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A Recent Gibbon Ape Leukemia Virus Germline Integration in a Rodent from New Guinea

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Abstract

As a result of germline colonization by retroviruses, large portions of vertebrate genomes are composed of endogenous retroviruses (ERVs). Most colonization events occurred millions of years ago and the processes remain obscure. However, in the Australo-Papuan region (Australia and New Guinea) some of the most recent germline colonization events have been discovered providing important models to study the transition from exogenous retroviruses (XRVs) to ERVs. The Wallace Line demarcates the biogeographical boundary separating much of Southeast Asia from the Australo-Papuan region. Faunal dispersion and natural pathogen transmission are restricted by this boundary. West of the Wallace Line, horizontally transmitted gibbon ape leukemia viruses (GALVs) have been isolated exclusively from captive gibbons in Thailand. Two Yinpterochiroptera microbat species from China (Guangxi and Sichuan provinces) appear to have been infected naturally. East of Wallace Line, the woolly monkey virus (WMV, a member of the GALV clade) and closely related viruses such as the koala retrovirus (KoRV) have been detected in eutherians (rodents and bats) and marsupials (koalas) in the Australo-Papuan region and are often vertically transmitted. The number of detected vertically transmitted GALV-like viruses in Australo-Papuan fauna compared to sporadic findings of horizontal transmission in Southeast Asia and China, suggest the origin of the GALV-KoRV clade may be in the former region and further models of early stage genome colonization may be found there. Using high throughput molecular methods, we screened 278 samples representing seven bat and one rodent family endemic to the Australo-Papuan region and representative bat and rodent species found on both sides of the Wallace Line. We identified two rodents (*Melomys*) from Australia and Papua New Guinea and no bat species harboring GALV-like retroviruses. *Melomys leucogaster* from New Guinea harbored a genomically complete replication-competent retrovirus with a shared integration site among individuals. However this integration was only present in some individuals of the species indicating this retrovirus is at the earliest stages of germline colonization of the *Melomys* genome, providing a new small wild mammal model of early stage genome colonization.

Significance Statement

Retroviruses colonize vertebrate genomes forming endogenous retroviruses. With very few exceptions, these colonization events are ancient. After screening 278 samples representing seven bat and one rodent family endemic to the Australo-Papuan region (Australia and New Guinea) we report the discovery of genomically intact and infectious retroviruses currently colonizing the genome of a *Melomys leucogaster* in New Guinea. This represents the second example, after the koala retrovirus (KoRV), of a retrovirus that has colonized the genome but retains a functional viral life cycle identified in the Australo-Papuan region.

Introduction

Retroviruses integrate into the host genome as part of their replication cycle. While the majority of integrations occur in somatic cells, several families of retroviruses have frequently integrated into the host

germline, resulting in the development of vertically transmitted endogenous retroviruses (ERVs). Approximately 8-10% of vertebrate genomes are composed of ERVs (1–3). Not all retroviral families colonize vertebrate genomes with equal frequency. Gammaretroviruses, for example the murine leukemia virus (MuLV) related viruses, frequently colonize the germline in vertebrates when compared to other retroviral groups (4, 5). Whereas most vertebrate retroviral colonization events were completed millions of years ago, Gibbon ape leukemia virus (GALV) and the closely related koala retrovirus (KoRV) represent gammaretroviruses that have colonized or have recently begun to colonize the genomes of a variety of mammals in Southeast Asia, the Australo-Papuan region (Australia, New Guinea) and Wallacea (including the Philippines apart from Palawan) (6). These regions represent historic biogeographical realms that have limited natural faunal dispersion among them as demarcated by the Wallace (1863), and Huxley (1868), and Lydekker (1896) Lines (Fig. 1). To date, according to the IUCN (The International Union for Conservation of Nature Red List of Threatened Species. v2020-3, <http://www.iucnredlist.org>), approximately eight bat families and three genera of murine rodents from the following two tribes have distributions that span the Wallace Line: (i) Hydromyini: *Haeromys* (Borneo and Sulawesi), *Chiropodomys* (West of the Wallace Line in the Asian continental shelf), (ii) Rattini: *Rattus* (from Asia to Philippines and Australia), *Maxomys* (from Asia to Sulawesi) and (iii) Murini: *Mus musculus* (West of the Wallace line in Sunda shelf) (7).

GALVs are oncogenic viruses consisting of seven isolates (8–14), initially detected in captive white-handed gibbons (*Hylobates lar*) in Thailand. The basal GALV strain, the woolly monkey virus (WMV-previously known as simian sarcoma associated virus-SSAV) was isolated from a brown woolly monkey (*Legothis lagotherica*) that was co-housed with infected gibbons and likely represents a gibbon to woolly monkey transmission (15, 16). GALVs are closely related to the recently identified ERVs in *Melomys* rodents, endemic to Australia (MbRV) (17) and the North Moluccas (MelWMV) (18, 19), forming a monophyletic clade with KoRV, which thus far is found exclusively in koalas (*Phascolarctos cinereus*). Extensive screening for GALV in wild gibbons failed to detect the virus (20), suggesting all isolates in primates are derived from horizontal transmission during captivity. The recently characterized gammaretroviruses (FFRV1, HPG, MmGRV and SaGRV) from wild-caught Australian bats (*Pteropus alecto*, *Macroglossus minimus* and *Syconycteris australis*), form a clade basal to GALV-KoRV, while HIGRV and RhGRV, isolates from the Chinese bats (*Hipposideros larvatus* and *Rhinolophus hipposideros*) are a GALV sister clade (21, 22). The number of identified GALV-KoRV related viruses in Australo-Papuan wildlife is greater than in Southeast Asia or China with only two isolates from bat species that have historically spanned the Wallace line (23, 24). Therefore, this complex distribution suggests the GALV-KoRV clade derives from the Australo-Papuan side of the Wallace Line and that the related viruses found in Southeast Asia or China represent either human-mediated spillover or infection of taxa in the Australo-Papuan region that can cross the Wallace Line.

To identify potential reservoirs for GALV-KoRV viruses and evidence of germline colonization events in the Australo-Papuan and Wallacean regions, we used pan-GALV-KoRV PCR, hybridization capture viral enrichment and a high throughput sequencing to screen bat and rodent species from both sides of the Wallace line (19). We identified genomically intact and infectious WMV, denoted complete melomys woolly monkey retrovirus (cMWMV) in some but not all populations of *Melomys leucogaster* endemic to New Guinea. Among individual *M. leucogaster* which were cMWMV positive, the retrovirus was found at a single shared integration site, indicating that it has been transmitted vertically and is a part of the *M. leucogaster* germline but has not become fixed in the species. No bats, including members of the same species found harboring GALV relatives in Australia and China, were positive. Structural modeling of the few variable amino acids among the retrieved sequences and WMV, and *in vitro* infection models suggest cMWMVs identified are replication-competent. Our data suggest that cMWMV represents an additional model to KoRV for exploring the earliest stages of retroviral germline colonization.

Results

WMVs in the Australo-Papuan region

The degenerate oligonucleotide primer set (KOGAWM-1) was designed to amplify the *gag* gene of any GALV, KoRV and WMV. The DNA of *R. norvegicus* was used as a negative control as these viral clades are absent from *Rattus*. Various tissue samples ($n = 278$) from seven bat families, and the murine rodents *Rattus* (spanning the Wallace Line), *Hydromys* and *Melomys* (spanning the Lydekker Line) were PCR-screened for the presence of GALV and KoRV-like sequences (Fig. 1, Table 1 and *SI Appendix Dataset S1*). None of the 156 bat samples yielded an amplicon. However liver and kidney tissue samples of five *M. leucogaster* ($n = 10$), a *R. verecundus* ($n = 5$) and a *R. niobe*_sp.B ($n = 5$) collected between 1985 to 2014, in Western and Southern Highland Provinces and two *M. burtoni* ($n = 7$) from Queensland of Australia each yielded an amplicon which had 89% to 100% sequence identity (Sanger sequencing) to WMV (*SI Appendix Dataset S1* marked *). *Melomys* is one of the most speciose genera (*Melomys* = 23 species, *Pseudomys* = 23 species, *Rattus* = 26 species) (25, 26) in the Australo-Papuan region with ongoing taxonomic revisions (27–30). Though currently confined to the east side of the Wallace line, the nearest relatives of the Australo-Papuan *Rattus* are found on Sulawesi Island, while the nearest relatives of *Melomys* (*Solomys*, *Protochromys*, *Paramelomys*, and *Uromys* in the *Uromys* division) are found among the Australo-Papuan “old endemic” rodents (27–30). The distribution and diet of the Papuan white-bellied melomys (*M. leucogaster*) overlap with those of the moss-forest rat (*R. niobe*) and slender rat (*R. verecundus*), especially along the New Guinean Central Cordillera (31).

Hybridization capture viral enrichment

Samples (89, 201, 204, 246, 249, 290, 291, 292 and 300) that yielded an amplicon were used for Illumina library preparation and subsequent hybridization capture enrichment. The target enrichment factor for each

sample was calculated from the VIP coverage information of gammaretroviruses output plot ([SI Appendix, Table S1](#)).

The novel retroviral sequences identified here were assembled into contigs (refer to materials and methods) and aligned to all the KoRVs, GALVs (including MbERV and MelWMV), Australian and Asian GALV-like bat sequences and the related gammaretroviruses ([SI Appendix, Table S2](#)). These alignments were used to perform phylogenetic analysis to infer the evolutionary relationships among the viral sequences. Contigs from *R. niobe*_Sp.B (89) and *R. verecundus* (246) formed a clade with *R. norvegicus* (LOC102557044), while partial sequence retrieved from one of the *M. burtoni* (201, contig-4 ~ 1,300 bp) grouped with viral outgroup sequences from the rodents *C. griseus* and *M. coucha*. The remaining *Melomys* consensus contigs formed a clade with WMV, while the Asian HIGRV and RhGRV formed a sister clade ([Fig. 2](#)). As described in Hayward *et al.* (22), we applied Gblocks to the full genome alignments, eliminating the divergent and poorly aligned regions and further compared it to our initial alignment. Tree topology was largely congruent for the full-genome alignment, individual genes, and the amino acid sequences ([SI Appendix, Fig. S2](#)). Thus, we conclude that the poor node support (ranging from 30 to 58) for the WMV-HIGRV-RhGRV clade is due to the low sequence diversity of these sequences rather than phylogenetic approaches employed.

Characterization of viral integration flanking sites

We extended the viral sequences into the host genome integration site for samples 249, 291, 292 and 300. Identical host flanking sequences were found each with the same target duplication site (TSD) for one integration site ([SI Appendix, Text file S1](#) and [Fig. S3A](#)). The data suggests there is only one cMWMV shared integration in all of the samples for which integration site flanking sequence could be identified. The results derived from different tissue samples of different *M. leucogaster* that were collected in 1985, 1987 and 2014 from four different collection sites and two different provinces in Papua New Guinea (PNG). An identical integration site in multiple tissues in multiple individuals can most parsimoniously be explained by vertical transmission indicating the WMV-like sequence is an endogenous retrovirus (ERV). Flanking sequences could not be extended for one of the *M. leucogaster* (290) and *M. burtoni* (201 and 204) and therefore it could not be determined if these viral sequences are ERVs or XRVs. However, the greater divergence of the *M. burtoni* sequence from *M. leucogaster* sequences, the closer relationship of the WMV-like sequence obtained from *M. burtoni* to the Indonesian MelWMV, and the observation that the MelWMV integration site is different from that of cMWMV in *M. leucogaster* ([SI Appendix Fig. S3B](#) and [Text file S1](#)), suggests that the *M. burtoni* sequence obtained is not cMWMV but a related retrovirus like the defective *M. burtoni* viruses MbRV and MelWMV. We termed this MelWMV variant MelWMV-NG (MelWMV New Guinea).

Structural characteristics of cMWMV

The cMWMV has retained the typical gammaretroviral structure with a genome of ~ 8.5 Kb, and unlike MelWMV has a potentially functional *env* gene. The coding region is flanked by 5' and 3' untranslated LTRs (*SI Appendix Fig. S4*). The conserved CETTG motif often found in highly infectious gammaretroviruses was identified in cMWMV (*SI Appendix, Fig. S5*). Further, cMWMV exhibited intact structural polyproteins (GAG, ENV) and functional POL with 97.3% pairwise identity to WMV and 57.6% to KoRV. The latter finding indicates a closer phylogenetic relationship of cMWMV to WMV. This outcome is consistent with the results from the BLAST search, multiple sequence alignments, and phylogenetic topology compared to KoRV-A.

Receptor binding, mediated by the ENV is vital to the viral cellular entry process and the determinant factor in viral tropism. To predict the functional effects of the identified mutations a computational strategy with five major criteria (*SI Appendix, Methods*) was used. Our prediction was that the probability of a mutation to have functional consequences increases with the number of criteria fulfilled. The results of these analyses, which are summarized in *SI Appendix Table S3*, strongly suggest that the identified mutations most probably do not functionally alter the proteins.

Specifically, out of the 46 differences identified, only 10 are predicted to be radical substitutions according to both BLOSUM 65 and 80 scores. This finding suggests that only a few mutations are physico-chemically different enough to suggest functional changes. Furthermore, only four mutations were found in a highly conserved amino acid position based on conservation level analysis of each position. This finding also suggests that the positions of these mutations are highly variable, thus, they might have a lower probability of being functionally important. Additionally, the two different prediction algorithms (SIFT and PROVEAN) that were used, found that only eight substitutions are suggested to alter protein function with only two of them predicted with high confidence by both algorithms. Analysis to determine whether any of the mutations change an amino acid of known function, suggested that only 12 mutations were found in known functional motifs, but none was predicted to alter the amino acid properties of the motif. Lastly, homology modeling of parts of the proteins was to test whether a mutation alters the protein local conformation. The latter analysis revealed that only four of the identified mutations might affect local topology (*SI Appendix Fig. S6* and *Table S3*). Although a few of the identified mutations were predicted to alter some characteristics of the protein, none of them fulfilled more than three of the above mentioned criteria, supporting the notion that these mutations most probably do not drastically alter protein function. Only two changes each were observed in the VRA and VRB region of the ENV protein relative to WMV (*SI Appendix, Fig. S5*). Our ENV modelling suggests that like WMV (32), cMWMV, may employ PiT-1 cellular receptors. However, we cannot exclude that these changes or changes in other viral domains affect receptor usage by cMWMV.

To evaluate the computational predictions, we performed cell culture experiments employing viral vectors for cMWMV and KoRV-A as a control by infecting NIH3T3 and HEK293T cells in triplicates and culturing over five days. cMWMV and KoRV-A (*Fig. 3*) viral kinetics as measured by qPCR of viral RNA in the

supernatant demonstrated weak increase in viral titre. However, upon 24 hours post infection (hpi), representing the post-wash time point, production of viral copies increased by 2-3 fold at 48 hpi and peaked at 72 hpi resulting in a 3-5 fold increase for all virus inocula. These data suggest that cMWMV and KoRV-A are replication-competent in HEK293T cells (Fig. 3). In NIH3T3 cells, increase of viral RNA was documented 48 hpi (Fig. 3). Furthermore, infection in presence of the reverse-transcriptase inhibitor azidothymidine (AZT) effectively diminished viral RNA production (280-fold for cMWMV and 2-fold for KoRV-A) in HEK293T cells and NIH3T3 cells for both viruses, confirming *de novo* synthesis of viral DNA at indicated time points in the absence of AZT (Fig. 3). Recognizing that other studies have observed NIH3T3 cell resistance to KoRV-A infection (22), we confirmed replication through a variety of additional experiments (SI Appendix, Methods). First, the experiments were performed independently with two NIH3T3 cell lines, one cultured in the lab of the authors (Charité) for years and one a newly obtained culture from the American Tissue Type Collection (ATCC). Increased viral expression was detected in both cell culture batches (ATCC results for NIH3T3 shown in Fig. 3). A and B domains of PiT-1, which are essential for virus entry (33, 34), were sequenced to determine if available NIH3T3 cultures have diverged in sequence. However, the sanger sequences of our cultures were identical to the reported PiT-1 *Mus musculus* mRNA sequence (M73696.1) (SI Appendix Fig. S8). Finally, we used a long fragment inverse PCR fragment approach coupled with PacBio sequencing to determine if and where integration sites occurred for cMWMV and KoRV infected HEK293T and NIH3T3 cell lines. Evidence of retroviral integration would support that viral particles had infected the cells, reverse transcribed and integrated over the course of the cell culture infection experiments. Multiple integration sites were identified for both viruses for all cell lines demonstrating that reverse transcription and viral integration had occurred demonstrating retroviral replication had taken place (SI Appendix Dataset S2). The infection observed for NIH3T3 cells in contrast to similar studies using viral expression constructs may be attributable to the sensitivity of the TaqMan based RT-PCR approach employed in the current study which can detect even minute amounts of expression.

Immunostaining of NIH3T3 cells, with antibodies against PiT-1 and PiT-2 cellular receptors suggest both receptors are present (SI Appendix, Fig. S7). We cannot therefore determine whether cMWMV exclusively binds to PiT-1 or is able to use PiT-2 cellular receptors as well to infect mouse cells such as NIH3T3 cells. Given that single amino acid changes can have major functional consequences for virus-receptor interaction (35), we cannot exclude that cMWMV can additionally or exclusively use another receptor entirely. Electron microscopy of cMWMV in both human HEK293T and mouse NIH3T3 cell lines revealed an electron-dense polygonal core which is enclosed by a spherical envelope (Fig. 4A, SI Appendix, Fig. S9). The particle morphology is similar to the morphology of KoRV-A particles propagated in the same cell lines (SI Appendix, Fig. S9 A, D) and corresponds to the C-type morphology of retroviruses (36, 37). We rarely found budding structures at the surface of the cells (Fig. 4B) which indicate the typical virus particle assembly observed with this morpho-type (36, 37). However, the density below the membrane of the budding site is not separated by a clearly visible gap from the membrane like in typical C-type viruses and

appears more similar to the budding in Human T-lymphotropic virus 1 (HTL-1) or Bovine leukemia virus (BLV), which share a similar particle morphology than C-type viruses (36, 37). Overall, our results suggest that like exogenous GALVs, cMWMV is capable of completing a retrovirus life cycle and forming infectious virions (Fig. 3 and Fig. 4).

Discussion

The cMWMV was detected in *M. leucogaster* in a subset of provinces (Southern Highland and Western Provinces) within its distribution in PNG but was not detected in Chimbu, Gulf and Sandaun Provinces. Identical integration sites were detected among multiple tissues and multiple individuals from the same region which strongly indicates the virus is endogenous and that these individuals inherited the virus as a genomic locus. The absence of cMWMV from other populations indicates it is not yet fixed in *M. leucogaster* and unlike MelWMV, has managed to retain intact open reading frames (ORFs) for all protein coding and non-coding viral sequences. In addition to retaining intact ORFs, cMWMV encoded polypeptides that likely maintained their original functions. This is supported by the minimal alterations in the predicted function and structure of the GAG, POL and ENV proteins because none of the identified mutations are predicted to result in functional changes between the cMWMV and WMV. This high level of structural and functional conservation could suggest that cMWMV, similar to WMV, might employ the ubiquitous sodium-dependent phosphate transporter (PiT-1, also known as SLC20A1) protein as cellular receptor. While we were unable to determine if cMWMV uses PiT-1 exclusively or can also use PiT-2 as a receptor, its ability to infect both NIH3T3 and HEK293T cells productively, and to form viral particles, and bud from the cell membrane suggest that this ERV is potentially infectious *in vivo*. The conservation of the PiT-1 receptor across mammals (38) could also explain why such diverse species including, rodents, bats, primates and marsupials have been infected by relatives of this viral clade.

MelWMV is an endogenized WMV in an isolated *M. burtoni* subspecies (Halmahera) in Indonesia. It has large deletions in the *env* and *pol* genes suggesting it is no longer capable of producing viral particles nor re-integrating in the host genome without a helper virus. MbRV is a WMV-like sequence identified in *M. burtoni* in Australia. However, neither the full genome nor its status as an ERV has been determined. We detected a MelWMV-like virus in *M. burtoni* samples from Australia. However, it lacked the large scale deletions of MelWMV suggesting that both it and MbRV represent at least three distinct integrations into the *M. burtoni* genome. The closer relationship of MbRV, MelWMV and the *M. burtoni* sequence identified in this study and the observation that the MelWMV integration site is different from that of cMWMV in *M. leucogaster*, suggest that the three *M. burtoni* viruses represent distinct colonization events from that of cMWMV in *M. leucogaster*.

Broad-scale phylogenomics indicates that 53% of all gammaretroviral-derived ERVs come from rodents (39), suggesting that rodents have transmitted XRVs and their integrated counterparts among mammals for millions of years. In laboratory mice, ongoing colonization continues to contribute to endogenization,

whereas in most other species this process was completed millions of years ago (5). The frequency of cross-species transmission and that rodent-derived ERVs are not monophyletic, suggests that rodents may still be a source of novel endogenizing retroviruses both among rodents and non-rodent mammals. GALV is thought to have been iatrogenically transmitted to captive gibbons as a result of experimental contamination with human material from New Guinea (11, 40). Most viruses ancestral to GALV-KoRV derive from murine rodents such as Asian *Mus caroli* ERV (McERV) (41) and the frequency of GALV-KoRV ERVs and XRVs in Australo-Papuan rodents, suggests an overall rodent origin for this viral group.

In contrast, several bat families such as Hipposideridae (23), Pteropodidae and Rhinolophidae (24) are documented to have crossed the Wallace Line but only few GALV-like retroviral sequences have been reported in these species. In Queensland, Australia, two *P. alecto* (21, 22) were found to harbor a GALV-like retrovirus though the same species was negative for GALV and KoRV relatives in PNG samples tested in our study. The seven Australian bat species (*M. minimus*, *P. alecto*, *P. conspicillatus*, *P. macrotis*, *P. poliocephalus*, *P. scapulatus* and *P. vampyrus*) tested by Simmons *et al.* (2014) did not yield any GALV or KoRV-like gammaretrovirus. We found no evidence for any GALV-like sequences in bats from Australia, Indonesia, Laos, PNG or Timor-Leste ($n = 156$). While rodents in the Australo-Papuan region regularly have detectable GALV relatives, bats rarely carry such sequences and likely as a result of independent cross-species transmission, such as *R. ferrumequinum* retrovirus (RfRV) which may have a treeshrew origin (42). HIGRV and RhGRV were isolated from the pooled fecal and pharyngeal samples from Chinese *H. larvatus* and *R. hipposideros* bats such that the prevalence of the viruses is unclear (22). All 156 bat samples (ca. 120 species) in the current study, including 14 Australo-Papuan *Hipposideros* and eight Australo-Papuan *Rhinolophus*, tested negative for GALV or KoRV related viruses. Thus, WMVs in *Melomys* rodents are common but in bats exclusively exogenous and are sporadically detected, suggesting recent transmission.

Based on the phylogenetic analysis, bats and rodents are host to viruses in both basal and crown positions within the GALV-KoRV clade. Several bat sequences were successive sister lineages to the GALV-KoRV clade while the ancestral sequences of the GALV-KoRV clade are associated with rodent hosts. This interpretation is consistent with the evolutionary history of the entire gammaretroviral group, which shows a transition from rodent to non-rodent lineages (39). While viruses identified in bats are GALV related and those previously identified in *M. burtoni* are degraded ERVs, cMWMV is a derived, completely intact WMV with 98.9% nucleotide identity to WMV. The phylogeny indicates that all the GALVs either represent derived WMVs or a clade that recently split from WMV. KoRV represents an older lineage and its exogenous counterpart may no longer exist as KoRV began colonizing the koala genome at least 50,000 years ago (43). Why no other taxa within the region carry sequences with higher KoRV identity is unclear, a question that could be answered by further taxa screening. This outcome may have to do either with chance that led to lack of germline invasion in the original host, or some unknown aspect of WMV biology that allows for a more opportunistic endogenization in rodents than other members of the GALV or KoRV clades. It should be noted that applying various alignment and phylogenetic approaches failed to adequately resolve the

relationships between RhGRV-HIGRV bat derived clade, and WMV-GALV clade. The high sequence identity among viruses across the genome makes phylogenetic resolution difficult, emphasizing the very close relationship and minimal divergence among these viral sequences ([SI Appendix Fig. S4](#)).

In conclusion, multiple germline colonizing retroviruses have been detected in mammals in the Australo-Papuan region, a very rarely observed process outside this region. In particular, endogenous WMVs have been found frequently among *Melomys* species across their biogeographical distribution. In all cases these viruses have endogenized in their rodent hosts but only regionally. Our finding suggests that like KoRV in its koala host, the endogenization process in *Melomys leucogaster* is at the earliest stages, providing an additional wildlife model of the complex process of germline colonization by exogenous retroviruses. Bats show a more regionally distinct and discontinuous prevalence even within the same species and may only be sporadically infected by contact with rodent reservoirs. There is no evidence of endogenization of GALVs in bats. Nonetheless, GALV-like viruses have been transmitted as far from the Wallace Line as Southeastern China and New Guinea. GALV-like viruses appear to be circulating, evolving and endogenizing in the endemic New Guinean rodent population and occasionally transmitting to other vertebrates resulting in viruses that endogenize in non-rodent mammals such as KoRV in koalas. The biodiversity within New Guinea is immense including within *Melomys*, which contains many defined species ($n = 23$), taxonomically uncharacterized populations, and close relatives in the *Uromys* division (7) most of which have yet to be screened for viruses. Our results suggest the region will be of particular interest for further identifying germline integration events and assessing the limits to which the Wallace Line prevents viral spillover into Southeast Asia and beyond.

Materials and Methods

Samples and DNA extraction

A total of 278 rodent ($n = 122$) and bat ($n = 156$) samples from the South Australian Museum (SAM) were analyzed. These samples were collected between 1981 and 2017, and represent seven bat families from 37 genera and ca. 120 species, three rodent genera from the family Muridae, representing ca. 38 species with six of them found on both sides of the Wallace Line (details in the [SI Appendix](#)). DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Germany) according to the manufacturer's protocol for frozen or ethanol-preserved blood, hair and tissue samples.

GALV-KoRV PCR screening

Degenerate primer set KOGAWM-1F 5'-CCCCTYAATCGACCTCASTGG-3' and KOGAWM-1R 5'-RTATCTCCTATARGCCTCCAT-3' (product size ~200 bp) were used to screen samples for GALV and KoRV related retroviruses by PCR and visualized using electrophoresis. Details are in the [SI Appendix](#).

Illumina library construction and target enrichment hybridization capture and sequencing

Illumina libraries were generated using standard methods and were hybridized with customized 70-mer biotinylated oligonucleotide meta-viral-baits (probes). Sequencing of enriched libraries was performed with the Illumina MiSeq platform with v2 reagent kit. Details are described in the [SI Appendix](#).

Bioinformatics analysis and virus classifications

The raw sequencing reads were demultiplexed, adaptor sequences, low-quality reads (quality cutoff 20 and minimum read length of 30 nt) and duplicates removed and merged using Cutadapt v1.15 (44), Trimmomatic v0.27 (45), Picard v1.4 (<http://broadinstitute.github.io/picard>), and BBMerge (46) respectively. Two pipelines were applied for identification and assembly of viral reads ([SI Appendix Fig. S1](#)): Virus Integrated Pipeline (VIP) (47) in sense mode and Genome Detective (48), a web-based bioinformatics pipeline (further details are found in the [SI Appendix](#)).

Phylogenetic analysis and integration site mapping

Multiple nucleotide alignments of the consensus sequences with thirty seven genome sequences of gammaretroviruses ([SI Appendix, Table S2](#)) were performed using default settings in MUSCLE (49). Statistical selection of the best-fit model for the phylogenetic analysis performed using jModelTest (50). Bayesian phylogenetic inference produced using Markov Chain Monte Carlo (MCMC) for 1,000,000 iterations in MrBayes v3.2.7 (51). A Maximum likelihood (ML) tree was constructed with rapid bootstrapping (1000 replicates) and GTRGAMMA substitution rate in Randomized Axelerated Maximum Likelihood (RAxML v8.2.11) (52). Retroviral flanking read sequences were mapped using Geneious (further details are described in the [SI Appendix](#)).

Retroviral protein structure modeling

The structure characteristics of the cMWMV viral genome were examined in comparison to the WMV genome. SWISS-MODEL server (53) was used for the prediction of the three-dimensional (3D) structures of WMV and cMWMV (249, a representative sequence with high sequence coverage). From the output structures predicted, only high quality protein models as defined by QMEAN4 (54) values were considered for further analysis. Both WMV and cMWMV genomes produced high quality structures in various domains for all three viral polypeptides (GAG, POL and ENV). Pairwise structural alignment, superimposition, and figure design were performed using PyMol v2.4 (55, 56). Further details are described in the [SI Appendix](#).

Virus production and immunofluorescence microscopy of cells

The 8459 bp construct for cMWMV ([SI Appendix Text file S2](#)) was based on a majority consensus sequence of all individual identified consensus sequences (shown in FIG 1) from *M. leucogaster* and the sequence identified from *M. burtoni*. While, minor sequence variation was observed among the consensus sequences

used to generate the overall consensus sequence, none altered any amino acids and therefore, the functional results resulting from the use of the overall consensus sequence should be representative for the individual isolates. The cMWMV and KoRV-A (AB721500) genomes were chemically synthesized and sub-cloned in pUC57 vector (GenScript, China). These constructs were used to transfect NIH Swiss mouse embryonic fibroblasts (NIH3T3) and Human embryonic kidney (HEK293T) cells ([SI Appendix](#)). To determine if tropism of cMWMV is comparable to GALVs or, similar to WMV, is restricted to PiT-1, HEK293T and NIH3T3 cells were immunostained for PiT-1 and PiT-2 proteins as described in the [SI Appendix](#).

Taqman RT-qPCR

We designed primers and fluorescent probes on *pol* gene of cMWMV and *env* gene of KoRV-A. TaqMan primers and probes with 5'-6-FAM and 3'-BBQ650 modifications were synthesized (Biomers, Germany). Viral RNA extraction and complementary DNA (cDNA) constructions performed as described in [SI Appendix](#). Quantification of absolute viral copies was performed with the LightCycler 480 II system (Roche, Germany). Previous cell culture infection studies of KoRV-A have suggested NIH3T3 cells are resistant to infection (57, 58). However, we hypothesize that the Taqman RT-qPCR assay is more sensitive than the previously employed reporter systems used, which may explain why we detected weak replication of KoRV-A in NIH3T3 cells.

Thin section electron microscopy (EM)

HEK293T and NIH3T3 cells were infected with indicated volumes of cMWMV- and KoRV-A-containing supernatants ([SI Appendix](#)). At 48 hpi, cells were fixed as described in [SI Appendix](#). Thin section microscopy and image processing was performed at the Laboratory for Diagnostic Electron Microscopy of Infectious Pathogens Robert Koch-Institut.

PiT-1 sequencing from NIH3T3 cells

We designed primers based on *Mus musculus* mRNA of PiT-1 (GenBank accession M73696.1) and amplified motifs A and B of the receptor as indicated in [SI Appendix](#).

DNA sonication inverse PCR to identify cMWMV and KoRV-A integration sites in HEK293T and NIH3T3 cell line infection experiments

Sonication based inverse PCR, followed by PacBio sequencing was conducted as described in [SI Appendix](#) to identify virus integration sites.

Data Availability

All data are included in the main text and the *SI Appendix*. Raw sequence data is deposited to SRA and is accessible with BioProject [PRJNA870749](#) (59). Sequences of cMWMV have been deposited in the GenBank database with accession [ON903268](#) (isolate 249), [OP921767](#) (isolate 290), [OP921768](#) (isolate 291), [OP921769](#) (isolate 292), [OP921770](#) (isolate 300) and [OP921771](#) which represents MelWMV-NG (isolate 204) (60, 61). BioSample accessions [SAMN37868416](#), [SAMN37868417](#), [SAMN37868418](#), [SAMN37868419](#), linked to the same BioProject shows PacBio sequences of HEK293T cells infected with cMWMV, NIH3T3 cells infected with cMWMV, HEK293T cells infected with KoRV-A and NIH3T3 cells infected with KoRV-A, respectively, which were used to identify the integration sites of the viruses. The alignment for [Fig. 2](#) is accessible at [10.6084/m9.figshare.21704216](#) (62). The consensus sequence used for *in vitro* experiments is provided in *SI Appendix, Text file S2* along with the retrieved flanking sequences (*SI Appendix, Text file S1*).

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Figures

Fig. 1

The approximate locality for rodent (orange circles) and bat (blue triangles) samples tested, corresponding to sample details in [SI Appendix Dataset S1](#). The closeup map shows the approximate locality of the *M. leucogaster* (249, 290, 291 and 292) and *M. burtoni* (204) harboring cMWMV. Map created using the free and open source QGIS version 3.16.10-Hannover. The Lydekker (1896) (red dashes), Wallace (1863) and Huxley's extension to the Wallace Line (1868) (white line) were drawn manually.

Fig. 2

The maximum likelihood phylogenetic relationship of cMWMV inferred from complete genomic nucleotide sequences of 46 gammaretroviruses. Node support was assessed by 1000 rapid bootstrap pseudoreplicates and is indicated at each node. The newick file is visualized with the Interactive Tree Of Life (iTOL) v5 (63). Branch length is with an average of 0.6 nucleotide substitutions per site. The avian reticuloendotheliosis virus (REV) was used as an outgroup and the sequences used for alignments and phylogenetic analysis are listed in [SI Appendix, Table S2](#). Silhouettes represent the host species. The viral contigs identified in this study are marked with a red asterisk. The cMWMV clade is marked with blue and *M. burtoni* 204 represents MelWMV-NG which is displayed in orange.

Fig. 3

Replication kinetics of KoRV-A (top panel) and cMWMV (bottom panel) in the mouse NIH3T3 (obtained from ATCC) and human HEK293T cells, based on two biological replicates. Both cell lines were either treated with 10 μ M of the reverse transcriptase inhibitor AZT prior to infection with 100 μ l of virus-suspension or infected with increasing volume of virus-suspension without AZT pre-treatment. At indicated time points, virus-containing supernatant was harvested for cDNA synthesis, following qPCR. *De novo* synthesis of viral DNA was measured and concentrations of the samples were calculated using standards of known DNA concentrations.

Fig. 4

Electron microscopy of thin sections through cMWMV-infected NIH3T3 cells. **(A)** A single cMWMV particle in the extracellular space close to a cell surface. The particle is limited by a distinct bi-layered bio-membrane, which is studded by dot-like outer surface structures and underlaid by a thin dense matrix layer. The core (= capsid) is dense and polygonal, like in other gammaretrovirus-like particles. **(B)** Budding of a virus particle at the tip of a microvillus (*mv*) with the characteristic semi-annular density (*arrowheads*) below the plasma membrane. Scale bars = 100 nm.