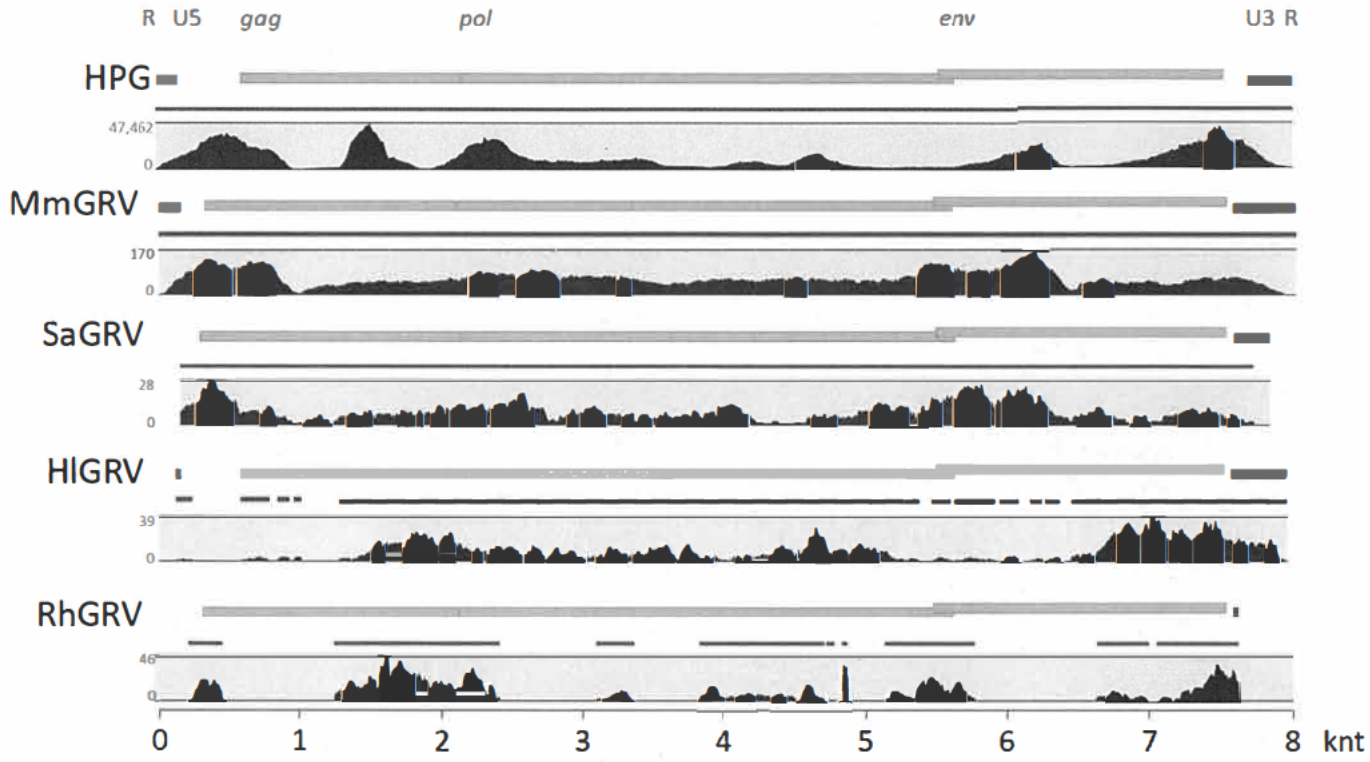
Serological assay for the presence of anti HPG antibodies in bats

To assess bat sera for the presence of anti-HPG-Envelope immunoglobin, 88 bat samples collected from the East coast of Australia and the Daintree rainforest between 2007 and 2014 were analyzed using a serological assay as previously described (15), with the following modifications: HPG Envelope gp120 proteins were coupled to magnetic Luminex beads (Thermo Fisher Scientific) using a mixture of Protein A/Protein G-biotinylated, and samples were analyzed using a Bio-Plex instrument (Bio-Rad). HIV SOSIP-Envelope proteins were utilized as a negative control (16). Previous studies published using this platform have used a threshold of at least three times the mean MFI of negative sera from other bat species with values below 250 MFI considered negative (17-20). The same principle was used here to establish a threshold based on an MFI of 250 corresponding to a negative sample with sample MFIs above 1000 considered positive.

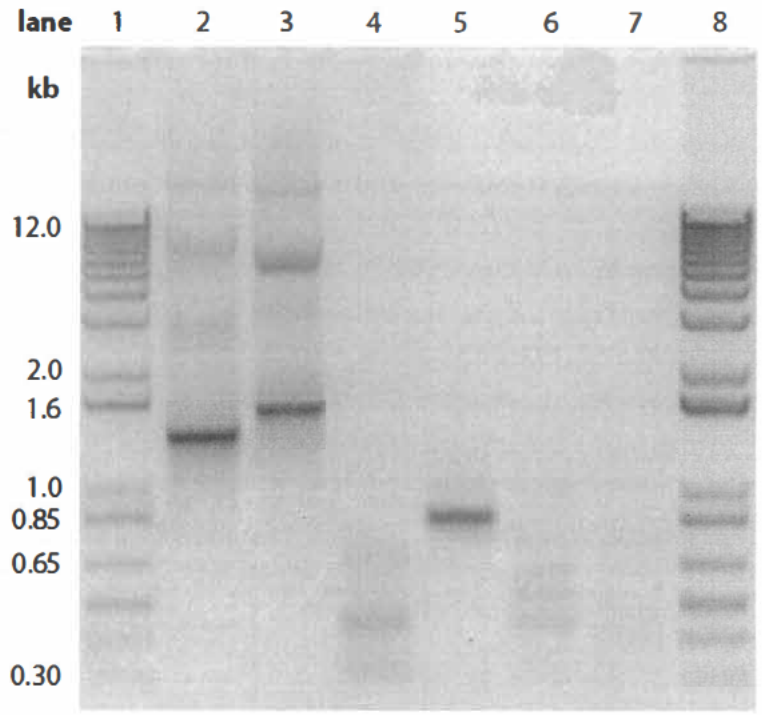
Supplementary Figures



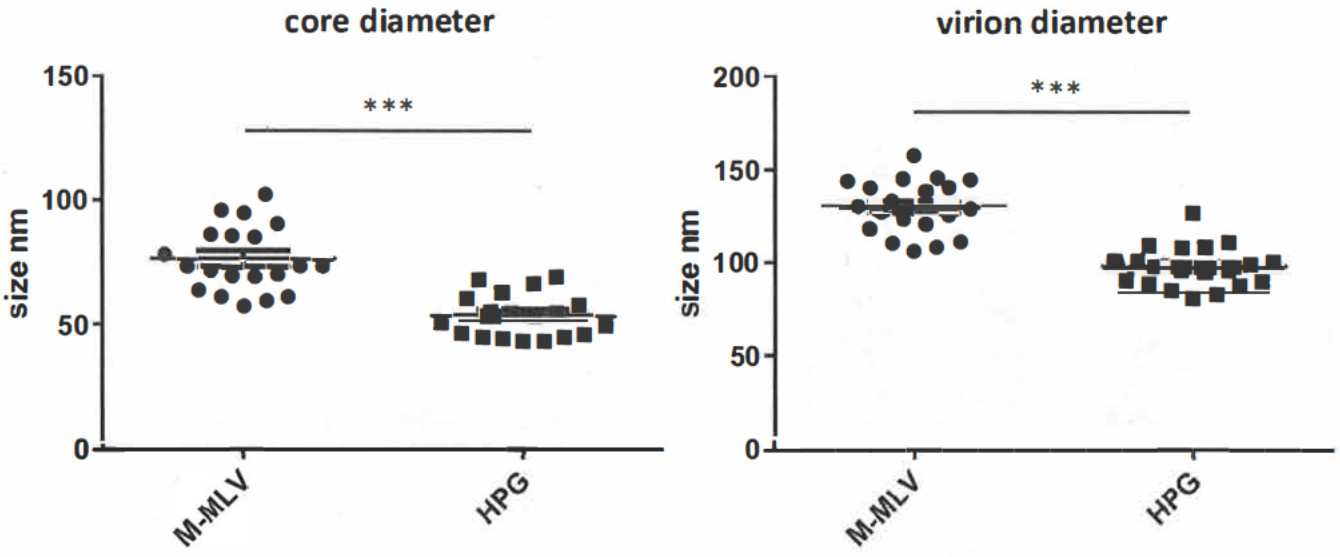
Supplementary Figure 1. *Schematic diagrams of the Hervey pteropid gammaretrovirus (HPG) expression construct and genome.* (A) Annotated HPG genome. Colors represent open reading frames (ORF; grey), nucleotide motifs (dark purple), polyprotein coding sequences (yellow), putative mature proteins (orange), structural and enzymatic protein domains (red), unique 5'/3' regions (U3/U5; green), and repeated regions (R) at both ends of the RNA genome (salmon). Scale is in nucleotides. PBS, primer binding site; PPT, polypurine tract; MHR, major homology region; CAE, cytoplasmic accumulation element; PolyA, polyadenylation signal. (B) The HPG expression plasmid pCC1-HPG. Colors indicate the plasmid backbone (grey), inserted proviral sequence (blue), HPG polyprotein coding sequences (yellow), long terminal repeats (LTRs; pink), unique 5'/3' (U3/U5) regions (green), and repeated regions (R) at both ends of RNA genome (salmon). Scale is in base pairs.



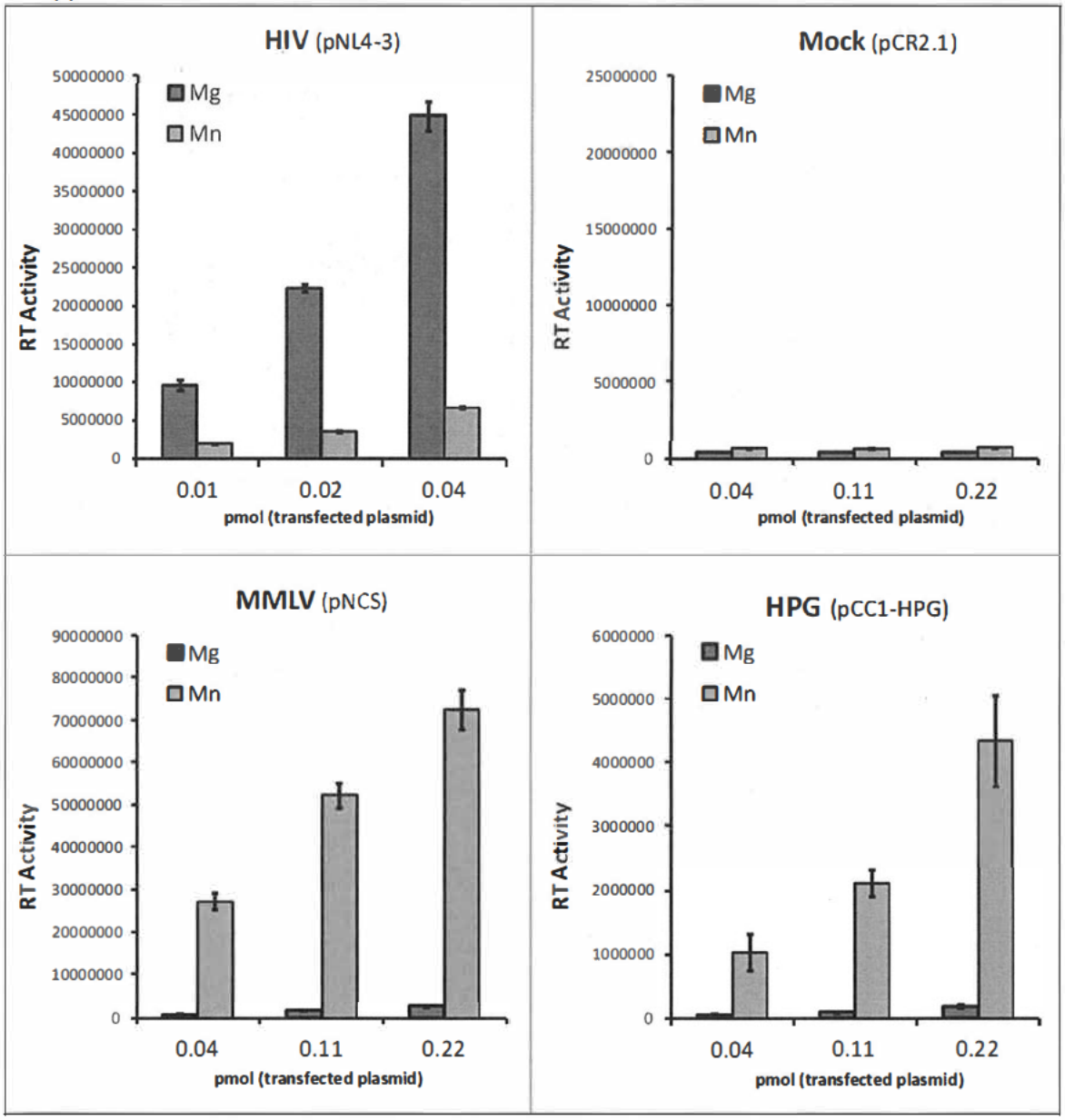
Supplementary Figure 2. *Sequencing read maps for KoRV-related retroviruses in Australian bats.* Colored bars indicate the genomic regions. Red: R, repeated regions at both ends of RNA genome; Dark blue: U5/U3, unique 5' & 3' regions; Light blue: retroviral genes *gag*, *pol*, and *env*. Black graphs represent the read coverage across the retroviral genomes, and gaps in the black lines above the graphs represent regions of zero coverage. Red numbers indicated the minimum and maximum read coverage. HPG, Hervey pteropid gammaretrovirus; MmGRV, Macroglossus minimus gammaretrovirus; SaGRV, Syconycteris australis gammaretrovirus; HlGRV, Hipposideros larvatus gammaretrovirus; RhGRV, Rhinolophus hipposideros gammaretrovirus.



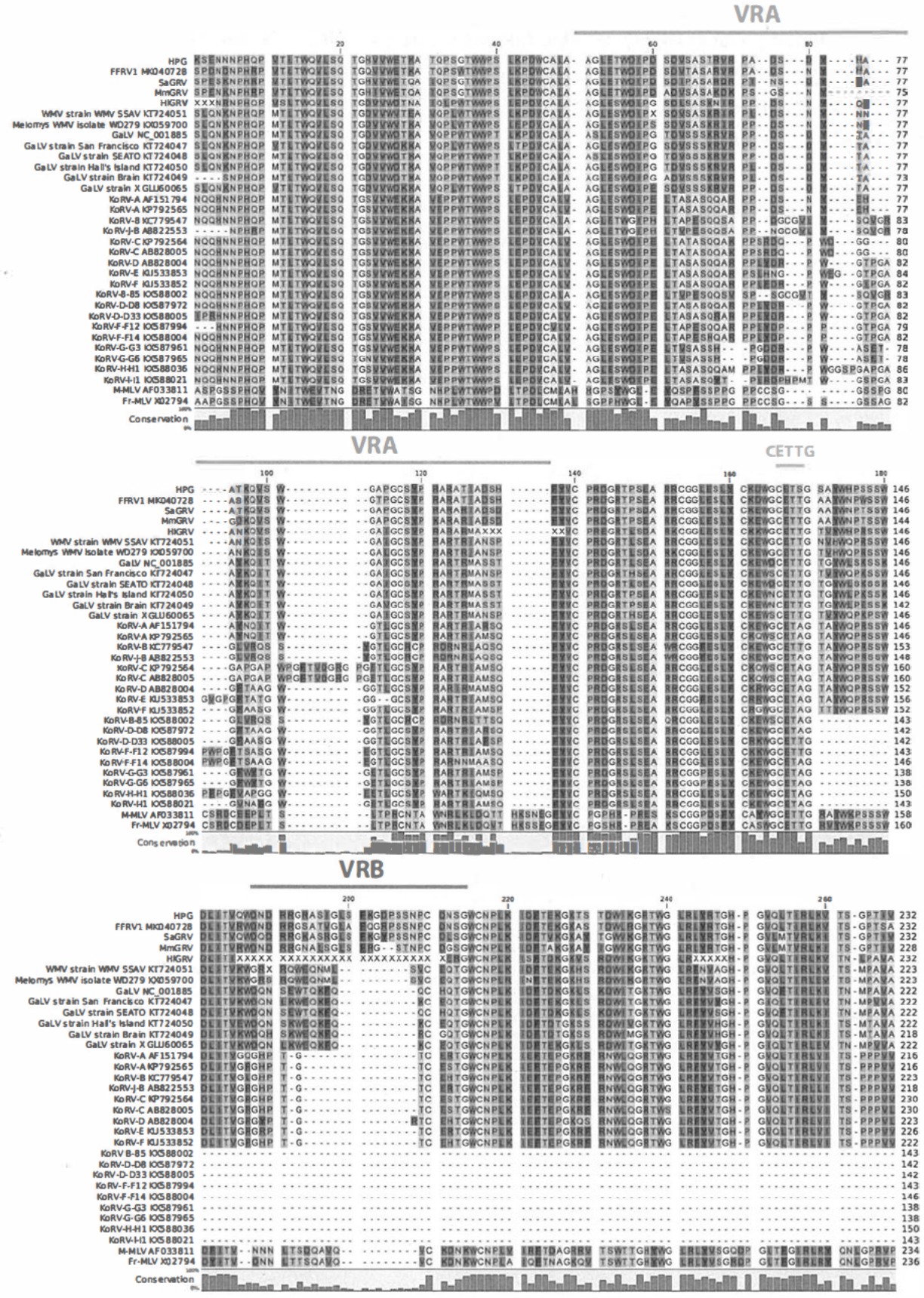
Supplementary Figure 3. *Assessment of the presence of endogenous Hervey pteropid gammaretrovirus (HPG) in the Pteropus alecto genome.* PCR primers (Supplementary Table 5) were used to amplify HPG DNA to generate HPG amplicons of 1.34 kb or 1.55 kb and were visualised on a 1% (w/v) agarose gel. Lane 1, 1 Kb Plus DNA Ladder; Lane 2, 80 ng of HPG plasmid was used as template to amplify a 1.34 kb HPG amplicon; Lane 3, 80 ng of HPG plasmid used as a template to amplify a 1.55 kb HPG amplicon; Lane 4, negative control where *P. alecto* genomic DNA (gDNA) was subjected to PCR using M13 primers (M13F/R, Supplementary Table 5); Lane 5, positive control for amplification of a single-copy gene, *APOBEC3Z3* (824 bp) using *P. alecto* gDNA as template; Lane 6, *P.alecto* gDNA subjected to PCR amplification using primers that generate the 1.34 kb segment of HPG; Lane 7, *P. alecto* gDNA subjected to PCR amplification using primers for the 1.55 kb segment of HPG; Lane 8, 1 Kb Plus DNA Ladder. Template gDNA was derived from *P. alecto* tissue and a kidney cell line. A representative gel using *P. alecto* kidney cell line gDNA as the template is shown.



Supplementary Figure 4. *The diameters of the virion core and virion of Hervey pteropid gammaretrovirus (HPG) and Moloney murine leukemia virus (M-MLV) viral particles.* Statistical significance was calculated using the Mann-Whitney test. ***p value < 0.001, N = 20.



Supplementary Figure 5. *Divalent cation preferences of Human immunodeficiency virus (HIV), Moloney murine leukemia virus (MMLV), and Hervey pteropid Gammaretrovirus (HPG) virion-associated reverse transcriptase.* Human or murine cell lines were transfected with infectious molecular clones of HPG, MMLV, and HIV (indicated in brackets), or the empty plasmid pCR2.1. Virions collected from the cell culture supernatant were assessed for virion-associated reverse transcriptase (RT) activity assay. Mg, magnesium; Mn, manganese. Error bars represent the standard error of the mean (SE, n = 3).



Supplementary Figure 6. *Multiple sequence alignment of the receptor binding domains of KoRV related viruses.*

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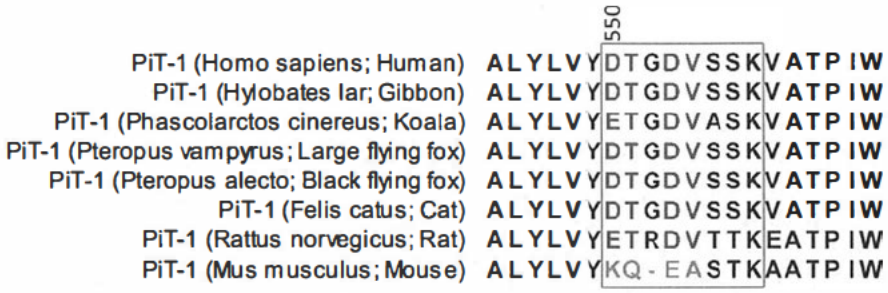
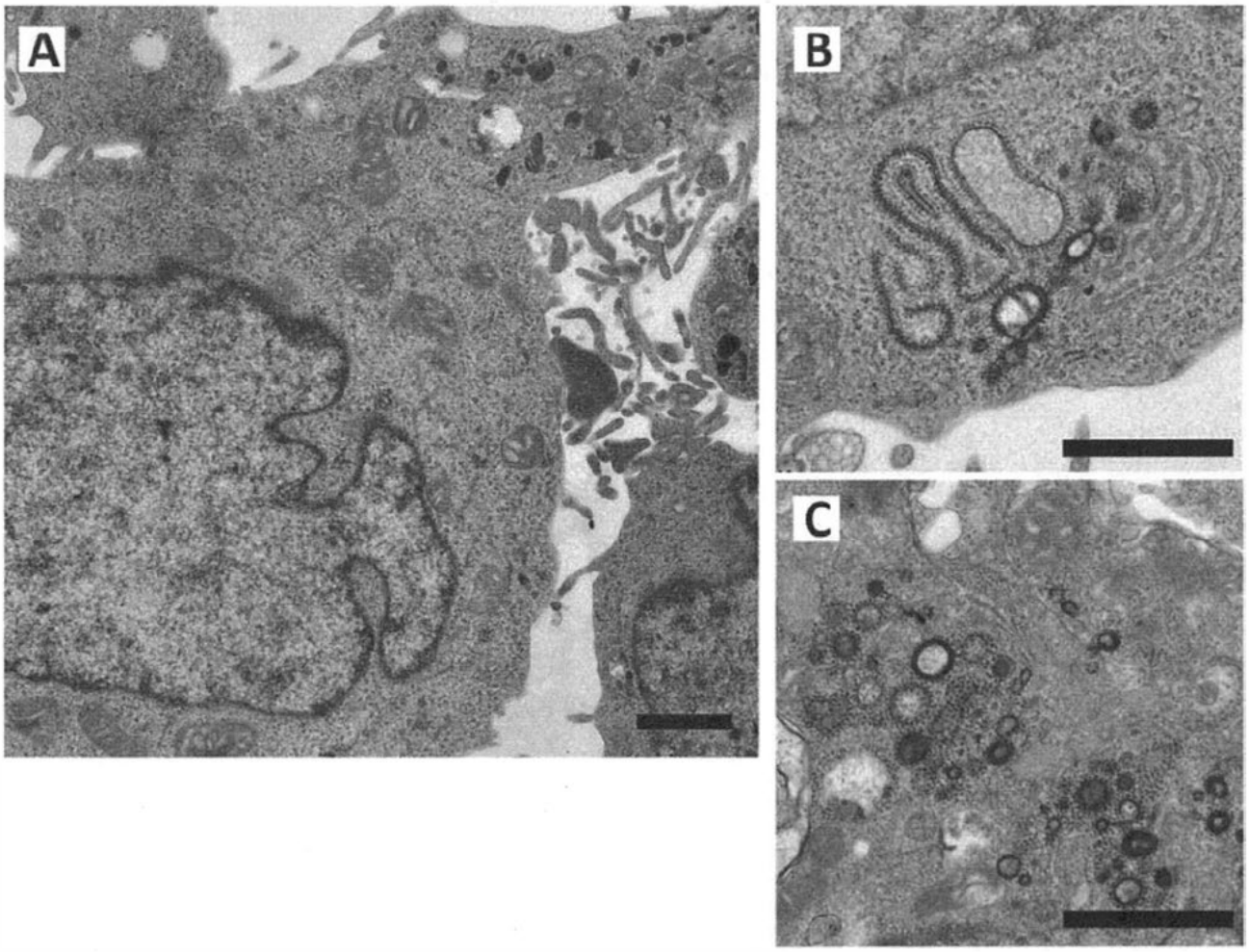
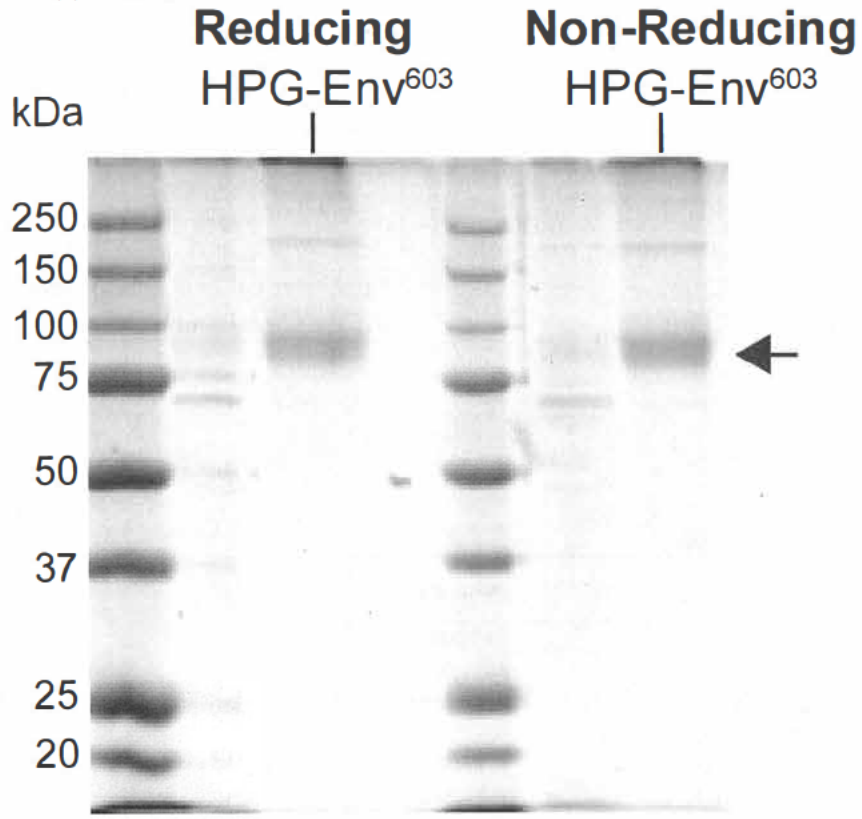
The alignment was generated using MUSCLE (9). The non-KoRV-related gammaretroviruses M-MLV and Fr-MLV are included for comparison. Highly variable regions A (VRA) and B (VRB) indicated by the green and red lines, respectively. The CETTG motif is denoted with an orange line. For HlGRV, 'X' indicates regions of zero sequence coverage. The sequence of the region downstream of the CETTG motif, which includes the VRB, is not available for some KoRV sequences.



Supplementary Figure 7. *Multiple sequence alignment of residues of the PiT-1 Region A of mammals permissive and resistant to GALV infection.* The Region A motif of mammalian PiT-1 (SLC20A1) is shown in the red box (amino acid positions 550-557). Residues highlighted in blue and red denote residues in GALV-infection susceptible and resistant mammalian PiT-1 homologs, respectively (21).



Supplementary Figure 8. *Electron micrographs (EM) of control untransfected cells and cells mock transfected with the empty vector pcDNA3.1.* A) Transmission electron micrograph of untransfected 293T cells. Cell morphology and ultrastructure is consistent with healthy cells in tissue culture. The cells have typical filopodia extensions of the plasma membrane. Cut in transverse, the filopodia appear round and are in the same size range as retrovirus particles. However, they can be clearly distinguished containing cytoplasmic material (including ribosomes). B – C) Transmission electron micrograph of 293T cells mock transfected with pcDNA3.1. Cells appear healthy and have typical filopodia. There are a few unusual structures (arrows) with appearance of altered endoplasmic reticulum membranes displaying and ordered density of ribosomes either attached to, or in close proximity to these structures. The structures do not show viral morphology and could be formed as an artefact from the transfection procedure. Scale bar represents 1 μm.



Supplementary Figure 9. *Hervey pteropid gammaretrovirus recombinant envelope protein expressed from the pcHPG-Env^{603} construct.* SDS-PAGE in the presence (reducing) and absence (non-reducing) of β mercaptoethanol and Coomassie blue staining revealed a major diffuse band with a molecular weight range of ~80-90 kDa.

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Supplementary Tables

Supplementary Table 1. The sources of KoRV-like viral genomes identified within Australian and Asian bats.

Virus	Bat species	Location	Sample type
HPG	*Pteropus alecto*	Hervey Bay, Australia	Fecal
MmGRV	*Macroglossus minimus*	Daintree Rainforest, Australia	Pooled oral and urine
SaGRV	*Syconycteris australis*	Daintree Rainforest, Australia	Pooled oral and urine
HlGRV	*Hipposideros larvatus*	Guangxi, China	Pooled fecal and pharyngeal
RhGRV	*Rhinolophus hipposideros*	Sichuan, China	Pooled fecal and pharyngeal

HPG, Hervey pteropid gammaretrovirus; -GRV = gammaretrovirus

Supplementary Table 2. GenBank accession numbers

Abbreviation	Name	Accession
FFRV1	Flying fox retrovirus isolate FFRV1	MK040728
GALV	Gibbon ape leukemia virus	NC_001885
GALV	Gibbon ape leukemia virus strain Brain	KT724049
GALV	Gibbon ape leukemia virus strain Hall's Island	KT724050
GALV	Gibbon ape leukemia virus strain San Francisco	KT724047
GALV	Gibbon ape leukemia virus strain SEATO	KT724048
GALV	Gibbon ape leukemia virus strain X	GLU60065
HlGRV	Hipposideros larvatus gammaretrovirus	
HPG	Hervey pteropid gammaretrovirus	
KoRV-A	Koala retrovirus	AF151794
KoRV-A	Koala retrovirus clone KV522	AB721500
KoRV-A	Koala retrovirus isolate Pci-maex1738	KF786281
KoRV-A	Koala retrovirus isolate Pci-SN265	KF786285
KoRV-B	Koala retrovirus isolate Br2-1CETTG	NC_021704
McERV	Mus caroli endogenous virus	KC460271
MmGRV	Macroglossus minimus gammaretrovirus	
RhGRV	Rhinolophus hipposideros gammaretrovirus	
SaGRV	Syconycteris australis gammaretrovirus	
WMV	Woolly monkey virus strain WMV SSAV	KT724051

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Supplementary Table 3. Summary results of HPG seroprevalence

Bat species	Samples tested	α-HPG-Env Ig-positive (MFU > 1000)	
Hipposideros ater	3	0	0.0%
Hipposideros diadema	1	0	0.0%
Macroglossus minimus	10	1	10.0%
Macroglossus syconycters	1	0	0.0%
Nyctimene robinsoni	4	0	0.0%
Nyctophilus bifax	1	0	0.0%
Pteropus alecto	32	12	37.5%
Pteropus conspicillatus	2	2	100.0%
Rhinolophus megaphyllus	23	0	0.0%
Syconycteris australis	11	0	0.0%

HPG, Hervey pteropid gammaretrovirus; MFU, mean fluorescence units

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Supplementary Table 4. Luminex data

Fluorescence intensity				Fluorescence intensity			
Sample	HPG Env[a]	HIV Env[b]	Species / Control	Sample	HPG Env[a]	HIV Env[b]	Species / Note
	1277.5[c]	270	Positive 1:50000[d]	45	1076.5	343.5	***Pteropus alecto***
	6397	269.5	Positive 1:5000[d]	46	543.5	305	*Pteropus alecto*
	184	246	Negative sera[e]	47	517.5	332	*Pteropus alecto*
1	162.5	184	*Hipposideros ater*	48	481	394	*Pteropus alecto*
2	91	252	*Hipposideros ater*	49	861	375	*Pteropus alecto*
3	334.5	265.5	*Hipposideros ater*	50	1217	321	***Pteropus alecto***
4	286.5	252	*Hipposideros diadema*	51	1429	311	***Pteropus alecto***
5	129.5	175	*Macroglossus minimus*	52	1448	304	***Pteropus alecto***
6	149	187	*Macroglossus minimus*	53	1581	191	***Pteropus conspicillatus***
7	141.5	183	*Macroglossus minimus*	54	4153	162	***Pteropus conspicillatus***
8	154	188	*Macroglossus minimus*	55	299.5	165.5	*Rhinolophus megaphyllus*
9	157	155	*Macroglossus minimus*	56	110	199	*Rhinolophus megaphyllus*
10	183	228	*Macroglossus minimus*	57	285.5	192.5	*Rhinolophus megaphyllus*
11	229	226.5	*Macroglossus minimus*	58	165	201	*Rhinolophus megaphyllus*
12	408	210	*Macroglossus minimus*	59	144	201	*Rhinolophus megaphyllus*
13	484	251	*Macroglossus minimus*	60	185	197.5	*Rhinolophus megaphyllus*
14	1349	216	***Macroglossus minimus***	61	128	182	*Rhinolophus megaphyllus*
15	196	177	*Macroglossus syconycters*	62	158	178	*Rhinolophus megaphyllus*
16	259	216	*Nyctimene robinsoni*	63	164.5	163	*Rhinolophus megaphyllus*
17	90.5	107	*Nyctimene robinsoni*	64	212	173	*Rhinolophus megaphyllus*
18	160	184	*Nyctimene robinsoni*	65	342	424	*Rhinolophus megaphyllus*
19	176	197	*Nyctimene robinsoni*	66	145.5	214	*Rhinolophus megaphyllus*
20	147	171	*Nyctophilus bifax*	67	151	200	*Rhinolophus megaphyllus*
21	599.5	167	*Pteropus alecto*	68	159	184.5	*Rhinolophus megaphyllus*
22	158.5	152	*Pteropus alecto*	69	168	223.5	*Rhinolophus megaphyllus*
23	153.5	156	*Pteropus alecto*	70	98	160	*Rhinolophus megaphyllus*
24	2813	200.5	***Pteropus alecto***	71	171	225.5	*Rhinolophus megaphyllus*
25	1473.5	140	***Pteropus alecto***	72	117	263.5	*Rhinolophus megaphyllus*
26	190	142	*Pteropus alecto*	73	176	202	*Rhinolophus megaphyllus*
27	178.5	152	*Pteropus alecto*	74	134	196	*Rhinolophus megaphyllus*
28	1715.5	160.5	***Pteropus alecto***	75	163.5	215	*Rhinolophus megaphyllus*
29	474.5	144	*Pteropus alecto*	76	120	173	*Rhinolophus megaphyllus*
30	1359.5	150.5	***Pteropus alecto***	77	133	210	*Rhinolophus megaphyllus*
31	318.5	193	*Pteropus alecto*	78	167	151	*Syconycteris australis*
32	835	157.5	*Pteropus alecto*	79	132	161	*Syconycteris australis*
33	341	164.5	*Pteropus alecto*	80	185	251	*Syconycteris australis*
34	193	198	*Pteropus alecto*	81	138	163	*Syconycteris australis*
35	516	191	*Pteropus alecto*	82	133	197	*Syconycteris australis*
36	218.5	138	*Pteropus alecto*	83	150.5	187	*Syconycteris australis*
37	115	128	*Pteropus alecto*	84	185.5	184	*Syconycteris australis*
38	574	348.5	*Pteropus alecto*	85	261	316.5	*Syconycteris australis*
39	768.5	349.5	*Pteropus alecto*	86	235.5	206	*Syconycteris australis*
40	853.5	259	*Pteropus alecto*	87	265.5	196.5	*Syconycteris australis*
41	1157.5	306	***Pteropus alecto***	88	428.5	397	*Syconycteris australis*
42	1925	248	***Pteropus alecto***				
43	3435.5	304	***Pteropus alecto***				
44	3669.5	282	***Pteropus alecto***				

[a]HPG Env, Hervey pteropid gammaretrovirus trimeric Envelope protein coupled to beads; [b]HIV Env, Human immunodeficiency virus SOSIP Envelope coupled to beads; [c]Red highlighted numbers define positive values defined by fluorescence values >1000; [d]Positive sera, rabbit anti HPG Env; [e]Negative sera, rabbit prebleed serum.

Supplementary Table 5. PCR amplification primers used in this study

Target	Primers	Coordinates	Primer sequence (5' > 3')
pCR4-TOPO plasmid	M13F		GTAAAACGACGGCCAG
	M13R		CAGGAAACAGCTATGAC
Mammalian cytB gene	FM-up		CCCCHCCHCAYATYAARCCM
	FM-down		TCRACDGGNTGYCCTCCDATT
***Pteropus alecto* APOBEC3Z gene**	A3Z3F	(2300..2317)	CAGCTCCGAGTCAAAAAG
	A3Z3R	(3104..3123)	AGCGGATCTTGTTGATAAAG
HPG *pol* 1.34 kb sequence	HPG-pol-F1	(4184..4204)	GAACTCATCGCCTTGACTCAG
	HPG-pol-R1	(5521..5500)	AGCAATACCGTCGACCTTTACC
HPG *pol* 1.55 kb sequence	HPG-pol-F2	(2967..2988)	TCTTCTGCCTCAAACTGCATCC
	HPG-pol-R2	(4511..4491)	CTGTGGTTTCAGCCAGTACTC
HPG *env* gene	HPG-env-F	(5354..5375)	GGAAGAATTCAAAGAGGTATACAGACCTGG
	HPG-env-R	(7998..8020)	GCATTCTAGAAGAGGTTTATTAGGTACACGGG
HPG *gag* 'specific'	HPG-gag-F	(512..532)	AACTCGCTACCGCTTTCCATT
	HPG-gag-R	(683..664)	CTTCCACGGACAGGTTGTGA
HPG-related leader 'broad'	HPG-rel-F	(192..212)	CCATCGACGGGAGGTAAGC
	HPG-rel-R	(389..373)	CTGATCCTGGGGCGTCC

cytB, cytochrome B; HPG, Hervey pteropid gammaretrovirus

Supplementary Table 6. RT-qPCR survey of Australian bat scat for HPG and related viruses

Site (North to South in descending order)	Samples collected	1st Assay[1] (DNA and RNA amplification)				2nd Assay (DNA amplification only)			
		Positive for HPG-related nucleic acids (DNA/RNA)		Positive for HPG-specific nucleic acids (DNA/RNA)		Positive for HPG-related RNA[2]		Positive for HPG-specific RNA[2]	
Hervey Bay	76	8	10.5%	5	6.6%	2	2.6%	5	6.6%
Dalby	16	1	6.3%	0	0.0%	0	0.0%	0	0.0%
Redcliffe	17	2	11.8%	0	0.0%	0	0.0%	0	0.0%
Sandgate	37	1	2.7%	0	0.0%	0	0.0%	0	0.0%
Boonah	31	12	38.7%	5	16.1%	5	16.1%	5	16.1%
Byron Bay	32	2	6.3%	1	3.1%	1	3.1%	1	3.1%
Alstonville	84	24	28.6%	12	14.3%	4	4.8%	12	14.3%
Nambucca Heads	80	7	8.8%	2	2.5%	1	1.3%	2	2.5%
Total samples	373	57	15.3%	25	6.7%	13	3.5%	25	6.7%

[1]57 samples that were positive in the 1st assay for HPG-related nucleic acid sequences (DNA or RNA) were prioritized for analysis to detect the presence of HPG-specific nucleic acids (DNA or RNA). 25 of which tested positive and were then analyzed in the 2nd assay.

[2]Samples are inferred as RNA positive through a failure to generate amplicons in the absence of reverse transcriptase.

HPG, Hervey pteropid gammaretrovirus

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s22

From: Edward Holme s22
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To: Joshua Hayward
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Subject: Re: HPG Paper Fwd: PNAS MS#2019-15400 Submitted

Great work Josh!

Eddie

Professor Edward C. Holmes FAA FRS
The University of Sydney

Duplicate Email - Removed

Robinson, Karen (Governance, Black Mountain)

From: Gary Crameri [redacted]
Sent: Thursday, 5 September 2019 2:41 PM
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Terrific Josh thanks for all your hard work
Cheers
Gary

[redacted]
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From: Heidi Drummer [redacted]
Sent: Thursday, 5 September 2019 3:05 PM
To: Joshua Hayward
Cc: mary.tachedjian; Kohl, Claudia; Adam Johnson; Dearnley, Megan (AAHL, Geelong AAHL); Brianna Jesaveluk; Christine Langer; Solymosi, Philip; Hille, Georg; Andreas; Cecilia Sanchez; Adam Werner; dimitri.kontos[redacted] Gary Crameri; Andy Poumbourios; Glenn.Marsh; Michelle Baker; Edward Holmes; Wang Linfa; <lna.Smith[redacted]; Gilda Tachedjian
Subject: Re: HPG Paper Fwd: PNAS MS#2019 15400 Submitted

Thanks to everyone for their contributions
Good Luck
H

On Thu, 5 Sep 2019 at 15:00, Joshua Hayward [redacted] wrote:

Hi all,
Just a heads-up that I will also be submitting an abstract regarding this work for the AVS10 meeting in December.
Cheers,
Josh

Joshua Hayward PhD

Research Officer
Retroviral Biology and Antivirals Research Laboratory

Burnet Institute
Disease Elimination and Maternal & Child Health Programs

[redacted]
GPO Box 2284, Melbourne, Victoria, Australia 3001
[redacted]

[redacted]
Duplicate Email - Removed

[redacted] s22

From: Smith, Ina (H&B, Black Mountain) [redacted] s22
Sent: Thursday, 5 September 2019 3:22 PM
To: 'Joshua Hayward'; Tachedjian, Mary (H&B, Geelong AAHL); Kohl, Claudia; Adam Johnson; Dearnley, Megan (AAHL, Geelong AAHL); Brianna Jesaveluk; Christine Langer; Solymosi, Philip; Hille, Georg; Andreas; Cecilia Sanchez; Adam Werner; dimitri.kontos [redacted] s22 ; garycrameri [redacted] s22 Heidi Drummer; Andy Poumbourios; Marsh, Glenn (H&B, Geelong AAHL); Baker, Michelle (H&B, Geelong AAHL); Edward Holmes; Wang Linfa; Gilda Tachedjian
Subject: RE: HPG Paper Fwd: PNAS MS#2019-15400 Submitted

Well done Josh

Thanks everyone.

Cheers
.na

Ina Smith, PhD
Senior Research Scientist | Risk Evaluation and Preparedness Program | Health and Biosecurity | CSIRO, Clunies Ross St, Black Mountain ACT 2601 Ph. 02 6218 3579

Duplicate Email - Removed

[redacted]

From: Gilda Tachedjian [redacted]
Sent: Thursday, 10 October 2019 7:53 AM
To: Edward Holmes
Cc: Joshua Hayward; Muzza Work; Kohl, Claudia; Adam Johnson; Dearnley, Megan (AAHL, Geelong AAHL); Brianna Jesaveluk; Christine Langer; philip daniel.solymosi [redacted] Hille, Georg; Andreas; Cecilia Sanchez; Adam Werner; dimitri.kontos [redacted] Gary Crameri; Heidi Drummer; Andy Poumbourios; Glenn.Marsh; Michelle Baker; Wang Linfa; Black Mountain Smith Ina
Subject: Re: PNAS MS# 2019 15400 Decision Notification

Terrific thanks Eddie!

On 10 Oct 2019, at 7:12 am, Edward Holmes [redacted] wrote:

Perfect Josh! You send em, I'll run em.

Cheers,

Eddie

Professor Edward C. Holmes FAA FRS
The University of Sydney

On 9 Oct 2019, at 10:04 pm, Joshua Hayward [redacted] wrote:

Hi Gilda,
I had done those using amino acid sequences however. For consistency we may consider generating nucleotide gag, pol and env trees with the same methodology and software as the full genome tree. Would that be ok, Eddie? I'm happy to split the processed alignment into the relevant genes.
Cheers,
Josh

Joshua Hayward PhD

Research Officer
Retroviral Biology and Antivirals Research Laboratory

Burnet Institute
Disease Elimination and Maternal & Child Health Programs

[redacted]
GPO Box 2284, Melbourne, Victoria, Australia 3001
[redacted]

On Wed, 9 Oct 2019 at 21:52, Gilda Tachedjian [redacted] wrote:

Thanks Eddie,

Josh has done the ENV and POL the former looks like the data in the paper, the latter lacks resolution (low boot strap values). Are you happy to do the recombination analysis?

Good to know the evolutionary time scale is irrelevant!

Cheers

Gilda

Sent from my iPad

> On 9 Oct 2019, at 8:48 pm, Edward Holmes [redacted] wrote:
>
> Sounds good Gilda.
>
> 1. The time issue. Irrelevant. The evolutionary time scale depicted in this phylogeny is clearly far older than the time span of sampling. Accordingly, sampling viruses a decade apart will no impact. This can just be argued with a statement such as this.
>
> 2. Individual tree genes. Easy and we sort of have most of these anyway. We could add gag. Easy to upload as a Supplementary Figure. If there is any phylogenetic movement this will be due to a lack of signal/resolution. Can't believe there will be much/any evidence of recombination. Easy to deal with though.
>
> Cheers,
>
> Eddie
>
> ---
>
> **PROFESSOR EDWARD C. HOLMES FAA FRS**
> ARC Australian Laureate Fellow
>
> **THE UNIVERSITY OF SYDNEY**
> Marie Bashir Institute for Infectious Diseases & Biosecurity,
> Charles Perkins Centre,
> School of Life & Environmental Sciences and Sydney Medical School,
> The University of Sydney | Sydney | NSW | 2006 | Australia
> [redacted]
>
> > On 9 Oct 2019, at 5:58 pm, Gilda Tachedjian [redacted] wrote:

Dear All,

Please see attached reviews on our manuscript which overall are favourable!!

Reviewer #2 has requested additional experiments - which we are already doing in anticipation (i.e Pit1 receptor).

Eddie, appreciate your input on the queries re: phylogeny i.e. How does time impact relatedness, request for RT and Env trees l.

Josh in response to the query re: HPG replication in cell culture - didnt you take soup from infected cells and use them to reinfect fresh cells?

Ina/Heidi/Andy - for the query re: HPG seropositivity we could modify what we are claiming or express MLV envelope and repeat the serology?

The revision is due 7th of December, although we can request for more time.

Cheers

Gilda

Professor Gilda Tachedjian BSc (Hons) PhD
Head, Life Sciences Discipline
Group Head, Retroviral Biology and Antivirals Lab
NHMRC Senior Research Fellow

Burnet Institute

s22

The Macfarlane Burnet Institute for Medical Research and Public Health Ltd,
85 Commercial Road, Melbourne, VIC 3004, Australia
burnet.edu.au

Equity through better health

Duplicate Email - Removed

[redacted]

From: Edward Holmes [redacted]
Sent: Tuesday, 3 December 2019 7:13 AM
To: Gilda Tachedjian
Cc: Joshua Hayward; Muzza Work; Kohl, Claudia; Adam Johnson; Dearnley, Megan (AAHL, Geelong AAHL); Brianna Jesaveluk; Christine Langer; Solymosi, Philip; Hille, Georg; Andreas; Cecilia Sanchez; Adam Werner; dimitri.konto [redacted]; Gary Crameri; Heidi Drummer; Andy Poumbourios; Glenn.Marsh; Michelle Baker; Wang Linfa; <Ina.Smith [redacted]
Subject: Re: PNAS MS# 2019 15400 Decision Notification

Many thanks for handling all this Gilda.

Cheers,

Eddie

PROFESSOR EDWARD C. HOLMES FAA FRS
ARC Australian Laureate Fellow

THE UNIVERSITY OF SYDNEY
Marie Bashir Institute for Infectious Diseases & Biosecurity,
Charles Perkins Centre,
School of Life & Environmental Sciences and Sydney Medical School,
The University of Sydney | Sydney | NSW | 2006 | Australia
[redacted]

On 3 Dec 2019, at 5:47 am, Gilda Tachedjian [redacted] wrote:

Dear all,

A short note to let you know that we have made excellent progress toward completing additional experiments requested by the reviewers however I have requested and have been granted an extension to submit our revised manuscript early next year. See email correspondence below for details.

Best regards

Gilda

Professor Gilda Tachedjian BSc (Hons) PhD
Head, Life Sciences Discipline
Group Head, Retroviral Biology and Antivirals Lab
NHMRC Senior Research Fellow

Burnet Institute
[redacted]

The Macfarlane Burnet Institute for Medical Research and Public Health Ltd,
[redacted]
burnet.edu.au

Begin forwarded message:

From: "Myers, Zoe" [redacted]
Subject: RE: PNAS MS# 2019-15400 Decision Notification
Date: 3 December 2019 at 5:00:23 am NZDT
To: "gilda.tachedjian [redacted]

Dear Dr. Tachedjian,

Thank you for your email. It will not be a problem to grant your extension until February 8, 2020. We understand it is a busy time of year and we look forward to receiving your revised manuscript.

Best wishes,

Zoe Myers

PNAS Editorial Office
[redacted]

From: PNAS [redacted]
Sent: Monday, December 2, 2019 10:56 AM
To: Myers, Zoe [redacted]
Cc: PNAS [redacted]
Subject: FW: PNAS MS# 2019 15400 Decision Notification

-JN

From: Gilda Tachedjian [redacted]
Sent: Friday, November 29, 2019 4:54 PM
To [redacted] pnascentral.org; PNAS [redacted]
Cc: Gilda Tachedjian [redacted] oshua Hayward [redacted] Smith, Ina (H&B, Black Mountain) [redacted] Mary Tachedjian [redacted] Adam Johnson [redacted]
Subject: Re: PNAS MS# 2019-15400 Decision Notification

Dear PNAS Editor-in-Chief,

I would like to request a 9 week extension from the current due date of our revised manuscript (requested new due date 8th Feb 2020)

We have been asked to undertake additional experiments by the reviewers which we agree will improve the manuscript.

While we are making excellent progress towards completion of these studies, we will need more time due to circumstances out of our control.

These include key staff being on leave including having to serve on a jury for almost a month and the upcoming holiday season where our institute is closed

over the break and staff are expected to take their annual leave up to mid January.

Thank you for considering our request.

We look forward to hearing from you soon.

Best Regards

Gilda

Professor Gilda Tachedjian BSc (Hons) PhD
Head, Life Sciences Discipline
Group Head, Retroviral Biology and Antivirals Lab
NHMRC Senior Research
Fellow

Burnet Institute
s22
The Macfarlane Burnet Institute for Medical Research and Public Health Ltd,
s22
burnet.edu.au

Equity through better health

On 9 Oct 2019, at 6:56
am, s22 pnascentral.org wrote:

October 8, 2019

Title: "**Infectious KoRV-related retroviruses circulating in Australian bats** "
Tracking #: 2019-15400
Authors: Hayward et al.

Dear Dr. Tachedjian,

The expert who is serving as editor for your manuscript [MS# 2019-15400] has obtained 2 reviews, which are included below. The editor requests that you constructively address the concerns of the reviewers in a revised manuscript. Please note that multiple revisions are rarely permitted and there is no guarantee that the paper will be accepted.

PNAS allows 60 days to submit a revision. Your revision is due by December 7, 2019. If you require additional time, please contact the PNAS office.

When submitting revised materials, we require that you include a cover letter with a point-by-point response to the reviewers' comments. If you submitted a single PDF at initial submission, you must submit individual publication-ready files (e.g., Word

file for manuscript text; EPS, TIFF, or high-resolution PDF for figures; Word file for tables; etc.)

Please note that statements such as "data not shown" and "personal communication" cannot be used to support claims in the work and should be removed prior to submission. Authors are encouraged to use supporting information to show all necessary data, or to deposit their data in a publicly accessible database if posting as supporting information is not possible. Authors should include a statement in their methods section describing how readers will be able to access the data, associated protocols, code, and materials.

You may submit your revised manuscript here: https://www.pnascentral.org/cgi-bin/main.plex?el_A2B7DVBw3A1BbFN2I7A9ftdMpCfRHgvFpmlE6PZCqVwQZ.

Adding, removing, or reordering your author list requires approval from all coauthors before we can proceed. If you wish to add an additional corresponding author, please note this in the "Comments for Editorial Staff" box when completing your revision.

We recommend that authors submit ORCID IDs. If you provide your ORCID ID when you submit your manuscript, you can opt in to have your ORCID record automatically updated if your article is published. Watch for an email from Crossref in your ORCID inbox requesting permission to access your ORCID record.

Thank you for submitting to PNAS. We look forward to receiving your revision.

Sincerely yours,
May R. Berenbaum
Editor-in-Chief
s22

****
Editor Comments:

Our reviewers were overall positive about the paper, but had specific suggestions for improvement. Both made very good points. Some weakening of claims (about possible relation to endogenous viruses, for example) might be in order. Reviewer #2 had several requests for additional experiments, and many were not difficult. Adding as many as feasible would strengthen the paper.

We would look forward to a revised draft that addresses as many as possible of the reviewers' points.

Reviewer Comments:

Reviewer #1:

Sufficient General Interest?:
Yes

Conclusions Justified?:
Yes

Clearly Written?:
Yes

Procedures Described?:
Yes

Supplemental Material Warranted?:
Yes

Willingness to Re-review?:
Yes

Comments:
Here Hayward et al. identify and characterize the a full-length, replication-competent gammaretrovirus genome isolated from a bat in Australia, called HPG. In addition, four other related, partial retroviral genomes were isolated from other bat species in Australia and China. These retroviruses are closely related to koala retrovirus (KoRV) and gibbon ape leukemia virus (GALV) with similar sequence, tropism, and structure. HPG envelope antibodies were detected in multiple bat serum samples and HPG-like nucleic acids were detected in multiple bat feces samples. The authors propose that this novel gammaretrovirus is actively replicating in bats and that bats having overlapping habitats with koalas and gibbons may have led to interspecies transmission. The work is solid and novel, but a few key questions were not fully addressed in the study that could strengthen the manuscript.

Major Comments:
1. It appears that KoRV-related retroviruses were only detected in bat mucosal excretions and not in blood. Is it known where these viruses may replicate in koalas (or bats based on receptor expression) and if this tropism would explain this finding? Related to this, how do the authors envision that interspecies transmission occurred? This would be particularly useful to include in the Discussion, as it is not clear when and how transmission(s) between the 2 species occurred.
2. On page 9, line 15 and 17, the authors state, " contain endogenous HPG-related sequences" and "...suggesting evidence of endogenization or latent infection with HPG-related viruses." However, on page 7, they report that HPG is

unlikely to be an endogenous virus. First, these statements are contradictory and should be reconciled. Second, in the BLAST analysis, presumably bat ERVs were identified. What sequence identity do they have with HPG? The authors state that "sequences with high percent nucleotide similarity" were not observed. However, "high" is not defined.
3. Regarding phylogenetic analysis with related gammaretroviruses, how much does time impact relatedness? For example, HPG was isolated from a bat obtained in 2011, but it is unclear when the other viruses were obtained and how this could affect evolution, particularly in new host species (i.e. species adaptation after transmission from bats to koalas).
4. On page 9, the authors describe 12 samples that were positive only for HPG DNA and not HPG RNA and state that they represent animals that are "latently infected with other HPG-related virus(es) or contain endogenous HPG-related sequences." An alternative explanation would be low quantity and/or low quality RNA present in these samples. It is unclear that RNA and DNA quantity or quality were controlled in the analysis.

Minor Comments:
1. The Introduction (page 5, line 1) and the Discussion (page 10, line 3) state "the east coast of Australia including the Daintree rainforest," which implies that the Daintree rainforest is part of the Australian east coast. However, the Results section (page 6, lines 6-7) state "373 bats along the east coast of Australia and 106 bats from the Daintree Rainforest (Queensland)" that seems to imply that the Daintree rainforest is separate from the east coast. The text should be consistent throughout the manuscript.
2. Supplementary Table 2: it is assumed that Genbank accession numbers for the bat retroviruses will be forthcoming and included in the final version of the manuscript.
3. It is recommended that data presented in Supplementary Figure 7 be described in the Results section after Figure 5 (page 8, 2nd paragraph).
4. Supplementary Figure 9 is not discussed anywhere in the text. It is suggested that it be removed or appropriately described in the text.

Reviewer #2:

Sufficient General Interest?:
Yes

Conclusions Justified?:
No

Clearly Written?:
Yes

Procedures Described?:
Yes

Supplemental Material Warranted?:
Yes

Willingness to Re-review?:
Yes

Comments:
This is an interesting paper submitted by Hayward and colleagues, describing the discovery and biological characterization of KoRV-related gammaretrovirus sequences in samples from different Australian bat species. The bulk of the results focuses on sequences obtained from scat of P. Alecto, and referred to here as Hervey pteropid gammaretrovirus (HPG). A consensus is used to reconstruct a full HPG viral genome, and a variety of biochemical methods and EM is used to confirm production of gamma-like virions. If true, this may be the first description of an exogenous gammaretrovirus of bats. While the study is likely to be of broad interest, there are several caveats to interpretation that should be addressed, as well as some minor points.

Page 7, results first paragraph - An important caveat is that this could also be a recent, rare and unfixed ERV insertion, similar to many KoRV loci in Koalas, especially in southern koalas - that is to say, a similar approach in southern koalas might "miss" detecting a rare enKoRV sequence. The intact nature of the HPG is also consistent with something that could be present in both exogenous and endogenous forms. Effectively, this doesn't change the impact of the manuscript - either its an exogenous gammaretrovirus of bats, a very recently endogenized gammaretrovirus of bats, or both. My suggestion is to stay open to all possibilities - present it as an exogenous virus, but acknowledge that the actual samples might have detected a germline insertion (ERV).

Page 7, results - phylogenetic analysis. While a tree based on the full genomes is potentially robust, the authors should also analyze RT and env separately. Do they give the same/similar results as one another, and are they consistent with the tree based on the entire genomes? Recombination can obscure phylogenetic relationships, especially when one part of the genome is more divergent or has had a very different evolutionary trajectory. For example, it could be one gene, such as env, that separates one branch from the others, but is the result of a single recombination event and not of divergence over time. Gene-specific phylogenies could be added to supplemental data, and wouldn't be necessary in the main text (unless they reveal a more complex phylogenetic history, in which case the authors will want to make it part of the story).

Page 8, serological analysis - the negative control (HIV Env) rules out general background, but does not rule out cross-reactivity with other gamma-type retroviruses or ERV expression. How specific is this assay? Since the claim is "HPG seropositivity", it should include Env proteins from a distant relative (GaLV, KoRV) and even a different gamma lineage altogether (e.g., MLV Env). The conclusion could then be "HPG-seropositivity" or "KoRV-related retrovirus seropositivity" depending - either result fits the story being described in the manuscript. But as is, it's not clear they can claim specificity for HPG.

page 17, figure 2 - Need to explain the ratios at the nodes (e.g., 1/100, 1/97, etc). Are these bootstrap values, and if so, why are they presented this way (usually they are given as percentages)? This info should be in the figure legend.

Page 18, Figure 3 and related results secion - The negative control (which is shown in Supp Fig 8) to confirm that the particles are produced by the transfected plasmid is not mentioned here or referred to in the main text. Authors also need to include some indication of how many images or fields were needed to detect the particles or, more specifically, to explain with what certainty the negative controls can be said to have less/no particles compared to the composites in figure 3. For example, were sufficient fields analyzed or similar numbers of cells visualized/ is this the result of comparing similar numbers of images/cells for transfected and control cells?

Page 20, figure 4: the HPG result is distinctive - the MLV infection results in the expected plateau consistent with ongoing replication, whereas HPG replication peaks and drops quickly to background. This raises the possibility that the data don't represent ongoing replication cycles, but rather a burst of production from initially infected cells. Another possibility is that the HPG retrovirus is replicating, but is toxic to cells, similar to lentivirus replication in cell culture. An experiment to examine these possibilities and to definitively establish successive rounds of replication is important (e.g., passaging filtered supe to a second plate/flask followed by RT assay, or replication with and without inhibiting RT, etc).

General - The manuscript proposes that HPG uses the same receptor as KoRV-A and GaLV, and even includes a supplemental figure depicting the conserved binding site motif in PiT-1 of the relevant host species. This is presented as part of the argument in referring to this as a "KoRV-related retrovirus", as in the title of the manuscript and elsewhere in the text. Given how easy it is to do, why not formally prove this? It should be straightforward, and there is plenty of precedent in the literature - either by adding PiT-1 expression to null cells (such as the NIH3T3 cells used in figure 5), or by

means of a standard superinfection cross-interference assay. Either experiment can be done with existing reagents in a relatively short period, and would strengthen the manuscript.

Page 32, line 21 supplemental methods refers to "Supp Figure 8" but probably is supposed to refer to Supp Figure 9.

Page 43, Supp figure 9 - legend could use some additional info - Are there control lanes (non-transfected or mock transfected) in the image? If so, are the controls the basis for establishing that the indicated bands are HPG Env? The lanes should be labeled or mentioned in the legend. Alternatively, If there are no control lanes, how can the authors claim that this isn't an unfortunate background band?

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PUBLICATION INFORMATION

High resolution figure files are required for the final version of your manuscript. PNAS figure preparation guidelines state that no specific feature within an image may be enhanced, obscured, moved, removed, or introduced. The grouping or consolidation of images from multiple sources must be made explicit by the arrangement of the figure and in the figure legend. Adjustments of brightness, contrast, or color balance are acceptable if they are applied to the whole image and if they do not obscure, eliminate, or misrepresent any information present in the original, including backgrounds. Please note that our production editors may flag figures that are not in compliance with our figure policy, resulting in delays. For more information on submitting high resolution figures please review the

s22

From: Wang Linfa s22
Sent: Tuesday, 3 December 2019 11:22 AM
To: Gilda Tachedjian
Cc: Joshua Hayward; Muzza Work; Kohl, Claudia; Adam Johnson; Dearnley, Megan (AAHL, Geelong AAHL); Brianna Jesaveluk; Christine Langer; Solymosi, Philip; Hille, Georg; Andreas; Cecilia Sanchez; Adam Werner; dimitri.kontos s22 Gary Crameri; Heidi Drummer; Andy Poumbourios; Glenn.Marsh; Michelle Baker; Edward Holmes; <Ina.Smith s22
Subject: Re: PNAS MS# 2019 15400 Decision Notification

Dear Gilda,

Thanks for the update and good luck!

Linfa

Sent from my iPhone

Duplicate Email - Removed