

# 1 Extensive longevity and DNA virus-driven 2 adaptation in nearctic *Myotis* bats

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## 41 Abstract

42 The genus *Myotis* is one of the largest clades of bats, and exhibits some of the most extreme variation  
43 in lifespans among mammals alongside unique adaptations to viral tolerance and immune defense. To  
44 study the evolution of longevity-associated traits and infectious disease, we generated near-complete  
45 genome assemblies and cell lines for 8 closely related species of *Myotis*. Using genome-wide screens of  
46 positive selection, analyses of structural variation, and functional experiments in primary cell lines, we  
47 identify new patterns of adaptation contributing to longevity, cancer resistance, and viral interactions in  
48 bats. We find that *Myotis* bats have some of the most significant variation in cancer risk across mammals  
49 and demonstrate a unique DNA damage response in primary cells of the long-lived *M. lucifugus*. We also  
50 find evidence of abundant adaptation in response to DNA viruses - but not RNA viruses - in *Myotis* and  
51 other bats in sharp contrast with other mammals, potentially contributing to the role of bats as reservoirs  
52 of zoonoses. Together, our results demonstrate how genomics and primary cells derived from diverse  
53 taxa uncover the molecular bases of extreme adaptations in non-model organisms.

## 54 Keywords

55 Aging, Bats, Cancer, Evolutionary Biology, Functional Genomics, Immunity, Infectious Disease

## 56 Introduction

57 Bats (order *Chiroptera*) represent approximately 20% of all known mammalian species and are  
58 one of the most phenotypically diverse clades of mammals<sup>1,2</sup>. Since their emergence 60 million years  
59 ago<sup>3-5</sup>, many bat lineages have independently evolved a wide variety of life history strategies and  
60 phenotypic traits, including exceptional changes in longevity, viral tolerance, and immune defense<sup>6-11</sup>.  
61 Such systems, in which shared traits have evolved *de novo* multiple times, are powerful resources for  
62 dissecting the genetic basis of phenotypes. Rigorous approaches to studying these traits, however,  
63 depend on high-quality, well-annotated genomes to test evolutionary and genomic hypotheses, and on  
64 experimental functional systems to validate these hypotheses.

65 The largest genus of bats - *Myotis* - is estimated to have emerged approximately 33 million years  
66 ago<sup>12,13</sup>, and encompasses over 139 described species spanning six continents and a wide range of  
67 ecological niches<sup>1,12-14</sup>. *Myotis* species demonstrate some of the most extreme variation in lifespan  
68 amongst mammals<sup>6,15-18</sup>, including a six-fold difference in lifespan between the longest-lived species (*M.*  
69 *brandtii*, 42 years<sup>15,19</sup>, **Figure 1A**) and the shortest-lived species (*M. nigricans*, 7 yrs<sup>15,20</sup>) which diverged  
70 approximately 10.6 million years ago<sup>5,14,21,22</sup>. In addition, *Myotis* species have been used as systems for  
71 investigating virus tolerance and other pathogen resistance<sup>23-25</sup> associated with the expansion and

72 contraction of antiviral defenses<sup>26–29</sup>, which have contributed to bats' ecological role as zoonotic  
73 reservoirs<sup>10,11,30–33</sup>.

74 The origin, evolution, and functional basis of these phenotypes can be studied experimentally in  
75 model organisms as well as via comparative evolutionary methods. The power of comparative  
76 evolutionary studies is constrained by several factors including incomplete phylogenetic coverage; poor  
77 temporal resolution; the quality and composition of gene annotations; and availability of functional data  
78 and tools for validation. Rapidly evolving genes, such as those associated with adaptations to pathogens  
79 <sup>34–36</sup>, present particular challenges for homology and alignment based methods. Similarly, poor  
80 phenotypic resolution and long divergence times between study species hinders the power of statistical  
81 approaches to identify patterns of selection and diversification<sup>37–40</sup>. Meanwhile, model organism-based  
82 approaches contribute a different, complementary perspective and provide the power of functional  
83 analyses; however, these studies can suffer from issues related to the suitability and diversity of the  
84 model species' genotype and phenotype.

85 While studies on the genetic basis of longevity in short-lived model organisms have been crucial  
86 for identifying and dissecting several key aging pathways, comparative studies of exceptionally long-lived  
87 organisms have uncovered novel genes and pleiotropic effects governing lifespan<sup>36,41–53</sup>. The  
88 comparative approach, however, has historically been hindered by limitations in available genomic  
89 resources and genetic tools for study. Similarly, studies of infectious disease response are common and  
90 powerful in model organisms, but the lack of diversity and inbred lines limits their scope. Bats in particular  
91 present an important case study in, and opportunity to study, variation in virus adaptation strategies due  
92 to bats' role as zoonotic reservoirs and their specific resistance to viruses<sup>36,54</sup>. While previous studies  
93 have shown unique infectious disease adaptations in bats, including loss of important inflammatory genes  
94 and expansions of and adaptation in some immune gene families<sup>54–56</sup>, they are typically hampered by the  
95 breadth and number of species analyzed, and only rarely functionally validate results from genomic  
96 analyses.

97 Here we combined comparative and functional approaches in *Myotis* to uncover strong genomic  
98 and functional evidence of adaptation to both aging-related and infectious diseases. We present for the  
99 first time a robust quantification of relative intrinsic cancer risk across mammals, finding that *Myotis* are  
100 overrepresented at the extreme of increased cancer risk. Consistent with this observation, we identified  
101 pervasive selection of genes in longevity- and cancer-related processes, especially in lineages which  
102 have undergone the greatest changes in lifespan. Furthermore, we found strong evidence of adaptation  
103 in response to DNA viruses in *Myotis* and other bats. Genome-wide enrichment of adaptation being driven  
104 by DNA viruses is unique to bats in comparison with other large groups of mammals. Finally, using near-  
105 complete assemblies, we identified structural variations encompassing stress response, immunity, and  
106 inflammation genes, including a trans-species copy number polymorphism of protein kinase R (PKR).  
107 Together, our results suggest that pleiotropy and co-evolution of traits in *Myotis* has played a key role in  
108 the evolution of exceptional longevity and infectious disease resistance.

## 109 Results

### 110 High quality chromosome-level assemblies of 8 *Myotis* bat species

111 To study how lifespan and viral response have evolved in *Myotis*, we collected skin punches and  
112 derived primary cell lines from several North American (“Nearctic”)<sup>21</sup> species (**Figure 1A,C**), including  
113 from one of the longest-lived mammals, *Myotis lucifugus*<sup>15</sup>. Using these cell lines and flash frozen tissues  
114 we generated *de novo* haplotype-resolved, chromosome-scale genome assemblies for eight species  
115 (**Figure 1A**) using a combination of long-read PacBio HiFi sequencing and HiC scaffolding. These  
116 genomes are highly contiguous, with an average of 98.6% of nucleotides assembled into 22-23 syntenic  
117 chromosome-scale scaffolds corresponding to the published karyotype<sup>57</sup> with an average QV of 66.  
118 These genomes have among the lowest auNG scores of any *Chiroptera* genome published to date  
119 (**Figure 1A, E; Table S1**). Across all 8 genomes, each autosome has been completely assembled  
120 telomere-to-telomere (T2T) in at least one species (**Figure 1E**). Within assemblies, 29%-70% of  
121 chromosomes are fully assembled with an average of less than one gap per chromosome (**Table S1**).  
122 When comparing the assemblies of species generated from tissue samples versus primary cell lines, we  
123 found that they were broadly comparable and structurally similar. However, genomes assembled from  
124 cell lines had slightly improved statistics likely attributable to the increased quality and molecular weight  
125 of extracted DNA (**Figure 1A, D, E; Table S1**).

126 Genomes were annotated using well-established pipelines<sup>36</sup> leveraging multiple lines of evidence,  
127 including short-read RNAseq, gene prediction (AUGUSTUS-CGP<sup>58</sup>, GeneMark-ES<sup>59</sup>; gene projections<sup>60</sup>,  
128 TOGA<sup>61</sup>); and homology (miniprot<sup>62</sup>). In total, we identified an average of 27,536 protein coding genes  
129 per species. We benchmarked our annotations using BUSCO<sup>63,64</sup> (V5.4.3) mammalian ortholog sets  
130 indicating these annotations are 98.2%-98.5% complete (**Figure 1C**). We also annotated a recent  
131 assembly of *Myotis yumanensis*<sup>65</sup> for inclusion in downstream analyses. Overall, these fully annotated  
132 genomes represent some of the most contiguous mammalian assemblies to date.

### 133 Resolving the phylogeny and the evolution of body size and lifespan in 134 nearctic *Myotis*

135 The phylogenetic relationships within *Myotis* have been the subject of much debate, with a  
136 number of conflicting phylogenies described in the literature based on different choices of genetic  
137 markers<sup>14,66-69</sup>. To resolve the phylogeny of Nearctic *Myotis*, we identified single copy orthologs of 17,509  
138 protein genes present in 536 mammalian genomes resulting in 30.6M aligned nucleotides. These  
139 alignments were used to build a maximum likelihood tree of *Eutheria*. The *Chiroptera* sub-clade was then  
140 time-calibrated using available fossil-based node calibrations (**Figure 1B; Figure S1; Table S2**). Our  
141 results conclusively recapitulate known sister species pairs including *M. lucifugus* and *M. occultus*; *M.*  
142 *yumansis* and *M. velifer*; and *M. evotis* and *M. thysanodes*. Our proposed phylogeny resolves the  
143 complex relationship between these sister taxa, with 100% bootstrap support at all nodes throughout  
144 *Chiroptera*.

145 Using our resolved Nearctic *Myotis* phylogeny, we re-examined the evolution of body size and  
146 lifespan in *Chiroptera*. In mammals and other metazoans, there is a strong allometric scaling (positive  
147 correlation with body size) of lifespan. Bats have been noted as an exception to this rule: they are  
148 exceptionally long-lived for their body size<sup>17,18,70</sup>, and this exceptional longevity has evolved *de novo*  
149 multiple times<sup>6,70,71</sup>. However, these observations have not been tested using phylogenetically corrected  
150 statistics leveraging well-resolved phylogenies. To test the hypothesis of non-allometric scaling of  
151 lifespan in bats, we modeled the evolution of body size and lifespan across a supertree of over 1000  
152 placental mammals (*Eutheria*)<sup>67</sup> (**Figure 2; Table S2**). In agreement with previous studies in  
153 vertebrates<sup>7,17,44,50,72–80</sup>, changes in body size are pervasive across mammals, with extreme changes  
154 seen in whales (*Cetacea*)<sup>78,79</sup>, elephantids (*Proboscidea*)<sup>42,44,72</sup>, and in sloths and armadillos  
155 (*Xenarthra*)<sup>73,80–82</sup> (**Figure 2A; Table S2**). Within bats, major changes in body size are only observed at  
156 the root of the lineage and within *Yinpterochiroptera* (megabats including genera *Pteropus*, *Eidolon*,  
157 *Megaderma*, and *Rhinolophus*). Outside of these clades, only minor changes in body size were observed  
158 (**Figure 2A**). The evolution of lifespan across mammals mirrors the evolution of body size; branches with  
159 large increases in body size (e.g. *Cetacea* ancestor, *Primate* ancestor) have also experienced large  
160 increases in lifespan (**Figure 2B**), leading to an overall positive association between lifespan and body  
161 size (**Figure S2A**). However, despite little change in body size in bats (**Figure 2A, C**), we observed some  
162 of the largest changes in lifespan across mammals towards the tips of the tree (**Figure 2B, D**), consistent  
163 with the theory of multiple independent increases in lifespan across bats. This is especially true in *Myotis*,  
164 where we saw many of the fastest increases in lifespan, including for *Myotis grisescens* (4.15x increase,  
165 100th percentile), *Myotis brandtii* (2.25x increase, 100th percentile), *Myotis lucifugus* (1.56x, 98th  
166 percentile), *Myotis myotis* (1.1x increase, 79nd percentile), and the *Myotis* common ancestor (1.26x  
167 increase, 92rd percentile) (**Figure 2D; Figure S2C; Table S2**). We next used phylogenetically-corrected  
168 generalized linear models and ANCOVA to study the relationship between body size and lifespan across  
169 mammals. While we find that non-bat mammals experience a 0.159% increase in lifespan per 1%  
170 increase in body size on average, bats experience a 0.223% increase in lifespan years per 1% increase  
171 in body size; these rates were not significantly different, however, suggesting that lifespan allometry is  
172 conserved in bats after accounting for phylogeny (**Figure S2E-F**; pANCOVA, p=0.29).

173 Rapid changes in body size and lifespan can have major implications for the evolution of cancer  
174 risk and resistance across mammals. The lifetime cancer risk of an individual is modeled as the product  
175 of body size (i.e. the number of cells within an individual), lifespan, and a constant representing the  
176 intrinsic cancer risk per cell per unit time. Within species, lifetime cancer risk scales linearly with body  
177 size, and with lifespan by a power-law of exponent 6<sup>83–86</sup>. In contrast to this *within-species* relationship,  
178 there is no significant correlation between body size, lifespan, and cancer risk *across species*<sup>86–89</sup> - a  
179 phenomenon known as Peto's Paradox. The observation of similar lifetime cancer incidence rates across  
180 mammals<sup>73,89,90</sup> suggests that species with more cells or longer lifespans have adapted to reduce their  
181 cancer risks (i.e. increased cancer resistance) (**Figure 2E**).

182 We hypothesized that the very rapid evolution of increased lifespan in *Myotis* would thus result in  
183 a dramatic increase in their expected cancer risk compared to other mammals. This can be quantified by  
184 the Reduced Intrinsic Cancer Risk per cell (RICR) between an extant mammal and its most recent  
185 ancestor, calculated as the log<sub>2</sub> ratio of body size and lifespan<sup>44,86</sup> between the two nodes (**Figure 2E**)<sup>44,86</sup>.  
186 Decreases in RICR correspond to an increase in the expected cancer risk. We used estimates of body  
187 size and lifespan across *Eutheria* to quantify changes in (RICR) across placental mammals (**Figure 2F**)<sup>44</sup>.

188 Bats overall were slightly overrepresented in the bottom 10% of RICR with an odds ratio of 1.15,  
189 highlighting the impact of rapid lifespan evolution on cancer risk. The longest-lived *Myotis* (*M. grisescens*,  
190 39 yrs & 1st pct; *M. brandtii*, 42 yrs & 2nd percentile; *M. lucifugus*, 36 yrs & 4th pct) and their most recent  
191 common ancestors (*lucifugus-occultus*, ~26 yrs & 8th pct; *Myotis* common ancestor, ~22 yrs & 14th pct)  
192 demonstrated some of the most pronounced decreases in RICR among mammals (**Figure 2F**; **Figure**  
193 **S2D**; **Table S2**). Similar to other extreme cases of body size and lifespan in vertebrates<sup>44,46,50,52,73,91-94</sup>,  
194 the pronounced changes in RICR seen in *Myotis* imply an extraordinarily strong selective pressure to  
195 evolve cancer resistance mechanisms at multiple points across *Chiroptera* in general, and within *Myotis*  
196 in particular.

## 197 Evolutionary signatures of cancer resistance in *Myotis*

198 We next set out to identify genes under positive selection across our phylogeny of Nearctic *Myotis*.  
199 We used aBSREL<sup>95</sup> to test for branch-specific positive selection among 15,734 single-copy orthologous  
200 genes identified in 536 mammalian genomes. We found that on average, 22.7% of genes were under  
201 selection across the 9 nearctic *Myotis* species and their internal branches after multiple testing correction  
202 at FDR≤5%; and 5.23% of genes were significant and had omega values above 1, signaling positive  
203 selection (**Table S3**). These genes were enriched for several pathways involved in immunity, cancer, and  
204 aging (**Table S3**). Many of these genes lie at the intersection of these two processes, including members  
205 of the Cluster of Differentiation (CD) family, Serpin family, insulin signaling pathway, redox repair, and  
206 iron storage (**Figure 3A**; **Table S3**), suggesting possible pleiotropic influences on genes under selection.

207 To test this, we quantified the contribution of genes under selection to pathways associated with  
208 the hallmark of cancer<sup>96-98</sup> by measuring the proportion of cancer-associated pathways overrepresented  
209 among genes under selection throughout the phylogeny (**Figure 3A**; **insets**). Many nodes within nearctic  
210 *Myotis* were enriched for cancer hallmark pathways, especially at the recent ancestors of the longest-  
211 lived species (e.g. *M. lucifugus*, *M. occultus*; **Figure 3A**). Testing the overall contribution of genes that  
212 have undergone selection in each species since the common *Myotis* ancestor, we observed significant  
213 enrichments in the representation of cancer-associated pathways only in species lineages with reductions  
214 in RICR (*M. lucifugus*, *M. occultus*, *M. evotis*, *M. thysanodes*, *M. yumanensis*; **Figure 3B**). This suggests  
215 that while genes under selection in nearctic *Myotis* frequently contribute to cancer-associated pathways,  
216 cancer resistance has only driven consistent selection in the longest-lived lineages with the greatest  
217 increases in cancer risk.

218 We also observed that many key genes involved in ferroptosis - specifically in iron transport,  
219 glutathione metabolism, and lipid peroxidation - were under both positive and negative selection at  
220 multiple instances throughout the phylogeny (**Table S3**). Many of these genes were recurrently under  
221 selection in each species' lineage, such as with ferritin (both heavy and light chains) at three distinct  
222 points in the evolutionary history of *M. yumanensis*. Genes under selection in iron transport are  
223 specifically involved in the regulation of free iron in the cell, specifically in the export and reduction of the  
224 free radical catalyst Fe<sup>2+</sup> (ferroportin, *HMOX1*) and the import, storage, and maintenance of Fe<sup>3+</sup> (ferritin  
225 and transferrin receptors 1 and 2). Additionally, we observe selective signatures in glutathione  
226 metabolism and oxidative stress response including: *SLC3A2* and *SLC7A11*, a heterodimer pair  
227 facilitating cystine import and glutamate export; glutathione synthetase; and glutathione peroxidase 3

228 (*GPX3*). Finally, we observed a pattern of selection in genes involved in synthesizing and maintaining  
229 key polyunsaturated fatty acids involved in ferroptosis, including *LPCAT3*, *ALOX15*, and *PRDX5*.

230 To test for intensified and relaxed selection in genes in long-lived or short-lived *Myotis*, we ran  
231 RELAX<sup>99</sup> on 12,438 genes present across 11 *Myotis* species, identifying 263 genes under intensified  
232 selection ( $k > 1$ ) and 101 genes under relaxed selection ( $k \leq 1$ ) after multiple testing correction ( $p_{adj} \leq 0.05$ ).  
233 Among genes of note showing significant intensified selection were *USP9X* (an X-linked ubiquitin  
234 protease associated with cancer and T cell development<sup>100,101</sup>,  $k=48.6$ ); *CDK16* (an oncogenic cyclin-  
235 dependent kinase that regulates autophagy<sup>102,103</sup>,  $k=44.9$ ); and *FGFR2* (a cell growth receptor associated  
236 with human cancers that is also a viral interacting protein<sup>104,105</sup>,  $k=26.1$ ) (**Figure S3B; Table S4**).  
237 Performing a gene set enrichment analysis for the 364 significant genes, we find a strong association  
238 among genes under intensified selection with FGF2 signaling, chromatin remodeling, and pathways  
239 associated with both retroviruses and coronaviruses (**Figure S3C; Table S4**). Finally, using  
240 RERConverge<sup>106</sup>, we investigated how genes' evolutionary rates correlated with the evolution of body  
241 size, lifespan, or the first two principal components of body size and lifespan across *Myotis*, and found a  
242 number of genes enriched in pathways associated with innate immunity, gamete production, and various  
243 metabolic processes, consistent with our other results (**Figure S3D-E; Table S4**).

244 The longest-lived bat in our study, *M. lucifugus*, had an overrepresentation of pathways  
245 specifically associated with DNA double-strand break (DSB) repair when looking at both lineage-wide  
246 and node-specific enrichments in positive selection using the Reactome database<sup>107</sup> (**Figure 3C; Table**  
247 **S3**). This includes 35 out of 65 genes in the high-fidelity Homologous Recombination Repair pathway,  
248 and 21/37 members of the Homology-Directed Repair via Single Strand Annealing (**Figure 3C; Table**  
249 **S3**). These results suggest that *M. lucifugus* might have an enhanced response to DNA DSBs relative to  
250 other bats. To test this hypothesis, we assessed the tolerance of *M. lucifugus* to neocarzinostatin, a  
251 potent radiomimetic agent that induces DNA double-strand breaks (**Figure 3D**), compared to *M. evotis*,  
252 three non-*Myotis* bats (*Eidolon helvum*, *Pteropus rodrigensis*, and *Rousettus lanosus*), and humans. At  
253 low doses of neocarzinostatin, *M. lucifugus* was the only species tested showing sensitivity to  
254 neocarzinostatin after 24 hours, with a drop in viability and concomitant increase in apoptosis. At high  
255 doses, *M. lucifugus* had the highest level of apoptosis and the greatest drop in viability of all the bats  
256 tested, although all bats were more resistant to DNA damage than humans. This is consistent with other  
257 long-lived species, including elephants<sup>42,43,90</sup>, naked mole rats<sup>51</sup>, and bowhead whales<sup>46,108</sup>, where  
258 longevity and RICR are associated with an increased ability to clear out damaged cells. Together, these  
259 results support the hypothesis that *M. lucifugus* has evolved an enhanced DNA double-strand break  
260 response as predicted by genes exhibiting signatures of positive selection in this species.

## 261 Adaptation to DNA viruses

262 Amongst genes under selection, a substantial portion were involved with immunity, including  
263 members of the immunoglobulin and Cluster of Differentiation gene families. These genes exhibited some of  
264 the highest evolutionary rates ( $\omega$ ) in our dataset, suggesting that they are under strong selection in *Myotis* (**Table**  
265 **S3; Table S4**). Because immune pathways are only one aspect of host viral adaptation<sup>109</sup>, we tested for  
266 adaptive signatures in virus-interacting proteins (VIPs) in *Myotis* and other bats. VIPs are host proteins  
267 that physically interact with viral proteins (e.g. *CD45*, **Figure 4A**), and can be proviral (contributing to viral

268 infection, e.g. viral receptors), antiviral (protective against viral infection, e.g. interferons), or both  
269 depending on infection stage and virus type. Previous studies investigating positive selection across  
270 mammals have found an enrichment for adaptation among a set of 5,528 manually curated VIPs, defined  
271 as host proteins that have at least one experimentally verified physical interaction with a viral protein,  
272 RNA, or DNA<sup>109</sup>.

273 By calculating an enrichment score from the ratio of positive selection in VIPs compared to their  
274 matched control genes using BUSTED-MH<sup>110</sup>, we found that, like other mammals, *Myotis* show an  
275 enrichment for adaptation at VIPs (**Figure 4B**; **Table S5**). Physical host-virus interactions may not always  
276 result in fitness effects in the host. We therefore repeated our analysis using a gene set restricted to VIPs  
277 with experimental evidence of specific pro- or anti-viral effects, and thus with a stronger expectation of  
278 fitness effects. We observed an even stronger significant elevation in the ratio of positive selection in  
279 these proviral and antiviral VIPs (**Figure 4C**; **Table S5**), but no elevation in this ratio in other VIPs (**Figure**  
280 **4D**; **Table S5**). This is consistent with the expectation of viral interaction as the cause of enrichment of  
281 positive selection in VIPs in bats<sup>111</sup>. We repeated this analysis using a dataset of 47 publicly-available  
282 non-*Myotis* bat genomes, and confirmed these same patterns across bats more broadly, even when  
283 excluding *Myotis* genomes (**Figure 4B** inset).

284 Previous work has suggested that bats may have different physiological responses to DNA and  
285 RNA viruses<sup>112</sup>. To determine if this was reflected in genomic VIP adaptation, we compared the  
286 enrichment of positive selection in VIPs that interact only with DNA viruses (DNA VIPs) to those that  
287 interact only with RNA viruses (RNA VIPs). Remarkably, we found that VIP adaptation in *Myotis* and  
288 other bats is driven by selection in DNA VIPs (**Figure 4E** and inset). This is in marked contrast to the  
289 observed pattern in RNA VIPs, which show no evidence of enrichment in adaptation (**Figure 4F** and  
290 inset). Note that this difference between DNA and RNA VIPs cannot be explained by a difference in the  
291 conservation of VIP status between the two. The vast majority of VIPs were discovered between human  
292 proteins and viruses that infect humans<sup>111</sup>, and a concern could then be that those proteins that are RNA  
293 VIPs in humans have evolved faster than DNA VIPs in bats, ultimately resulting in the more frequent loss  
294 of their VIP status in bats. We can however exclude this possibility, since DNA and RNA VIPs have very  
295 similar average dN/dS ratios (*Myotis*, 0.2 vs. 0.18 respectively; non-*Myotis* bats, 0.163 vs. 0.153  
296 respectively).

297 In contrast to what we observe in bats, VIP adaptation in humans is driven by positive selection  
298 in RNA - and not DNA - VIPs<sup>109,113</sup>. To investigate if DNA VIP-driven adaptation in bats is exceptional  
299 among mammals, we replicated these analyses across four other large mammalian orders that are well  
300 represented among publicly-available mammalian genomes: *Primates*, *Glires*, *Eeungulata*, and  
301 *Carnivora*. We found that while other mammalian orders show a mix of adaptation enrichments in both  
302 RNA and DNA VIPs, none show an absence of genome-wide enrichment of adaptation in RNA VIPs as  
303 observed in bats (**Figure S4**). These results highlight that bats, including *Myotis*, may have faced greater  
304 selective pressures from DNA viruses than from RNA viruses, in contrast to other mammals.

## 305 Evolution of structural variation within constrained karyotypes

306 With only six known exceptions, all *Myotis* species with cytological data have a conserved  
307 karyotype (60+ *Myotis* spp.: 2n = 44<sup>114–118</sup>; *M. annectans*: 2n = 46<sup>116</sup>; *M. laniger*: 2n = 48<sup>117</sup>; *M. bechsteinii*:



308  $2n = 42$ <sup>119</sup>; *M. daubentoni*:  $2n = 42$ <sup>120</sup>; *M. davidii*:  $2n = 46$ <sup>121</sup>; *M. macrodactylus*:  $2n = 44/45$ <sup>122,123</sup>). This  
309 conserved *Myotis* karyotype, shared among species spread across six continents<sup>1,2</sup>, consists of three  
310 large autosomes and one small metacentric autosome; 17 small telocentric autosomes; and metacentric  
311 X and Y chromosomes<sup>57,124</sup>. Consistent with this broad cytological conservation, we find large scale  
312 synteny across the Nearctic *Myotis* in this study. However, structural variants (SVs) including inversions,  
313 duplications, and translocations are relatively common within chromosomes, especially in putative  
314 centromeric regions (**Figure 5A, B**).

315 We used SyRI<sup>125</sup> to identify SVs across pairwise alignments of Nearctic *Myotis* genomes relative  
316 to the outgroup *M. myotis* and identified 6,813 - 8,013 SVs per genome. Most of these events were small,  
317 with 97 - 99% of events under 10Kb. In the three large autosomes, which constitute ~30% of each  
318 genome, we cataloged an average of 509 SVs (**Table S6**). In contrast, in the small autosomes,  
319 constituting ~65% of each genome, we observed an average of 316 events, highlighting the distinct  
320 structural evolution between these chromosome types (**Table S6**). However, large ( $\geq 10$ Kb) duplications,  
321 large inverted duplications, and large inverted translocations were more common on small autosomes  
322 compared to the large autosomes (**Table S6**).

323 We also quantified the distribution of transposable elements (TEs) across chromosomes.  
324 Surprisingly, LINE elements were significantly enriched around the centromeres of all chromosomes,  
325 both metacentric and telocentric (**Figure 5B**); while this is rare in mammals, it has been recently  
326 described as a feature of Phyllostomid genomes<sup>126</sup>. In many cases, particularly in the 3 large metacentric  
327 chromosomes, LINE elements appear to have displaced other TEs. Rolling circle and SINE elements  
328 were particularly depleted concomitant with LINE enrichment. In contrast, SINE elements were enriched  
329 at telomeres. The concentration of segmental duplications is significantly correlated with TE density in  
330 each species (linear regression,  $p < 0.01$ ; **Figure 5B**; **Figure S5J**) highlighting the possible importance  
331 of TEs in facilitating structural evolution.

332 One particularly striking example of structural evolution we identified is a ~20-Mb block at the  
333 subtelomeric end of chromosome V15 undergoing frequent and recurrent inversions and translocations  
334 in nearctic *Myotis* (**Figure 5A**). This region spans several immune-related genes including multiple  
335 members of interleukin signaling pathways, including IL-1 and IL-36. A 10Mb portion of this block was  
336 recently identified as a potential target of recent selection by adaptive introgression<sup>69</sup>. We identified  
337 between 2-3 major (8+ kb) blocks in this region exhibiting inversions between Nearctic *Myotis*, which  
338 correspond to similarly sized regions in the outgroup *M. myotis* (**Figure 5A**; **Table S5**). Additionally, we  
339 noted a depletion of DNA transposable elements at the boundaries of each inversion (**Figure 5B**),  
340 particularly for rolling circle (RC) and SINE elements. Both of these elements can catalyze large-scale  
341 structural rearrangements via DNA damage repair and homologous recombination, respectively<sup>127-131</sup>.

342 Gene duplications and losses can be drivers of evolution via dosage modification<sup>132,133</sup>, sub- and  
343 neofunctionalization<sup>134,135</sup>, regulatory network remodeling<sup>136</sup>, and other processes<sup>132</sup>. We quantified gene  
344 gains and losses across *Myotis* relative to their single-copy human orthologs. Using CAFE<sup>137</sup>, we found  
345 38 gene families underwent significant expansions or contractions in at least one nearctic *Myotis* species  
346 (**Figure 5C**). However, gain and loss rates varied substantially across branches of the *Myotis* phylogeny.  
347 The terminal *M. auriculatus* and *M. velifer* branches had ~4-fold more significant gene family expansions  
348 (37 and 35 families, respectively; **Figure 5C**) than other *Myotis* branches. In contrast, the terminal *M.*

349 *californicus* and *M. yumanensis* branches had ~2-fold more significant contractions (24 and 23 families,  
350 respectively; **Figure 5C**) than other *Myotis* branches. We observe significant overrepresentation of  
351 pathways at FDR≤10% in only 4 gene sets: gene families that underwent significant expansions in *M.*  
352 *auriculus*, *M. velifer*, and *M. volans*; and genes that underwent significant contractions in *M. lucifugus*  
353 (**Figure S5A-H**). Many of these pathways were shared between all sets, including pathways involved in  
354 translation regulation; ROBO receptors and neuronal development; selenoprotein and selenocysteine  
355 metabolism; and influenza life cycle (**Figure S5A-H**).

356 Given that many of the genes in these pathways are VIPs, we used the method of Huang et al  
357 (2023)<sup>48</sup> to test if VIP genes in particular underwent significant copy number changes relative to non-VIP  
358 genes. We found that while the birth-death rate of VIP genes is similar to that of other genes ( $p = 0.071$ ),  
359 together VIP genes are significantly more likely to have undergone expansions and/or contractions on at  
360 least one branch of the *Myotis* family ( $p < 0.001$ ; **Figure S5I-J**). This suggests that there is variation in  
361 gene family birth rates across species, but that VIPs are more dynamic across the Nearctic *Myotis* as a  
362 whole than other types of genes.

363 To further explore the functional impact of gene duplications we ranked genes by their maximum  
364 copy number across all genomes. We found that the gene families with the highest copy numbers were  
365 concentrated in pathways associated with cancer, aging, immunity, and olfaction (**Figure 5D**). One  
366 striking case is *FBXO31*, with ~2.4x more copies on average than the next most duplicated gene in *Myotis*  
367 (20-48 copies). *FBXO31* is a SCF (SKP1-cullin-F-box) protein ligase involved in cell cycle regulation and  
368 DNA damage response, consisting of two functional domains: a F-Box domain and a CDK binding  
369 domain<sup>138</sup>, and has previously been speculated as a driver of longevity in *Myotis*<sup>93</sup>. Quantifying *FBXO31*  
370 copy number across over 500 mammals using reciprocal best-hit BLAT, we found that this gene was  
371 more highly duplicated in *Myotis* than in any other mammal genome (**Figure 5E**). Furthermore, while  
372 there were additional partial matches of non-canonical copies of *FBXO31* in non-*Myotis* species, all  
373 copies identified in *Myotis* are full-length genes with functional domains. To model the evolution of gene  
374 copy number, we used GeneRax<sup>139</sup> to reconcile the gene tree and species tree. GeneRax infers a gene  
375 family tree under scenarios of gene duplication and loss, taking into account the species tree. We found  
376 support for an original 14 duplications in the common ancestor of Nearctic *Myotis*, with subsequent gains  
377 and losses in each lineage (**Figure 5F**). These results highlight a massively expanded gene family in  
378 *Myotis* with potential consequences for the regulation of stress response and other processes.

## 379 An actively segregating, trans-species copy number polymorphism of 380 the antiviral factor Protein Kinase R, PKR

381 Our highly contiguous genome assemblies provide a unique opportunity to understand the  
382 evolutionary and functional dynamics of structural variation in adaptation. To illustrate this, we explored  
383 the antiviral innate immune Protein Kinase R (*PKR/EIF2AK2*), an interferon-stimulated gene with  
384 adaptive duplications unique to *Myotis*<sup>28</sup>. Among our Nearctic *Myotis* genome assemblies, we resolved the  
385 structure of the two known structural haplotypes: H1, containing a single copy of *PKR* (*PKR2*); and H2,  
386 containing two tandemly duplicated copies of *PKR* (*PKR1* and *PKR2*; **Figure 6A**). We also identified a  
387 third haplotype - H3 - with three tandem duplicates of *PKR* (*PKR1*, *PKR2*, and a third copy). While 7 out  
388 of 9 *Myotis* species carried duplicated haplotypes (H2 in 6 species, H3 in *M. californicus*), to our surprise,

389 5 of these cases were heterozygous for the duplicated haplotype: (i.e. H1/H2 or H1/H3; **Figure 6B**).  
390 Furthermore, two *Myotis* individuals (*lucifugus* and *evotis*) only encoded for *PKR1* (i.e. H1/H1; **Figure**  
391 **6B**). To determine the evolutionary history of the duplicates, we used GeneRax<sup>139</sup> to construct a tree from  
392 alignments of all *PKR* gene copies across Neartic *Myotis*, using *Pipistrellus pygmaeus* as a non-*Myotis*  
393 outgroup (**Figure 6C**). Our results suggest that *PKR2* is the ancestral copy of *PKR*, and that *PKR1*  
394 originated from a single duplication event at the root of *Myotis*. Intriguingly, we observed that in the  
395 heterozygous species, both *PKR1* and *PKR2* on the duplicated haplotype clustered with other duplicated  
396 haplotypes, resulting in species tree violations for the ancestral copy, *PKR2* (**Figure 6C**). These results  
397 highlight that both the duplicated and unduplicated haplotypes have likely been segregating for over 30  
398 million years, representing an ancient trans-species polymorphism.

399 PKR is a stress response and innate immune factor that interacts with viral or inverted Alu repeats  
400 dsRNAs via its dsRNA binding motifs (dsRBMs), leading to PKR auto-phosphorylation and  
401 dimerization<sup>140,141</sup>. Upon activation, PKR can then phosphorylate various molecules leading to protein  
402 translation shutdown and restriction of viral replication<sup>140,141</sup>. While the independent functional impacts of  
403 *PKR1* and *PKR2* were previously investigated<sup>28</sup>, the effects of co-expressing both copies remain  
404 unknown. This is important because their final effects may be additive, synergistic or dominant negative,  
405 providing clues into why the *PKR* duplication is polymorphic both within and between *Myotis* species. We  
406 therefore investigated the functional impact of the duplicates' co-expression on steady state protein  
407 levels, homo/hetero-dimer formation, cell viability, protein translation shutdown and antiviral restriction  
408 (**Figure 6D-G**). We used PKR-KO HeLa cells transfected with either *Myotis myotis* or *Myotis velifer* *PKR1*,  
409 *PKR2*, and *PKR1+2*. We found that the coexpression of *Myotis* Flag-*PKR1* and Flag-*PKR2* did not affect  
410 their protein expression levels (**Figure S6A**). Interestingly, co-immunoprecipitation (coIP) experiments  
411 show that *Myotis myotis* *PKR1* and *PKR2* do not interact (i.e. no heterodimers), even though *Myotis*  
412 *myotis* *PKR1* can dimerize (**Figure 6D, Figure S6B**). Furthermore, coexpression of *PKR1* and *PKR2* led  
413 to a simple additive effect in their translation shutdown activity (**Figure 6E**), suggesting that neither copy  
414 is dominant negative. Using non-toxic doses of *Myotis* PKRs in the context of VSV-GFP (Vesicular  
415 stomatitis virus encoding a GFP reporter<sup>142</sup>) infections, we found that, although *PKR1* and *PKR2* are both  
416 antiviral<sup>28</sup>, the coexpression of *PKR1* and *PKR2* is not beneficial against VSV (**Figure 6F**). Similar results  
417 were found with an unrelated virus, SINV-GFP (Sindbis virus encoding a GFP reporter) (**Figure S6C**).  
418 Finally, because duplicated haplotypes may lead to increased doses of PKR in *Myotis* cells, we tested  
419 PKR impact on cell viability. We found that at low doses none of the *Myotis* PKRs affected cell viability.  
420 However, higher doses of PKRs led to more cell toxicity, potentially resulting in a tradeoff (**Figure 6G**).  
421 Altogether, this may explain why PKR is rarely duplicated in mammals, and why both single- and duplicate  
422 haplotypes of the loci are segregating across several *Myotis* species. These genomic and functional  
423 results highlight the impact of an unfixed gene duplicate which may play a role in adaptation to viral  
424 infections.

## 425 Discussion

### 426 A functionally empowered approach to comparative genomics

427 Bats are widely known for their long lifespan, cancer resistance, and viral tolerance<sup>6,10,11,36,70,89,143–</sup>  
428 <sup>145</sup>. As highly complex and pleiotropic processes, the genes and mechanisms underlying these  
429 phenotypes can be challenging to identify. Comparative approaches to identify the genetic bases of these  
430 traits are constrained by the availability of high-quality genomes, annotations, and functional resources  
431 for validation. These challenges are exacerbated in the case of rapidly-evolving phenotypes, such as  
432 host-pathogen interactions.

433 Here we outline an approach that enables functional comparative biology by generating cell lines  
434 from wing punches of wild caught bats for genome assembly, comparative genomics, and functional  
435 follow up. Cell lines are generated from minimally-invasive biopsies collected in the field thus avoiding  
436 disturbing natural populations. Given the high density of bat species concentrated at single locations  
437 world-wide<sup>146,147</sup> it is feasible to collect wing punches from a large number of individuals across a wide  
438 phylogenetic range; these wing punches can be used to generate cell lines and sequencing libraries for  
439 reference genomes in a matter of weeks. This is an important advance, not only for efforts to expand  
440 genetic resources across the tree of life<sup>148–150</sup>, but for conservation genomics. As our approach can  
441 generate genomic resources from minimal material gathered via non-lethal sampling, it is well-suited for  
442 the study of rare or endangered species for which acquiring sufficient amounts of material can be  
443 challenging.

### 444 Evolution of lifespan and cancer risk in a new phylogenetic context

445 The evolution of body size and lifespan across mammals - and the rapid evolution of lifespan in  
446 *Yinpterochiroptera* in particular - has major implications for the co-evolution of cancer risk and resistance.  
447 While models of body size evolution are well-studied in mammals<sup>7,44,72,74</sup> the evolution of lifespan is less  
448 well understood. By explicitly modeling the evolution of lifespan separately from body size, we  
449 recapitulate the extant relationship between body size and lifespan across mammals in evolutionary time.  
450 Contrary to prior work, we show that bats exhibit relaxed allometric scaling of lifespan comparable to  
451 other mammals. However, *Myotis* demonstrates an increased rate of change in lifespan given body size  
452 compared to other mammals. This altered scaling of longevity in *Myotis* has dramatic consequences for  
453 their intrinsic, per-cell cancer risk and for the evolution of tumor-suppressor genes and pathways. While  
454 cancer risk scales linearly with body size, it scales over time as a power law of  $6^{83,86,87}$ . Meanwhile, while  
455 mammalian body sizes span a  $10^6$  range of masses, they only span a  $10^2$  range of lifespans<sup>16,151</sup>. Unlike  
456 other systems where the evolution of cancer resistance has been driven by rapid changes in body size<sup>42–</sup>  
457 <sup>44,50,91,94</sup>, the body size of *Myotis* has not significantly changed since their common ancestor. Instead, the  
458 rapid and repeated changes in lifespan across an order of magnitude in *Myotis* lead to some of the most  
459 significant changes in intrinsic cancer risk seen across mammals.

460 We found a number of genes under selection across multiple longevity-associated pathways,  
461 consistent with the pleiotropic nature of the aging process. These include members of canonical longevity  
462 pathways such as mTOR-IGF signaling, DNA damage repair, oxidative stress, and the senescence-

463 associated secretory phenotype. We additionally identified selection in various pathways that have likely  
464 emerged as a result of the unique biology of bats, including genes at the intersection of immunity and  
465 senescence, such as Serapin-family genes; genes in metabolic pathways including amino acid  
466 metabolism; and pervasive selection observed in the ferroptosis pathway, which sits at the intersection  
467 of bats' extreme oxidative challenges, metabolic demands, immune function, and cancer resistance. By  
468 quantifying the relative contributions of genes under selection to cancer-related pathways at each node,  
469 we found significant enrichment of these processes across the phylogeny, especially at nodes  
470 undergoing the greatest changes in lifespan and cancer risk.

471 While the implications of an increased cancer risk are clear, the implications of decreases in  
472 relative cancer risk are less so. As expected by Peto's Paradox, we observe an overrepresentation of  
473 cancer-related pathways among genes under selection at nodes experiencing high increases in relative  
474 cancer risk, consistent with patterns observed in other vertebrates<sup>44,46,50,52,73,91-94</sup>. However, we also  
475 observed an enrichment in cancer-related pathway representation among genes under selection in nodes  
476 with significant decreases in cancer risk (e.g: *M. thysanodes*, *M. velifer*). This combination of low intrinsic  
477 cancer risk alongside the persistence of cancer-related adaptations, has been observed previously in  
478 sloths and armadillos<sup>73</sup>. Intriguingly, these species demonstrate some of the lowest known rates of cancer  
479 among mammals. While no reports or studies of neoplasia rates have been published in *Myotis*, the use  
480 of *in vitro* models of carcinogenesis provides a promising avenue for comparative studies of cancer  
481 resistance under controlled conditions. In agreement with our results, *in vitro* and xenograft transplant  
482 models have shown that cells of long-lived bats, including *M. lucifugus*, are more resistant to  
483 carcinogenesis than shorter-lived bats and other mammals<sup>145</sup>. Such studies provide a reliable route for  
484 the experimental validation of the evolution of cancer resistance in species where *in vivo* work would  
485 otherwise prove ethically or practically intractable.

## 486 Viral adaptation and immunity

487 The nature of viral tolerance and infectious disease adaptation in bats has major implications for  
488 understanding their role as zoonotic reservoirs and mechanisms of infectious disease adaptation. Here  
489 we focus on Virus Interacting Proteins (VIPs) that influence viral response and contain vital information  
490 about the nature of host adaptation to viruses<sup>109</sup>. By integrating comparative analyses of VIP adaptation,  
491 VIP and immune gene family expansion and contraction, and functional experiments, we show that virus  
492 adaptation in bats is mostly driven by DNA viruses, as opposed to RNA viruses; we recapitulate and  
493 expand on previous results related to positive selection in immune genes and immune gene family  
494 expansion, contraction, and loss; and demonstrate complex patterns of structural variation, including a  
495 segregating duplication of protein kinase R (PKR), a major protein involved in the antiviral innate immune  
496 system, that has functional relevance in its activity against viruses.

497 The remarkable dominance of adaptation in response to DNA viruses in bats is in contrast with  
498 viral adaptation in humans and other primates, which is driven by RNA viruses<sup>113,152</sup>; and in other  
499 mammals, in which virus adaptation is driven by a combination of DNA and RNA viruses. Most zoonoses,  
500 including those hosted by bats, are RNA viruses<sup>10</sup>, making this especially important in understanding the  
501 dynamics of emerging infectious diseases. This novel finding complements previous observations that  
502 bats are more likely than other mammals to asymptotically harbor RNA viruses, while being more  
503 susceptible themselves to other pathogens, such as fungi<sup>112</sup>. This suggests multiple, non-exclusive,

504 possibilities. First, bats may have some other form of response to RNA viruses that sufficiently reduces  
505 the fitness effect of these viruses such that the associated VIPs did not adapt as strongly. Second, our  
506 result does not imply that bats have not adapted to RNA viruses, rather that adaptation to RNA viruses  
507 does not exceed the genomic baseline adaptation, while adaptation to DNA viruses does. Indeed, bats  
508 are known to mount adaptive immune responses to some RNA viruses and the strength of their immune  
509 response can have complex interactions with hibernation and reproduction<sup>10</sup>. It has been previously  
510 suggested that bats may rely more strongly on adaptive immunity in response to RNA viruses than to  
511 other pathogens<sup>112</sup>, though evolutionary functional analyses have also found evidence of innate immune  
512 adaptation to RNA viruses, including *RTP4* to flaviviruses<sup>153</sup> and *OAS1* to SARS-COVs<sup>154</sup>. This is  
513 consistent with our findings of positive selection and gene family expansion in adaptive immune proteins.

514 While previous work has shown associations between gene family size and certain phenotypic  
515 traits in bats<sup>36,54,155,156</sup>, confirmation of functional effects of copy number is rare. By resolving individual  
516 haplotypes in these nine *Myotis* species, we were able to confirm a single duplication event at the origin  
517 of *Myotis PKR1* and *PKR2*. We further demonstrate functional implications of copy number variation in  
518 Protein kinase R, as previously shown in functional evolutionary studies (eg. Jacquet et al. 2022). These  
519 results are especially interesting in the light of other studies that have found trans-species polymorphisms  
520 related to immune genes<sup>157</sup>. This further illustrates the importance of high-quality genome assemblies  
521 and annotations, to distinguish copy number variation between haplotypes, as well as between functional  
522 copies and pseudogenes<sup>158</sup>.

## 523 The role of agonistic pleiotropy in driving adaptations in bats

524 Multiple hypotheses have been proposed to connect the unique physiology and ecology of bats  
525 with the evolution of remarkable adaptations such as viral infection tolerance, stress tolerance, and  
526 exceptional longevity<sup>143</sup>. Hypothesized drivers of disease resistance and longevity evolution in bats  
527 include the evolution of flight (e.g. “flight as fever” hypothesis<sup>159</sup>, though this hypothesis has recently been  
528 critiqued<sup>160</sup>), the disposable soma hypothesis<sup>161</sup>; metabolic state<sup>162</sup>; torpor<sup>6</sup>; and other adaptations to  
529 specific environments<sup>9,156,163,164</sup>. Additionally, many studies have highlighted the intersection of one or  
530 more of these traits, including a relationship between hibernation and both longevity<sup>6</sup> and disease  
531 resistance<sup>112</sup>. Our results are consistent with an *agonistic* pleiotropy hypothesis, wherein genetic  
532 adaptations for many specific traits (e.g. physiological stress to flight, hibernation, DNA virus innate  
533 immunity) may prove beneficial to other seemingly-unrelated traits (e.g. cancer resistance, cellular  
534 homeostasis, longevity).

535 Consistent with this, many of the genes and pathways highlighted in this study have been found  
536 to play vital roles across physiological traits in bats and other species. For example, two genes under  
537 selection in nearctic *Myotis* - *FTH1* and *IGFN1* - have been implicated in functional studies as key  
538 hibernation genes<sup>165–167</sup>, viral interacting proteins<sup>168–171</sup>, and as pro-longevity genes<sup>172–174</sup>. Similarly, many  
539 DNA VIPs such as *BRCA1/2* and *POLG* represent core DNA maintenance genes essential for cancer  
540 resistance and longevity<sup>51,175–181</sup>; the existence of active DNA transposable elements such as *Helitron* in  
541 *Myotis* may provide another selective pressure on DNA repair genes<sup>182</sup>. Beyond individual genes, many  
542 of the overarching pathways under selection in *Myotis*, such as those associated with inflammation,  
543 senescence, and ferroptosis lie directly at the intersection of aging-related immune  
544 processes<sup>36,54,56,75,167,172,183–188</sup>. While these results suggest the possibility that traits such as cancer risk,

545 cellular homeostasis, and antiviral response have evolved in tandem due to pleiotropic selection at  
546 overlapping points in bats' evolutionary histories, further functional validation will be required to  
547 disentangle the functional impacts of these genetic changes and disambiguate the drivers of selection.

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## 1353 Materials and Methods

### 1354 Data availability

1355 All sequencing data and genomes generated in this study are available on NCBI under Bioprojects  
1356 PRJNA973719 and PRJNA1035541. Annotations generated in this study are available at  
1357 <https://github.com/docmanny/myotis-gene-annotations>. All other code is available at  
1358 <https://github.com/sudmantlab/MyotisGenomeAssembly>.

### 1359 Sample collection and cell line derivation

1360 All bats sampled for this study were wild caught under scientific collection permits for California  
1361 and Arizona (see Supplemental Table 1). Bats were sampled using standard mist-netting procedures,  
1362 including taking standard body measurements, following USGS recommendations for White-Nose  
1363 Syndrome and COVID-19 prevention<sup>189,190</sup>.

1364 For *M. lucifugus*, the donor individual was field-caught in California and transported to the  
1365 Genetics Laboratory of the California Department of Fish and Wildlife, where they were euthanized via  
1366 isofluorane. The *M. velifer* individual was caught in Arizona and euthanized in the field via isofluorane. For  
1367 both *M. lucifugus* and *M. velifer*, tissues were collected and preserved via flash-freezing in liquid nitrogen.

1368 For *M. volans*, *M. occultus*, *M. auriculus*, and *M. californicus*, two 3-mm wing punch biopsies were  
1369 taken from the left and right plagiopatagium of each donor individual and placed in a live cell collection  
1370 media consisting of DMEM/F12 (Gibco) supplemented with 15mM HEPES (Gibco), 20% FBS (Gibco),  
1371 and 0.2% Primocin (Invivogen) [yohe2019; curty2023; capel2023]. Wing punches were then  
1372 brought back to a cell culture facility in Berkeley, where they were used to generate cell lines as previously  
1373 described[yohe2019; curty2023; capel2023]. Additional cell lines for *M. lucifugus*, *M. velifer*, *M.*  
1374 *yumanensis*, *M. evotis*, and *M. thysanodes* were similarly collected and generated.

1375 Cell lines for the *M. evotis* and *M. thysanodes* genomes were generously provided by Richard  
1376 Miller. Cell lines for functional work in *Rousettus langosus*, *Pteropus rodrigensis*, and *Eidolon helvum*  
1377 were provided by the San Diego Frozen Zoo.

## 1378 Sequencing and assembly

1379 For 6 genomes (*M. evotis*, *M. thysanodes*, *M. volans*, *M. occultus*, *M. auriculus*, and *M.*  
1380 *californicus*) DNA was extracted from primary cell lines expanded from 3M cells at Passage 2-4 to  
1381 approximately 40M cells per line using a Circulomics BigDNA CCB kit following the UHMW protocol for  
1382 cells. DNA from *M. lucifugus* was extracted from flash-frozen tissue by the Genetics Lab of the California  
1383 Department of Fish and Wildlife. PacBio HiFi libraries were generated and sequenced on a Sequel II  
1384 (PacBio) by the Functional Genomics Core at the University of California, Berkeley. For cell-line-derived  
1385 genomes, Hi-C libraries for these genomes were generated from 1M cells at Passage 3 using the OmniC  
1386 for Illumina kit (Dovetail genomics); libraries were submitted for quality control and sequencing on the  
1387 Illumina NovaSeq platform (Novogene). For the *M. velifer* genomes, DNA was extracted from flash-frozen  
1388 tissues, and all DNA extraction, library prep, and sequencing was completed by Dovetail Genomics  
1389 following standard protocols. For *M. lucifugus*, a previously published Hi-C dataset from 4 pooled  
1390 individuals was used for scaffolding<sup>191,192</sup>.

1391 The PacBio reads were processed using SMRTTools (v6.0.0-1, PacBio) to generate the circular  
1392 consensus sequences using the settings --minPasses=3 --minRQ=0.99. Hi-C reads were processed  
1393 using trimmomatic<sup>193</sup> (v0.35-6) to remove adapter sequences and low-quality bases using the settings  
1394 ILLUMINACLIP:data/trimmomatic-adapters/TruSeq3-PE-2.fa:2:40:15 SLIDINGWINDOW:5:20. To  
1395 generate the primary contig assemblies, we used hifiasm<sup>194,195</sup> (v0.14-hd174df1\_0) in Hi-C mode,  
1396 providing both the CCS reads and the trimmed Hi-C reads as input, and purging duplicates using the -l2  
1397 option. For our reference genomes, we proceeded with the primary contig assembly  
1398 (\*.asm.hic.p\_ctg.gfa).

1399 All reference genomes were scaffolded with YAHS<sup>196</sup> (v1.1a.1s) and the Hi-C datasets. Dovetail  
1400 Omni-C data were processed and mapped to the genome following the manufacturer's instructions using  
1401 bwa<sup>197,198</sup> (v0.7.17-h5bf99c6\_8), pairtools<sup>199</sup> (v0.3.0-py37hb9c2fc3\_5), and samtools<sup>200</sup> (v1.12-

1402 h9aed4be\_1). YAHS was run using both default settings as well as with `--no-contig-ec`; after comparing  
1403 the outputs, we proceeded with the `--no-contig-ec` version for our final assemblies.

1404 To finalize the assemblies, we performed manual curation using PreTextView<sup>201</sup> and the Rapid  
1405 Curation toolkit<sup>202</sup> (version ff964069). The X chromosomes were identified based on size, synteny across  
1406 genomes, and half-coverage observed in XY genomes; putative Y chromosomes were similarly identified  
1407 in XY genomes. Mitochondrial genomes were identified and removed from the final assembly by running  
1408 mitohifi<sup>203</sup> (v3.0) in contig mode on the assembly and removing all scaffolds identified as mitogenomes.  
1409 The consensus mitogenome from mitohifi was designated as the representative mitogenome for the  
1410 assembly after manual curation. Finally, to eliminate spurious duplicates, we used FunAnnotate<sup>204</sup>  
1411 (v1.8.15) and the “clean” function to identify and remove any remaining scaffolds with 90% identical to a  
1412 larger scaffold.

## 1413 Identification and annotation of repetitive elements

1414 We used RepeatMasker<sup>205</sup> (version 4.0.7-open) to annotate repetitive elements in our genomes.  
1415 We first ran RepeatMasker using a curated database of transposable elements from 249 mammalian  
1416 species<sup>36,206</sup> (David Ray, pers. comm.) and the settings “*-engine ncbi -s -noisy -xsmall*” followed by a  
1417 second run using RepeatModeler<sup>207</sup> and RepeatMasker to identify *de novo* repeats missing from the  
1418 curated database. All repeats were then soft-masked in all genomes. To assess the repeat landscape,  
1419 we calculated the summary of divergence from the repeat alignments and created the repeat landscape  
1420 using auxiliary RepeatMasker scripts (calcDivergenceFromAlign.pl & createRepeatLandscape.pl).

## 1421 Structural variation

1422 To understand the distribution of structural variants, including segmental duplication events, we  
1423 used SyRI (Synteny and Rearrangement Identifier<sup>125</sup>) and BISER (Brisk Inference of Segmental  
1424 duplication Evolutionary stRucture<sup>208</sup>). We first masked telomere regions using TIDK (Telomere  
1425 Identification toolKit<sup>209</sup>), and mapped the primary 22 scaffolds of the nearctic *Myotis* genomes to each  
1426 other with minimap2<sup>210</sup>. The scaffold corresponding to the X chromosome was omitted because there is  
1427 no corresponding scaffold in the *M. yumanensis* assembly. To verify homologous chromosomes and fix  
1428 strand orientation, we used *fixchr* from the SyRI package and manually renamed scaffolds accordingly,  
1429 then re-mapped with minimap2. We ran SyRI on the resulting files and plotted the results with plots<sup>211</sup>.  
1430 We ran BISER on the primary 22 scaffolds of the nearctic *Myotis* genomes with `-keep-contigs` and default  
1431 settings to generate bed files with the inferred segmental duplication regions.

## 1432 RNA-seq

1433 To assist our annotation efforts, we generated mRNA-seq for 7 of the species sequenced *de novo*  
1434 in this study. For *M. velifer*, samples of heart, brain, kidneys, lungs, pancreas, and testis collected from  
1435 the donor individual were provided to Dovetail Genomics (CA, USA) for mRNA-seq library preparation  
1436 and sequencing. Using the same cell lines used for the genomes of *M. occultus*, *M. thysanodes*, *M.*

1437 *evotis*, *M. volans*, *M. auriculus*, and *M. californicus*, we generated rRNA-depleted total RNA-seq libraries  
1438 using the NEBNext rRNA Depletion Kit v2 and Ultra II Directional RNA Library Prep Kits. RNA and  
1439 libraries were quality controlled on an Agilent Bioanalyzer using the RNA 6000 Nano and DNA High  
1440 Sensitivity assays, respectively. Samples were sequenced on to 100M 150PE reads per sample using  
1441 the Novoseq platform (Novogene). For *M. lucifugus*, we used the following published RNA-seq data on  
1442 NCBI SRA generated using poly-A selection and paired-end sequencing: SRR6793287, SRR6793288,  
1443 SRR6793289, SRR6793290, SRR6793291, SRR6793292, SRR6793293, SRR6793294, SRR6793295,  
1444 SRR6793296, SRR6793297, SRR6793298, SRR6793299, SRR6793300, SRR6793301, SRR7064951,  
1445 SRR10512805, SRR10512806, SRR10512807, SRR10512808, SRR10512809, SRR10512818,  
1446 SRR10512829, SRR10512840, SRR10512845, SRR10512846, SRR10512847, SRR10512848,  
1447 SRR10512849, SRR10512850, SRR10512851, SRR10512852, SRR10083333, SRR10083334,  
1448 SRR10083335, SRR10083336, SRR10083337, SRR10083338, SRR10083339, SRR10083340,  
1449 SRR10083351, SRR10083352, SRR1916825, SRR1916826, SRR1916827, SRR1916830,  
1450 SRR1916832, SRR1916834, SRR1916836, SRR1916839, SRR1916841, SRR1916842, SRR18761564,  
1451 SRR18761566, SRR18761568, SRR18761571, SRR18761573, SRR18761563, SRR18761565,  
1452 SRR18761567, SRR18761569, SRR18761570, SRR18761572, SRR18761574, SRR1270869,  
1453 SRR1270914, SRR1270919, SRR1270921, SRR1270922, SRR1270923, SRR4249979, SRR4249988,  
1454 SRR5676382, SRR5676383, SRR5676395, SRR5676396, SRR5676402, SRR1869462, and  
1455 SRR1013468.

## 1456 Gene annotation and alignment

### 1457 Gene predictions

1458 To create optimal gene annotations, we combined *ab initio* gene predictions; orthology inferences;  
1459 and transcriptomic evidence for a total-evidence dataset facilitated using FunAnnotate<sup>204,212</sup> with manual  
1460 interventions. To generate high-quality orthology-based evidence, we downloaded the UNIPARC  
1461 database<sup>213</sup> of genes present in all Chiropteran genomes and mapped these proteins to our genomes  
1462 using miniprot<sup>62</sup> (v 0.6-r194-dirty). We assembled our transcriptome data into transcripts using  
1463 TRINITY<sup>214</sup> (v 2.13.2), and mapped these transcripts to our genomes using minimap<sup>210</sup> (v 2.24).

1464 Next, we ran BUSCO<sup>63,64</sup> (version 5.4.3) using the “eutheria\_odb10” database and AUGUSTUS<sup>58</sup>  
1465 to identify BUSCO orthologs in our genomes. GFFs describing the gene structure of single-copy BUSCO  
1466 orthologs was then used by FunAnnotate to train SNAP<sup>215</sup> and GlimmerHMM<sup>216</sup> (v 3.0.4) prior to gene  
1467 prediction. GeneMark-ES<sup>59</sup> (v 4.72) was run using its self-trained model. AUGUSTUS<sup>217</sup> (v 3.4) was run  
1468 using a previously-generated model jointly trained on 6 high-quality bat genome assemblies<sup>36</sup> and  
1469 supplemented with protein and transcriptome hints generated by FunAnnotate from the UNIPARC and  
1470 Trinity datasets.

1471 To leverage high-quality annotations from other genomes, we used TOGA<sup>61</sup> (version 1.0.1) to  
1472 generate gene annotations for each of our species, using inference from hg38 annotations. TOGA  
1473 outputs a table of genes (“reg” genes) associated with the projected transcripts from the reference  
1474 genomes, and a BED file describing the CDS structure of these projected transcripts. To generate a final

1475 GFF file summarizing these data, we converted the original BED file to a GFF file using [program];  
1476 removed the erroneous “Gene” level attributes; and added in new “Gene” entries describing the TOGA-  
1477 designated genes, modifying the “Parent” attributes of the mRNAs to refer to the correct parent gene.  
1478 Transcript projections that were not associated with a TOGA gene designation were then dropped.

1479 Finally, we used LiftOff<sup>60</sup> (v1.6.3) to lift over annotations from the *Myotis myotis* genome  
1480 (mMyoMyo1.0\_primary<sup>36</sup>). Using BUSCO and manual curation, we assessed both the original GenBank  
1481 (GCF\_014108235.1) and NCBI RefSeq (GCA\_014108235.1) annotations, and selected the NCBI  
1482 RefSeq annotation, as it had slightly improved BUSCO scoring and less erroneous intron-exon junctions  
1483 at select genes. We removed all non-protein-coding genes from the initial GFF file, then ran LiftOff using  
1484 the settings “-exclude\_partial -polish -cds”.

1485 We evaluated each line of evidence by assessing their completeness using BUSCO and  
1486 comparing the completeness score to the total number of predicted genes. We found that SNAP and  
1487 GLIMMERHMM performed the poorest for gene annotations, with both the lowest BUSCO scores and  
1488 the highest number of low-quality predictions. The miniprot-UniParc and TOGA-hg38 datasets generated  
1489 the highest quality gene prediction datasets, with near-complete BUSCO scores and reduced low-quality  
1490 protein predictions.

## 1491 Gene prediction curation

1492 We used EvidenceModeler<sup>218</sup> (version 2.0) to generate an initial consensus gene set using only  
1493 the best lines of evidence (AUGUSTUS, weight 2; high quality AUGUSTUS, weight 5; TOGA-hg38,  
1494 weight 12; miniprot-UniParc, weight 5; and LiftOff-mMyoMyo1, weight 5) with hints from protein orthology  
1495 (miniprot-UniParc, weight 6) and RNA-seq (TRINITY, weight 5) for alternative splicing. By default,  
1496 EvidenceModeler does not consider genes that are located within intronic regions of other genes. To  
1497 restore these genes, we intersected the EvidenceModeler consensus gene GFF with the TOGA-hg38  
1498 GFF to identify which genes were present in intronic regions and omitted from EvidenceModeler; these  
1499 genes were then added back to the EvidenceModeler gene set.

1500 To eliminate remaining spurious predictions, we cross-referenced our gene annotations against  
1501 the SwissProt<sup>219</sup> database using DIAMOND<sup>220</sup> (v. 2.1.4) with settings “--ultra-sensitive --outfmt 6 qseqid  
1502 bitscore sseqid pident length mismatch gapopen qlen qstart qend slen sstart send ppos evalue --max-  
1503 target-seqs 1 --evalue 1e-10”. We kept all genes that matched a protein on SwissProt with at least 80%  
1504 identity, matched over 50% of the target sequence, and coded for at least 50 amino acids. Of the  
1505 remaining genes, we kept them only if they contained both a start and stop codon with no internal stop  
1506 codons.

1507 Finally, we further curated our annotations by putting the EVM and TOGA gene predictions in  
1508 competition with each other when they both annotated the same locus, but with different overlapping or  
1509 neighboring annotations. In such cases, one of the gene annotations is likely closer to the truth. To  
1510 determine which, we compared EVM and TOGA gene models with their closest human gene BLAST hits.  
1511 Only proteins with a BLAST match to a human Ensembl v99 annotation with the lowest E-values below  
1512 0.001 were considered. These human homologs were used as a reference for curation as they are well-  
1513 defined and characterized. We observed that occasionally, either the EVM or TOGA model predicted a

1514 transcript much longer than their human closest homolog. Closer inspection revealed that such cases  
1515 represent artifactual mergers of neighboring genes during the annotation process, clearly visible from the  
1516 fact that they map to two distinct human homologs in succession. Such cases were resolved by choosing  
1517 the annotations (between EVM and TOGA) that were not affected by the artificial merger. We further  
1518 observed a specific class of mergers between neighboring, segmentally duplicated genes, with the  
1519 resulting annotations representing chimeric mixes of exons from the duplicates. In such cases we  
1520 selected the annotations that clearly stayed within the boundaries of the separate duplicates, as identified  
1521 by mapping to the closest human homolog. For the remaining annotations where both TOGA and EVM  
1522 both mapped to a single human homolog throughout their entire length, we selected the most complete  
1523 annotation that was closest in length to the human homolog.

## 1524 Orthologous Gene Alignments

1525 Phylogeny and selection analyses described in this manuscript rely on high-quality alignments of  
1526 bat orthologous coding sequences. To first find and align orthologous *Myotis* genes to the greatest extent  
1527 possible, we first complemented the gene annotations described above with likely missing annotations  
1528 that could still be found through BLAT homology searches. Missing gene annotations are always  
1529 expected in non-model species genomes and reflect a feature of annotation pipelines in general, not an  
1530 artifactual issue. For example if the first coding exon of a gene falls into a small local assembly gap, the  
1531 lack of a start codon may prevent the trigger of a CDS annotation, or may lead to the clearly incomplete  
1532 CDS being subsequently filtered out. Similarly, erroneous indels representing sequencing errors may  
1533 interrupt coding reading frames. Genes with missing annotations can still be detected in assemblies  
1534 through classic BLAST or BLAT homology searches, and then aligned with their annotated orthologs  
1535 from other species. To align orthologous *Myotis* genes from ten species (those sequenced here plus  
1536 *Myotis myotis* and *M. yumanensis*), we first decided to use *Myotis velifer* as the *Myotis* species of  
1537 reference, since the RNA-seq data we used was generated with *M. velifer* tissues.

1538 We first looked for missing homologs of *M. velifer* genes in the other *Myotis* genomes by blatting  
1539 *M. velifer* CDS to the other *Myotis* assemblies (BLAT command line including non-default options -q=dnax  
1540 -t=dnax -fine) to find matches outside of already annotated genomic segments. When multiple velifer  
1541 CDS matched to the same locus with multiple overlapping homologous BLAT matches, we selected the  
1542 match with the highest number of identical nucleotides. The remaining matching BLAT sequences were  
1543 further considered if they spanned at least 50% of the velifer CDS, and included 100 codons or more.  
1544 BLAT matches including stop codons were removed. This process added 1,837 putative CDS to consider  
1545 for orthologous alignments for *M. auriculus*, 1,785 for *M. californicus*, 1,796 for *M. evotis*, 1,505 for *M.*  
1546 *lucifugus*, 3,234 for *M. myotis*, 1,826 for *M. occultus*, 1,822 for *M. thysanodes*, 1,800 for *M. volans* and  
1547 1,729 for *M. yumanensis*. The correct reading frames for these putative CDS were then determined by  
1548 aligning to the velifer CDS that generated the initial match with MACSE v2. MACSE has the crucial  
1549 advantage over other aligners of being able to repair broken reading frames due to sequencing indel  
1550 errors or erroneous gene annotations. At this stage, we restricted any further analysis to those velifer  
1551 CDS with human homologs (BLASTP E-value<0.001 with at least one human canonical protein-coding  
1552 gene from Ensembl). One-to-one orthologs with the 23,030 remaining velifer CDS in other *Myotis* species  
1553 were then determined using Orthofinder v2.5.4<sup>221</sup>. The sequences of each group of ortholog were then  
1554 aligned with MACSE v2<sup>222</sup> with default settings. The resulting CDS with potentially repaired reading



1555 frames were then checked with PREQUAL<sup>223</sup> to exclude sequencing errors and erroneous inclusion of  
1556 non-homologous segments in annotations. The remaining parts of orthologous sequences that passed  
1557 PREQUAL filtering were then aligned again using MACSE v2 with default settings. The first round of  
1558 alignment with MACSE ensures that we do not remove portions of CDS that look like they have no  
1559 homology and would thus be removed by PREQUAL, just because of frameshifts that are easy to repair  
1560 first with MACSE. The second round of MACSE is to align the remaining codons once PREQUAL has  
1561 removed erroneous portions of CDS that could have otherwise disturbed the alignment process. We  
1562 further masked (i.e. replaced with indels) codons near indels with putative alignment errors as described  
1563 in Bowman et al.<sup>224</sup>. Of the 23,030 initial *M. velifer* CDSs, this process resulted in 21,756 alignments with  
1564 at least one ortholog in another *Myotis* species.

1565 We also aligned pan-Chiroptera orthologs from 47 non-*Myotis* genomes publicly available on  
1566 NCBI at the time of analysis, to test the generality of our observations to all bats. We used the same  
1567 strategy described above to complement *Myotis* gene annotations with BLAT matches, but this time  
1568 blatting velifer CDS on non-*Myotis* assemblies (with -q=dnax -t=dnax -fine again) to find all the potential  
1569 orthologs in the non-*Myotis* assemblies. We previously found that because BLAT represents a first filter  
1570 to include only portions of homologous CDS with good local similarity, using BLAT matches results in  
1571 higher quality alignments of orthologs than using existing gene annotations of disparate qualities that too  
1572 often include non-homologous portions of introns among other issues<sup>224,225</sup>. As before with only *Myotis*  
1573 species, we recovered putative one-to-one orthologs with Orthofinder. This process resulted in the  
1574 alignment (as previously described with two rounds of MACSE and PREQUAL in the middle) of 19,009  
1575 orthologous CDS with at least one non-*Myotis* orthologous CDS.

1576 To test whether the patterns of virus-driven adaptation observed in bats are unique across  
1577 mammals, we also prepared four more datasets of 70 primate orthologous CDS alignments, 138  
1578 euungulate alignments, 127 glires alignments, and 82 carnivora alignments (see supplementary files XY  
1579 for the species and their respective assemblies used). We used the same pipeline as the one used to  
1580 align 47 pan-chiroptera species as described above, except that instead of starting from velifer CDS, we  
1581 started from human Ensembl v109<sup>226</sup> CDS (the longest isoform available in each case) for primates, *Mus*  
1582 *musculus* Ensembl v109 longest CDS for glires, *Canis familiaris* Ensembl v109 longest CDS for  
1583 carnivores, and *Bos taurus* Ensembl v109 longest CDS for euungulates. These species were chosen for  
1584 the very high quality of their gene annotations.

## 1585 Gene Trees & Phylogeny

1586 A phylogeny of all 536 mammals in our alignments was generated using IQTREE<sup>227</sup> (version  
1587 2.3.1) using all gene alignments with the settings “-B 1000 -m GTR+F3x4+R6.” To generate gene trees,  
1588 we first filtered our gene alignments to exclude alignments with over 50% gaps in the sequence and less  
1589 than 4 species. With the remaining alignments, we used IQTREE to find the best-fitting substitution model  
1590 and tree using settings “--wbtl --bnni --alrt 1000 -B 1000 --safe”. The best substitution models for each  
1591 gene were saved as a NEXUS file. To generate the phylogeny of bats, we first concatenated all gene  
1592 alignments using *catfasta2phym* (<https://github.com/nylander/catfasta2phym>) to concatenate our  
1593 individual gene alignments into species-level alignments, filling in missing species in each sub-alignment

1594 with gap symbols to preserve the alignment structure. Furthermore, we generated a partition file  
1595 describing the region of each gene sub-alignment within the concatenated alignment.

## 1596 Time-calibration of 59 bat genomes

1597 Using our codon alignments of 59 bat genomes, we generated a time-calibrated phylogeny using  
1598 *mcmctree*<sup>228</sup> and PAML<sup>229</sup> (v. 4.10.0) using an approximate likelihood method. Using the pan-bat codon  
1599 alignments and our phylogeny as input, with fossil calibrations based on previously published work<sup>4,36,230–</sup>  
1600 <sup>237</sup>, we ran *mcmctree* twice to generate the Hessian matrix and confirm convergence. This was followed  
1601 by 10 independent chains using the “out.BV” file from the first run. Finally, the output files of all 10 chains  
1602 were combined to compute final divergence time estimates (see Table S2).

## 1603 Ancestral Body Size, Lifespan, and Cancer Risk reconstruction

1604 To explore how body size and lifespan have evolved over time in mammals, we used a super-  
1605 phylogeny of mammal species<sup>67</sup> subsampled to only contain species with extant body size and lifespan  
1606 data collected from AnAge<sup>15</sup> and PanTHERIA<sup>16</sup>. Ancestral body sizes and lifespans were simulated  
1607 separately using StableTraits<sup>238</sup>.

1608 To estimate ancestral longevity quotients (AncLQs), we followed the method of Austad and  
1609 Fisher<sup>18</sup> and used a linear model of lifespan given body size trained on non-flying mammals to predict  
1610 the lifespans at each ancestral node given median estimates of body size. AncLQs were then estimated  
1611 from the ratio of observed lifespan versus predicted lifespan for each node.

1612 Relative Incidence of Cancer Risk (RICR) was calculated across our mammalian phylogeny  
1613 following the method of Vazquez and Lynch (2021)<sup>44</sup>. The cancer risk  $K$  at a given node was calculated  
1614 using the log of the median predicted body size and lifespan. An organism's lifetime risk of cancer  $K$  is  
1615 proportional to  $Dt^6$ , where  $D$  is the body size and  $T$  is the maximum lifespan. RICR is then calculated as  
1616 the  $\log_2$  ratio of the cancer risk between a node and its direct ancestor.

## 1617 Selection Scans & Evolutionary Rates

### 1618 aBSREL

1619 To conservatively test for branch-specific selection, we used aBSREL<sup>95,239</sup> (version 2.5.48) to test  
1620 for selection at each branch within the Nearctic *Myotis* clade for 15,734 gene alignments spanning 536  
1621 mammals. These genes were identified as 1:1 orthologs across the full alignment, with no more than  
1622 50% sequence gaps and at least 4 species present in the alignment. We defined genes under selection  
1623 as those with an FDR-corrected p-value of less than 0.05; genes were specifically identified as under  
1624 positive selection if  $\omega > 1$ .

### 1625 BUSTED

1626 To quantify the total amount of positive selection across the *Myotis* tree or the different species  
1627 trees used in this manuscript, we used an improved version of the BUSTED<sup>110,239</sup> test called BUSTED-  
1628 MH. The original BUSTED test estimates for a given gene the proportion of codons that have evolved  
1629 under positive selection, with  $dN/dS > 1$ , summed over all the branches of a given tree, regardless of the  
1630 branch and regardless of the codons in a multi-species alignment. The version of BUSTED we used,  
1631 BUSTED-MH, includes two crucial improvements over the original BUSTED that make it much less likely  
1632 to generate false positive inferences of positive selection, albeit at the cost of becoming a very  
1633 conservative test. First, BUSTED-MH takes synonymous substitution rate variation into account, which  
1634 prevents mistaking cases where  $dN/dS$  is greater than one just because  $dS$  is low, with cases where  
1635  $dN/dS$  is greater than one because positive selection actually increased  $dN$ . Second, BUSTED-MH takes  
1636 complex substitutions that simultaneously involve more than one nucleotide into account in its likelihood  
1637 models. This prevents attributing positive selection to cases where  $dN/dS$  is greater than one where  
1638 instead a complex substitution changed multiple amino acids in a single event. BUSTED-MH has been  
1639 shown to strongly reduce the rate of false positives that typically plague  $dN/dS$ -based tests of positive  
1640 selection<sup>240</sup>.

1641 We applied BUSTED-MH to 19,646 *Myotis* orthologous CDS alignments with at least five  
1642 orthologs. These orthologs are cases where the Orthofinder gene trees coincide with the species tree.  
1643 This effectively removes issues regarding whether we should use the gene or the species tree, at the  
1644 cost of removing 2,110 genes from the *Myotis* selection analysis. Similarly, we applied BUSTED-MH to  
1645 17,469 non-*Myotis* bat alignments with at least five orthologs. This includes a subset of 14,091 alignments  
1646 with orthologs in two thirds of the non-*Myotis* bat species that we specifically used to show that patterns  
1647 of virus-driven adaptation are representative of all, and not just a limited subset of bats. We also tested  
1648 17,890 primate alignments with at least five orthologs with BUSTED-MH, as well as 19,311 glires, 18,000  
1649 carnivora and 18,504 ungulate alignments.

## 1650 RERConverge

1651 Between-species life history diversity may be undergirded by significant evolutionary rate shifts in  
1652 important genes, where evolutionary change across the gene tree correlates either positively or  
1653 negatively with changes in a particular life history trait across the trait tree. In *Myotis*, we were interested  
1654 specifically in testing whether or not longevity-related metrics could be correlated with evolutionary rate  
1655 shifts for particular genes, and if, among those, we could identify types of genes (gene ontologies) that  
1656 were enriched.

1657 To answer this question, we used RERconverge<sup>106</sup>, an R package which uses gene trees to  
1658 compute relative evolutionary rates (RERs), then tests for correlations between RERs and trait changes  
1659 between species. 40 bat genomes were aligned to produce MSAs, which were then split into three groups  
1660 to be tested independently: all bats ( $n=59$ ), non-*Myotis* bats ( $n=29$ ), and *Myotis* ( $n=11$ ). Gene trees were  
1661 constructed under the GTR+G model with the same topology as determined in our phylogenetic analysis,  
1662 across all 39 available bat species. After concatenating the gene trees, RERs were calculated in  
1663 RERconverge. Trait correlation analysis was performed by regressing these RERs against 4 distinct trait  
1664 axes. Two of the axes were maximum longevity and size, which were obtained from AnAge<sup>151</sup> and  
1665 PanTHERIA<sup>16</sup>; an additional two axes were obtained by plotting species along the first 2 principal

1666 components of size and maximum longevity. Since size generally correlates with longevity, even within  
1667 Myotis, PCA allows us to describe species using orthogonal trait axes that roughly correspond to size-  
1668 independent longevity and longevity-independent size. Using a Wilcoxon rank-sum test, we then tested  
1669 for enrichment in correlation significance amongst different gene sets.

## 1670 RELAX

1671 The evolution of life history diversity across a clade may also manifest in differential selection  
1672 regimes across relevant genes or types of genes. Specifically, the evolution of a particular life history  
1673 may be driven by either relaxation or intensification of selection in different genes. In Myotis, we were  
1674 again interested in whether we could identify genes and gene sets related to increased longevity within  
1675 the clade.

1676 RELAX<sup>99</sup> is used to identify genes under either relaxation or intensification of selection across  
1677 groups groups of species within a clade using MSAs and a labeled species tree. MSAs for 11 available  
1678 Myotis species across ~19,000 shared genes were fit using the BS-REL framework to a branch-site  
1679 model, using the species tree determined from our phylogenetic analysis. 4 longer-lived species, *Myotis*  
1680 *lucifugus*, *M. occultus*, *M. evotis*, and *M. myotis* were set as the foreground class with the remaining  
1681 species set as the background class. RELAX then used these branch classes to estimate a distribution  
1682 of  $\omega$  (dN/dS) for each branch class, constrained by the relaxation factor  $k$ . An LRT is performed for  $k \neq 1$   
1683 against  $k = 1$ , with  $k > 1$  implying relaxation of selection and  $k < 1$  implying intensification of selection.  
1684 The results from this test were then used to perform a Wilcoxon rank-sum test to identify enrichment in  
1685 the significance of the  $k$ -parameter amongst different gene sets.

## 1686 VIPs

1687 To determine if *Myotis* and other bats are enriched for adaptation at Virus Interacting Proteins  
1688 (VIPs), we conducted a test comparing levels of adaptation, inferred by BUSTED, in sets of VIP genes  
1689 compared to matched control genes. Sets of control genes were resampled in a bootstrap procedure  
1690 ([https://github.com/DavidPierreEnard/Gene\\_Set\\_Enrichment\\_Pipeline](https://github.com/DavidPierreEnard/Gene_Set_Enrichment_Pipeline)) to generate 95% confidence  
1691 intervals for sets of genes at progressively smaller BUSTED p-value thresholds<sup>109,111,113,152</sup>. When VIPs  
1692 are subject to greater levels of positive selection than expected relative to the sets of matched control  
1693 genes, we expect a pattern in which the high p-value thresholds show weaker enrichment but smaller  
1694 confidence intervals, because more genes are used in these calculations. As the p-value threshold gets  
1695 smaller, the signal of enrichment is expected to get stronger but at the expense of larger confidence  
1696 intervals.

1697 We generated five sets of VIP genes: A set of all VIP genes with aligned orthologs from at least  
1698 five species in the tested clade (Nearctic *Myotis* or pan-Chiroptera without *Myotis*); a set of VIP genes  
1699 with known pro- and/or anti-viral activity; a set of VIP genes with no known pro- and/or anti-viral activity;  
1700 a set of VIP genes that interact only with DNA viruses; and a set of VIP genes that interact only with RNA  
1701 viruses. Because both the number of species and genes included, as well as their level of homology,  
1702 influences the power of these tests we also tested the influence of the stringency of gene choice by  
1703 generating a separate set of genes for the pan-Chiroptera analyses that included only genes with aligned  
1704 orthologs in at least two thirds of the non-*Myotis* species. Analyses using this more limited set of genes

1705 show the same result in terms of enrichment of adaptation in VIP genes and comparing DNA VIPs and  
1706 RNA VIPs, showing that the observed patterns are valid across bats regardless of the stringency of  
1707 homology.

1708 The bootstrap procedure matches a tested gene set of interest such as VIPs with sets of control  
1709 genes (non-VIPs when testing VIPs) that have the same average values as the set of interest for multiple  
1710 potential confounding factors that could explain differences in adaptation instead of interactions with  
1711 viruses. For example, if the level of gene mRNA expression has an influence on the rate of adaptation,  
1712 we then need to match VIPs with control sets of non-VIPs that collectively have the same average  
1713 expression as VIPs. For each group of tested VIPs we build 1,000 control sets with randomly sampled  
1714 non-VIPs according to a matching procedure described in Enard & Petrov 2020<sup>152,241</sup>. We match the 27  
1715 following factors between VIPs and non-VIPs, for all tested groups of species:

- 1716 ● the length of the aligned CDS.
- 1717 ● the overall CDS GC content in each orthologous alignment.
- 1718 ● the GC content at aligned codons' position 1, 2 and 3 separately.
- 1719 ● the number of species with a one-to-one ortholog out of all the species included in an alignment,  
1720 where species with no ortholog are represented by gaps the whole length of the alignment.
- 1721 ● the number of species with an ortholog at least 90% of the length of the species of reference  
1722 (*Myotis velifer* in bats, human in primates, etc; see above).
- 1723 ● the overall proportion of each orthologous alignment made of indels.
- 1724 ● the three synonymous rates of evolution estimated by the likelihood model of HYPHY Busted.
- 1725 ● the proportions of codons that fall in the three latter synonymous rates.
- 1726 ● average human mRNA expression in 53 GTEx v7 tissues<sup>242</sup>, in  $\log_2$  of Transcripts Per Million  
1727 (TPM).
- 1728 ● lymphocyte human mRNA expression from GTEx v7, in  $\log_2$  of TPM.
- 1729 ● testis human mRNA expression from GTEx v7, in  $\log_2$  of TPM.
- 1730 ● mRNA expression in  $\log_2$  of TPM for six separate *Myotis velifer* tissues: heart, brain, kidneys,  
1731 lungs, pancreas, and testis.
- 1732 ● the number in  $\log_2$  of protein-protein interactions (PPIs) in the human protein interaction  
1733 network<sup>243</sup>.
- 1734 ● the proportion of genes that are immune genes according to Gene Ontology annotations of  
1735 the closest human homolog including Gene Ontology terms GO:0002376 (immune system  
1736 process), GO:0006952 (defense response), and/or GO:0006955 (immune response) as of  
1737 summer 2021<sup>244</sup>.
- 1738 ● the proportion of housekeeping genes defined as genes with stable expression across many  
1739 human tissues, listed in Eisenberg & Levanon<sup>245</sup>.
- 1740 ● the overall dN/dS ratio estimated by Busted for the orthologous CDS alignments.

1741 We match the overall dN/dS between VIPs and control non-VIPs to account for an important  
1742 issue of dN/dS tests: dN/dS-based tests tend to lose statistical power to detect positive selection  
1743 in CDS alignments with higher selective constraint<sup>246</sup>. The amount of positively selected sites  
1744 being equal, positive selection tests based on dN/dS tend to have lower statistical power and  
1745 tend to generate more false negative results when the rest of the coding sequence is more highly  
1746 constrained. VIPs tend to be much more strongly constrained than non-VIPs<sup>109</sup>, which gives a

1747 strong, unfair statistical disadvantage to VIPs when testing positive selection with BUSTED or  
1748 other HYPHY tests. We limit this issue by matching VIPs and control non-VIPs for dN/dS. Thus,  
1749 VIPs have an excess of adaptation compared to non-VIPs when they have a balance of the same  
1750 total amount of non-synonymous changes more tilted towards advantageous rather than neutral  
1751 amino acid changes. In this case non-VIPs still have less constraint (more neutral changes) than  
1752 VIPs, and thus still more power to detect positive selection, but not to an extent as severe and  
1753 unfair as if we did not match the overall dN/dS<sup>109</sup>. In the case where VIPs do not have an excess  
1754 of adaptation, then they have the same balance of advantageous and neutral amino acid changes  
1755 resulting in the same overall dN/dS. This is the case of RNA VIPs in bats in this study; this internal  
1756 negative control shows that the matching of dN/dS works as intended.

## 1757 Gene Duplications

1758 To quantify patterns of gene duplication and loss, we quantified the copy number of genes with  
1759 human orthologs from our gene annotations for each nearctic *Myotis* genome. To calculate per-gene  
1760 expansion and loss rates and their statistical significance, we ran CAFE<sup>137</sup> v5 on the previously described  
1761 set of copy number counts using our time-calibrated species tree pruned to include only the nine nearctic  
1762 *Myotis* species. *M. myotis* was excluded because of its lower quality assembly. We ran CAFE on the  
1763 subset of genes with two or more copies in at least one species using a Poisson distribution for the root  
1764 frequency ( $-p$ ), first generating an error model to correct for genome assembly and annotation error ( $-e$ ).  
1765 We compared the base model (each gene family belongs to the same evolutionary rate category) to  
1766 gamma models (each gene family can belong to one of  $k$  evolutionary rate categories) with different  
1767 values of  $k$ . A final gamma model with  $k=9$  was chosen to balance model log likelihood with the number  
1768 of gene families for which the optimizer failed. The model was run three separate times to ensure  
1769 convergence.

1770 To understand if genes in these pathways have higher birth rates or are more likely to have  
1771 significant changes in gene copy number than expected relative to other genes, we compared the gene  
1772 copy birth rate  $\lambda$  and number of genes that have significantly expanded or contracted in copy number on  
1773 at least one branch within our nearctic *Myotis* phylogeny. Following Huang et al.<sup>48</sup>, we tested if VIP genes  
1774 in particular underwent significant copy number changes or had significantly different birth/death rates  
1775 than non-VIP genes. For each category of VIP genes (all VIPs, DNA VIPs, DNA only VIPs, RNA VIPs,  
1776 and RNA only VIPs), we generated 100 bootstrap sets of control non-VIP genes with the same number  
1777 of genes as the corresponding VIP gene set. We ran CAFE on each set of VIP genes and the  
1778 corresponding control non-VIP genes to infer per-gene birth-death rates and per-gene, per-branch  
1779 expansion/loss events.

## 1780 Assessment of DNA Double-Strand Break Tolerance

1781 We assessed each species' tolerance to DNA double strand breaks using a by measuring viability,  
1782 cytotoxicity, and apoptosis across a range of doses of Neocarzinostatin, a radiomimetic drug. We  
1783 measured dose response curves in wing-derived primary dermal fibroblasts across 5 bat species (*Myotis*  
1784 *lucifugus*,  $n=8$ ; *Myotis evotis*,  $n=3$ ; *Rousettus langosus*,  $n=2$ ; *Eidolon helvum*,  $n=2$ ; *Pteropus rodrigenis*,  
1785  $n=2$ ) using the multiplexed ApoTox-Glo assay (Promega). Using 96-well plates, two individuals and 11

1786 doses were assessed simultaneously with four technical replicates. Results were normalized to treatment  
1787 controls for each individual in R as previously described<sup>42,43,46,50</sup>.

## 1788 Mapping PKR exons

1789 We further validated the annotations for the PKR locus by re-aligning the primary *M. velifer* coding  
1790 sequence back to the nine nearctic *Myotis* reference genomes, as well as a non-*Myotis* outgroup,  
1791 *Pipistrellus pygmaeus*, and the genome haplotypes for each of these species. Because the presence of  
1792 two copies makes this task challenging for most aligners, we independently aligned the *M. velifer*  
1793 reference PKR sequence to sequential sections of each genome in 50kb search regions surrounding the  
1794 known loci in each genome. This alignment search was conducted for 5 regions upstream (250 kb) and  
1795 5 regions downstream (250 kb) of the known loci. In species with two known copies, the location of each  
1796 copy was included in a separate search region. This was to prevent erroneous merging or loss of exons.  
1797 These regions were retrieved using bedtools getfasta<sup>247</sup> and alignment was performed using miniprot<sup>62</sup>.  
1798 Miniprot settings were optimized to retain secondary alignments (-p 0 -n 1 -outsc=0.0 -outc=0.0) and  
1799 find known exons with accurate boundaries (-J 18 -F 21 -O 15 -L 10). The resulting gff file was converted  
1800 to bed format using AGAT<sup>248</sup>, sequences retrieved with bedtools getfasta, and a custom script used to  
1801 remove identical duplicates. Finally, all sequences were aligned with MACSE v2.07<sup>222</sup>. We used  
1802 BISER<sup>125,208</sup> to confirm the presence of segmental duplications in these regions.

## 1803 PKR cell lines and vectors

1804 All PKR experiments were performed using HeLa PKR-KO cells (kindly provided by A. Geballe,  
1805 Fred Hutchinson Cancer Center, Seattle WA) that were plated either at densities of  $5 \times 10^4$  cells/mL in  
1806 24-well plates or at  $1 \times 10^5$  cells/mL in 12-well plates. The cells were maintained at 37°C with 5% CO<sub>2</sub>  
1807 and cultured in DMEM supplemented with 5% fetal bovine serum (FBS), 1% penicillin/ streptomycin mix  
1808 and 1 µg/mL puromycin (Sigma-Aldrich). All transfections were performed 24 hours after seeding, using  
1809 3 µL of TransIT-LT1 Transfection Reagent (Mirus Bio) per 1 µg of DNA and Opti-MEM media. We used  
1810 previously-generated pSG5-FLAGx2 vectors encoding either *M. myotis* PKR-1 (GenBank OP006550),  
1811 *M. myotis* PKR-2 (GenBank OP006559), *M. velifer* PKR-1 (GenBank OP006558), or *M. velifer* PKR-2  
1812 (GenBank OP006557)<sup>28</sup>. Plasmids encoding the interferon-stimulated gene ISG20<sup>241</sup> and a constitutively  
1813 active variant of the sterile alpha motif domain-containing protein 9-like SAMD9L-F886Lfs\*11 (here,  
1814 SAMD9L<sup>249</sup>) were used as controls in viral infections and cell translation experiments, respectively.

## 1815 Western blot

1816 We assessed for the steady state protein expression of *M. myotis* Flag-PKR<sub>s</sub> after transfection  
1817 of 350 ng and 700 ng of DNA plasmids encoding either PKR1 alone, PKR2 alone, or both PKR1 and  
1818 PKR2 (175 ng of each and 350 ng of each, respectively). Briefly, cells were re-suspended and lysed in  
1819 ice-cold RIPA buffer (50 mM Tris pH8, 150 mM NaCl, 2 mM EDTA, 0.5% NP40) with protease inhibitor  
1820 cocktail (Roche) and sonicated. 20 µL of the clarified fraction was denatured with 5 µL of 6x Laemmli  
1821 buffer at 95°C for 5 min and loaded into 4-20% BioRad Criterion TGX Stain-Free precast gel. The wet  
1822 transfer into a PVDF membrane was executed overnight at 4°C. The membranes were blocked in a  
1823 1xTBS-T buffer (Tris HCl 50 mM pH8, NaCl 30 mM, 0.05% of Tween 20) containing 10 % powder milk,

1824 and were incubated for 1h. The membranes were incubated with primary mouse anti-Flag (Sigma F3165)  
1825 and anti-Tubulin (Sigma T5168) antibodies and secondary anti-Mouse IgG-Peroxidase conjugated  
1826 (Sigma A9044). Detection was made using the Chemidoc Imagina System (BioRad) with SuperSignal  
1827 West Pico Chemiluminescent Substrate (ThermoFisher Scientific).

## 1828 PKR co-immunoprecipitation

1829 HeLa  $\Delta$ PKR cells were transfected with 1.25 $\mu$ g plenti6 HA-tagged *M. myotis* PKR1 plasmid per million  
1830 cells and 1.25 $\mu$ g of either plenti6 myc empty vector, myc-tagged *M. myotis* PKR1 or myc-tagged *M.*  
1831 *myotis* PKR2 plasmid using TransIT-LTI transfection reagent (Mirus Bio). The next day, some wells were  
1832 infected with Sindbis virus expressing GFP (SINV-GFP) at MOI 2 for 24 hours. Cells were then scraped  
1833 with cold PBS and pelleted. For the IP, cells were lysed in 500 $\mu$ l IP buffer (50mM Tris HCl pH7.5, 140mM  
1834 NaCl, 6 mM MgCl<sub>2</sub>, 0.1% NP40) supplemented with RNase (RiboLock, Fisher Scientific) and protease  
1835 (cOmplete EDTA-free protease inhibitor cocktail, Sigma) inhibitors for 10 minutes on ice, then centrifuged  
1836 at 12,000  $xg$  for 10 min at 4°C. 5% of the volume was kept for input, while the rest was incubated with  
1837 40 $\mu$ l  $\mu$ MACS anti-c-myc MicroBeads (Miltenyi Biotec) for 1h at 4°C with constant rotation. Samples were  
1838 then loaded onto  $\mu$ MACS columns placed in the magnetic field of a  $\mu$ MACS Separator (Miltenyi Biotec),  
1839 washed 4 times with cold IP buffer, and eluted with the  $\mu$ MACS denaturing elution buffer. Proteins were  
1840 denatured in elution buffer for 5 min at 95°C, then loaded onto a 4-20% BioRad Criterion TGX Stain-Free  
1841 precast gel and transferred onto an Amersham Protran nitrocellulose membrane (Sigma) for 1h.  
1842 Membranes were blocked for 1h in 5% milk in PBS (Euromedex) supplemented with 0.2% tween (Fisher)  
1843 and incubated with mouse anti-myc monoclonal antibodies (Abcam 9E10, cat# ab32) then secondary  
1844 anti-mouse IgG antibodies conjugated with HRP (Sigma, cat# A4416), or with rat anti-HA antibodies  
1845 conjugated with HRP (Roche, Sigma, cat# 12013819001). Images were taken on a Fusion FX imager  
1846 (Vilber) with SuperSignal West Femto Chemiluminescent Substrate (ThermoFisher Scientific).

## 1847 Cell viability assay

1848 HeLa PKR-KO cells were transfected 24h after plating in 96 well plates, with 100 or 200 ng of  
1849 pSG5 plasmid: empty or coding for *M. myotis* or *M. velifer* PKR1, PKR2 or PKR1+2 equal mix (50%-  
1850 50%). 24 hours post-transfection, positive control cells were treated with an apoptosis-inducing drug,  
1851 Etoposide, at different doses (250, 200 or 100  $\mu$ M). 48 hours post transfection, cells were harvested and  
1852 lysed to quantify luminescent signal according to CellTiter-Glo® Luminescent Cell Viability Assay  
1853 (Promega) kit protocol.

## 1854 VSV and SINV infections

1855 VSV infections. Cells were transfected 24 h after plating with 350 ng of pSG5 plasmid: empty, or  
1856 encoding *M. myotis* or *M. velifer* PKR1, PKR2, or equal input of PKR1 and PKR2 (175 ng per plasmid),  
1857 or a plasmid encoding interferon-stimulated exonuclease gene 20 (ISG20), due to its known antiviral  
1858 activity against VSV as positive control<sup>241</sup>. Cells were infected 24 h post transfection with replicative VSV-  
1859 GFP virus<sup>142</sup> at a MOI of 3. Cells were fixed with 4% paraformaldehyde 16-18 hours post infection. VSV  
1860 infection was quantified by measuring the percentage of GFP positive cell populations with BD



1861 FACSCanto II Flow Cytometer (SFR BioSciences). Fold change results were normalized to the empty  
1862 pSG5 condition across at least three independent experiments.

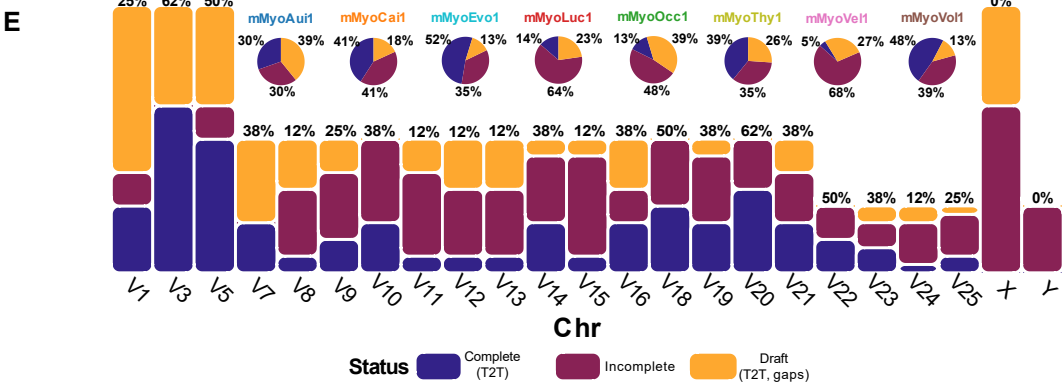
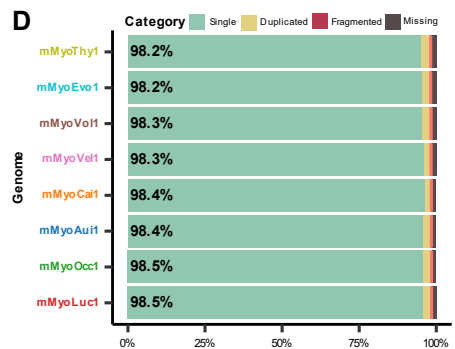
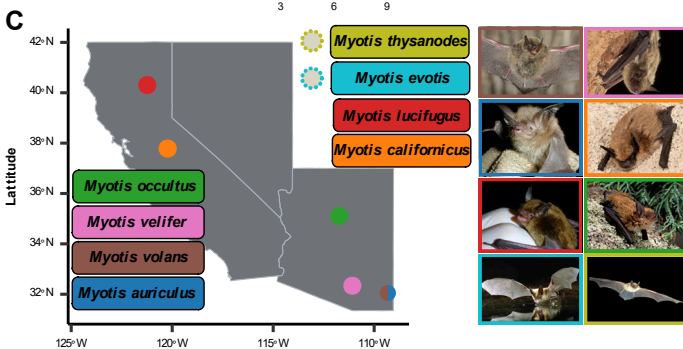
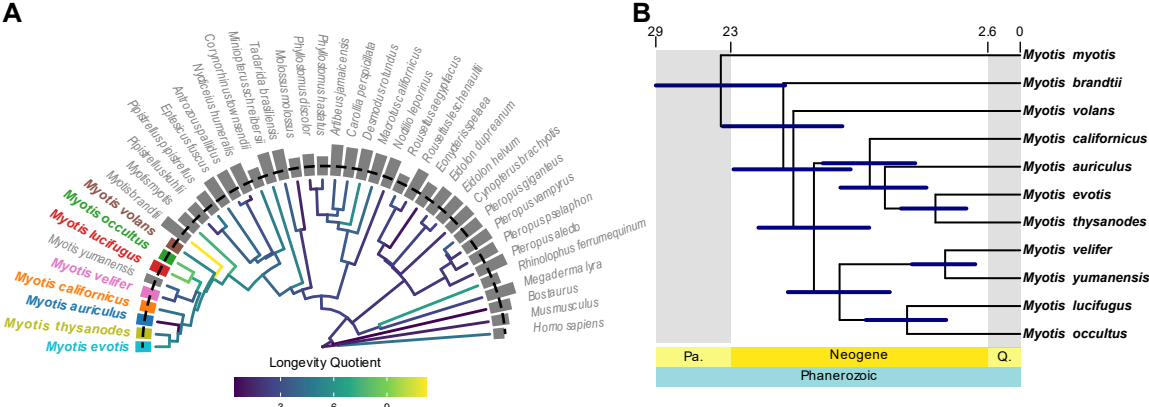
1863           SINV infections. Hela  $\Delta$ PKR cells were transfected with 5 $\mu$ g pSG5 empty vector, *M. myotis* PKR1,  
1864 *M. myotis* PKR2 or 2.5 $\mu$ g *M. myotis* PKR1 + 2.5 $\mu$ g *M. myotis* PKR2 per million cells using TransIT-LTI  
1865 transfection reagent (Mirus Bio). The next day, some wells were infected with SINV-GFP at MOI 0.2.  
1866 Cells were then placed into a CellCyte X live cell imaging system (Cytena) and pictures of every well  
1867 were taken every 2h for 48h. The fraction of GFP+ cells over the total cell area was measured and  
1868 averaged from six photos of 2 individual wells per condition, and repeated for a total of three independent  
1869 experiments.

## 1870 Luciferase reporter assays

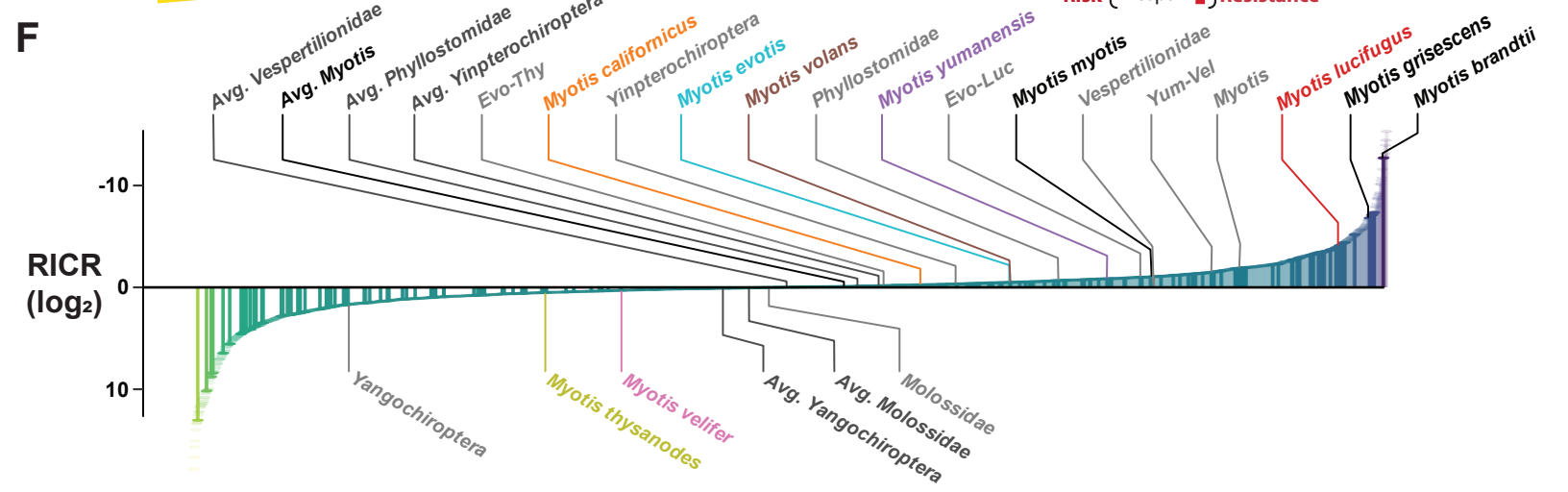
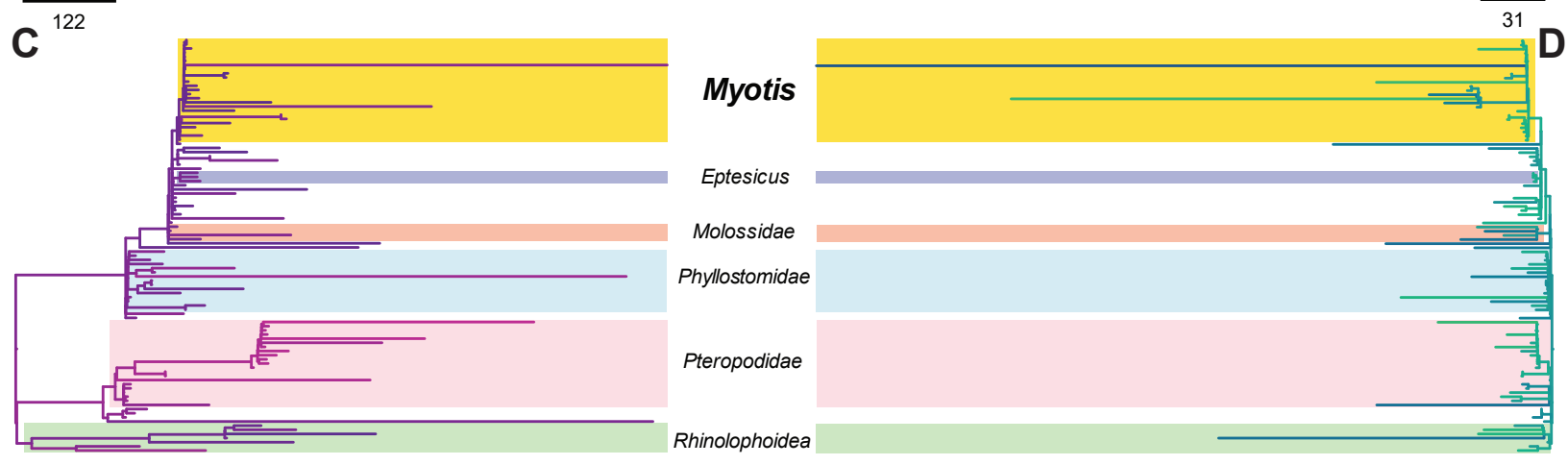
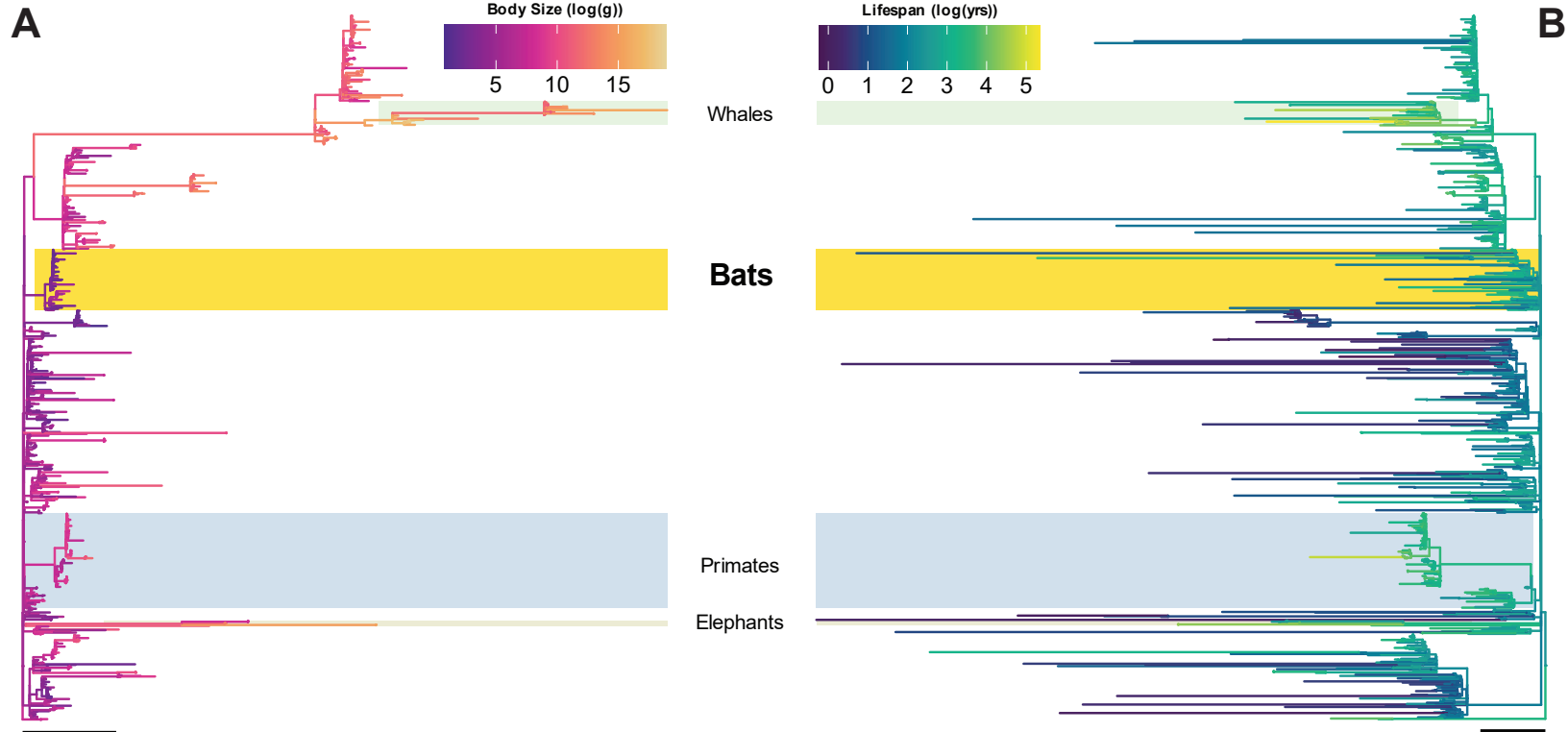
1871           Luciferase reporter assays were carried out to investigate whether the two PKR paralogs have  
1872 synergistic, additive or dominant negative effect in translation shutdown. Transfection was performed as  
1873 previously described with additional 50 ng of FFLuc firefly luciferase reporter plasmid per well. Sterile  
1874 alpha motif domain-containing proteins 9L (SAMD9L gain-of-function mutant) was used as a positive  
1875 control of translational repression<sup>249</sup>. 24 h post transfection, cells were briefly washed with PBS, lysed by  
1876 a 5 $\times$  reporter lysis buffer (Promega) and incubated overnight at -20°C. Cells were then collected and 100  
1877  $\mu$ l of the luciferase substrate (Promega) was added to 20  $\mu$ l of the lysis supernatant. Alternatively, cells  
1878 were lysed using BrightGlow Lysis Reagent (Promega E2620). The relative luminescence units (RLUs)  
1879 were immediately quantified with LUMIstar Omega microplate reader optima (BMG Labtech). All  
1880 luciferase assays were conducted in technical duplicates in at least five independent experiments. Fold  
1881 change results were normalized to the empty pSG5 condition within each independent experiment.

## 1882 Figures

- 1883 **Figure 1: 8 near-complete reference assemblies for North American (Nearctic) *Myotis*.**
- 1884 **Figure 2: Evolution of body size, lifespan, and cancer risk in bats and mammals.**
- 1885 **Figure 3: Selection in Nearctic *Myotis* is enriched for pleiotropic cancer resistance pathways.**
- 1886 **Figure 4: Adaptation to DNA viruses, but not RNA viruses, is enriched in *Myotis* and other bats.**
- 1887 **Figure 5: A varied structural variation landscape across 9 nearctic *Myotis* species.**
- 1888 **Figure 6: Evolutionary history and function of an actively segregating copy number polymorphism**
- 1889 **of *PKR* in *Myotis*.**

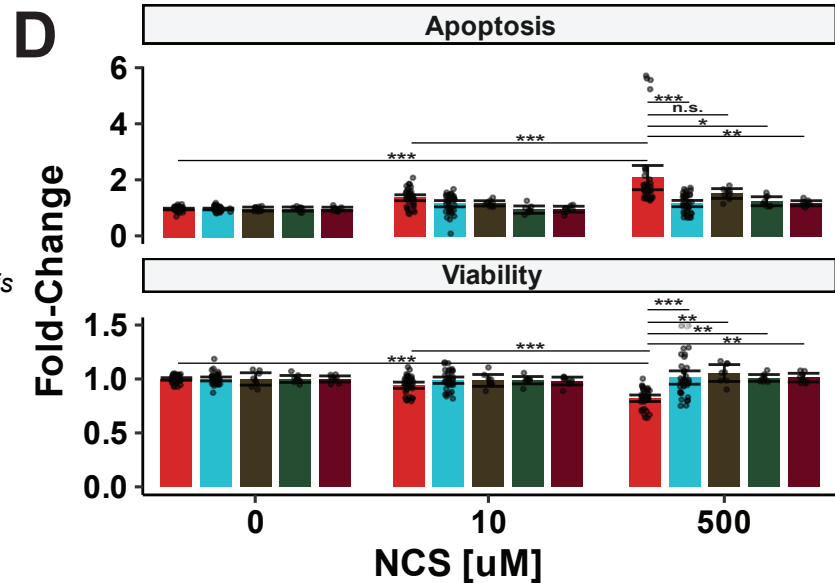
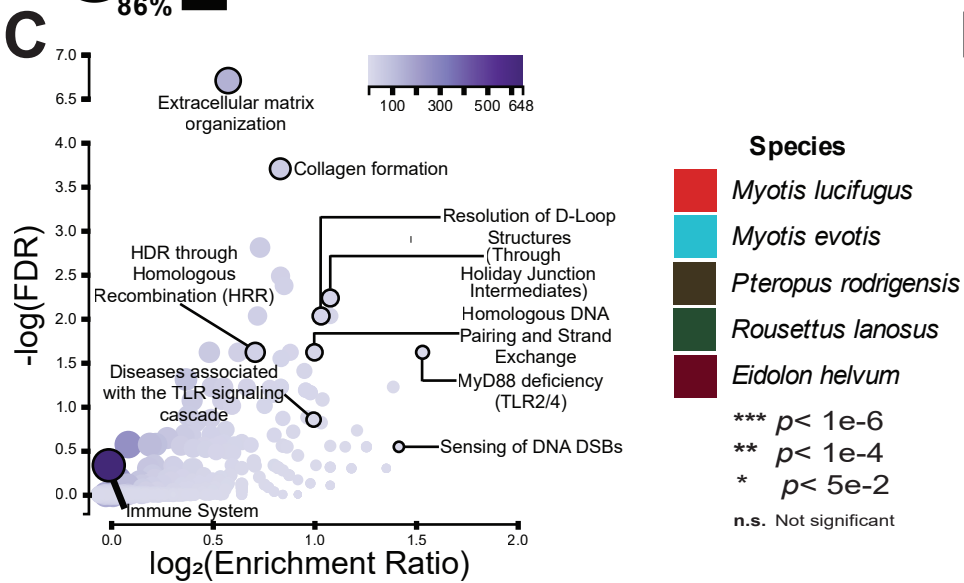
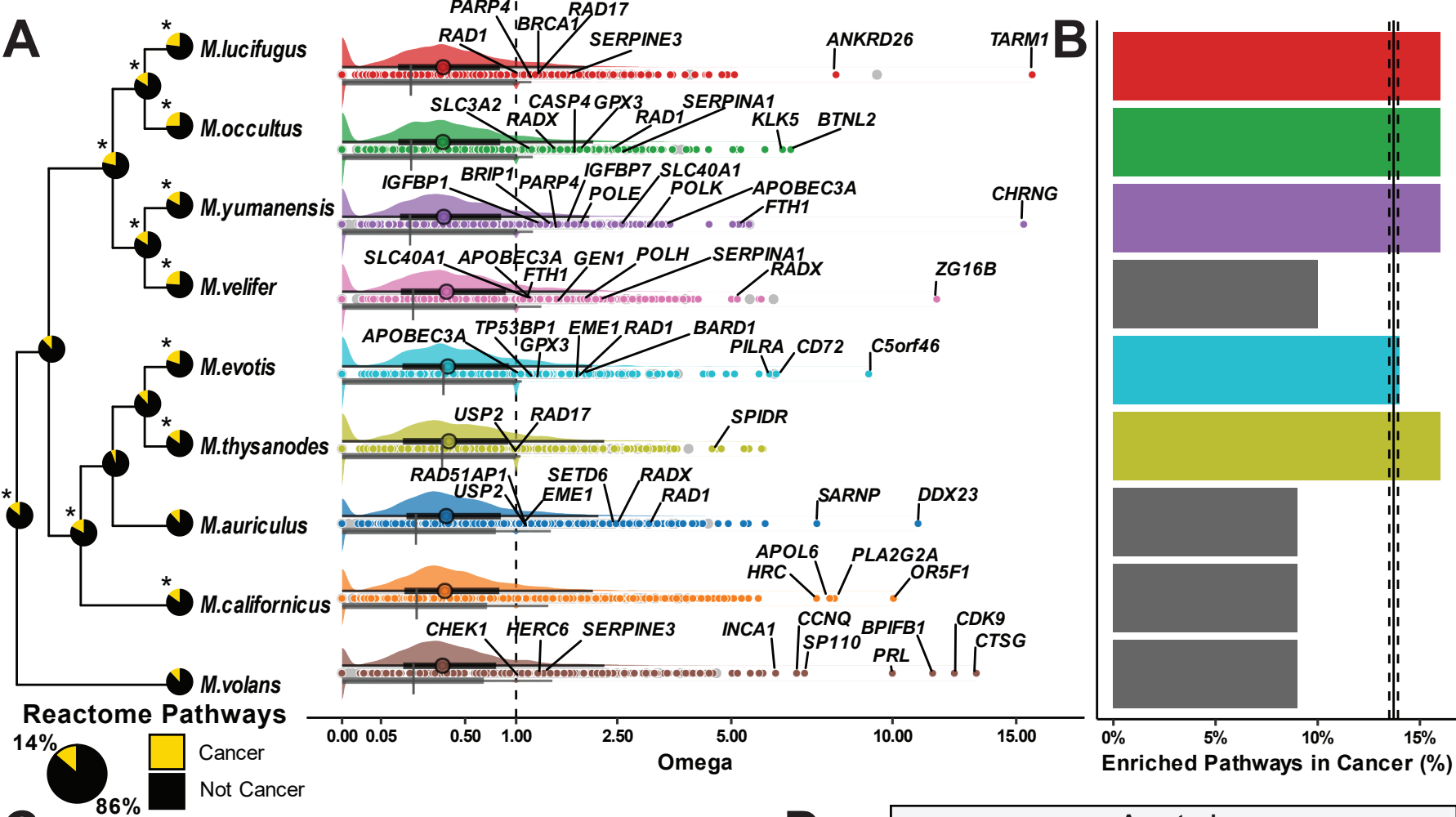


1890 **Figure 1: 8 near-complete reference assemblies for North American (Nearctic) *Myotis*.** **A)** Phylogeny  
1891 of 38 bat genomes with 3 outgroup species: cow (*bosTau9*), mouse (*mm39*); and human (*T2T-CHM13v2.0*).  
1892 Bars at the tips of the phylogeny indicate the AuNG score of each genome (lower values equal more  
1893 contiguous genomes); the dotted line represents the AuNG score for complete (T2T) genome assemblies  
1894 as represented by T2T-CHM13v2.0. **B)** The time-calibrated phylogeny of 9 Nearctic and two representative  
1895 Palaeartic *Myotis* species based on orthologous codon alignments. Blue bars represent age uncertainties.  
1896 **C)** Map of capture sites with representative images (see “Acknowledgements” for attributions) for the  
1897 individuals and species sequenced in this study; cell lines for *M. evotis* and *M. thysanodes* were provided  
1898 by Richard Miller and were not collected for this study. **D)** Mammalian BUSCO scores for annotations  
1899 generated for the 8 new *Myotis* genomes. **E)** Ideogram bar plot indicating completion status of each  
1900 chromosome in assembly. Pie graphs indicate completion status of all chromosomes in assembly. All  
1901 chromosomes were positively identified based on size, synteny, and homology to human chromosomes<sup>57</sup>.  
1902 “Complete (T2T)” status indicates that a chromosome is fully assembled telomere-to-telomere without gaps;  
1903 “Draft (T2T, gaps)” status indicates that a chromosome is fully scaffolded with both telomeres, but has one  
1904 or more gaps in the assembly; “Incomplete” status indicates that a chromosome was positively identified,  
1905 but was not scaffolded from telomere to telomere (only contains one telomere).



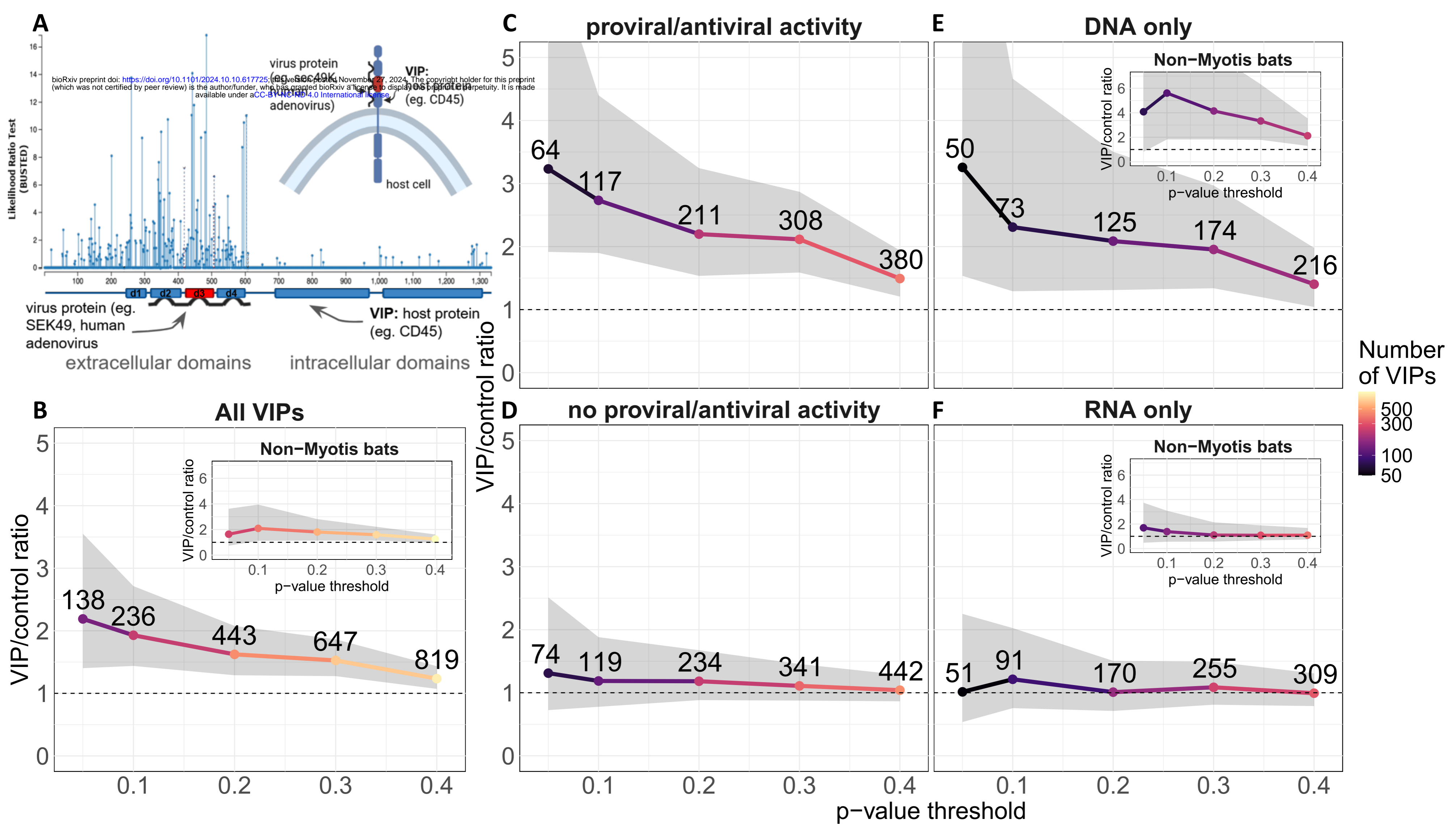
## 1975 Supplemental Information

- 1976 **Document S1.** Figures S1-S6
- 1977 **Table S1.** Genome Statistics
- 1978 **Table S2.** Phylogeny time calibration and evolutionary modeling data
- 1979 **Table S3.** aBSREL significant gene lists and Reactome enrichments
- 1980 **Table S4.** RERConverge and RELAX results and enrichments
- 1981 **Table S5.** List of VIPs and VIP subclasses
- 1982 **Table S6.** SyRI-identified structural variants (SVs)
- 1983 **Table S7.** Experimental data for Neocarzinostatin and PKR experiments
- 1984

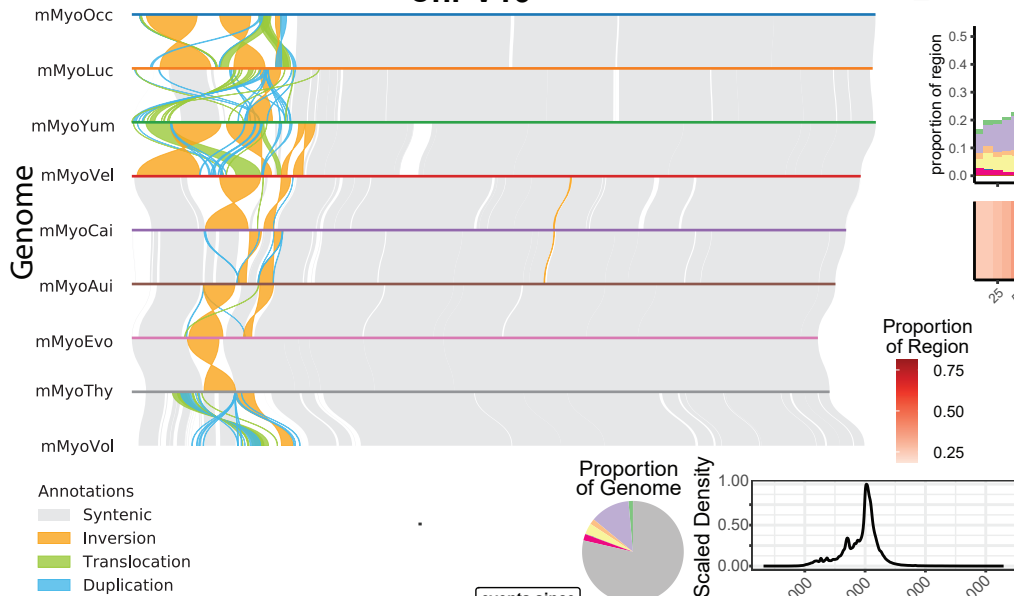
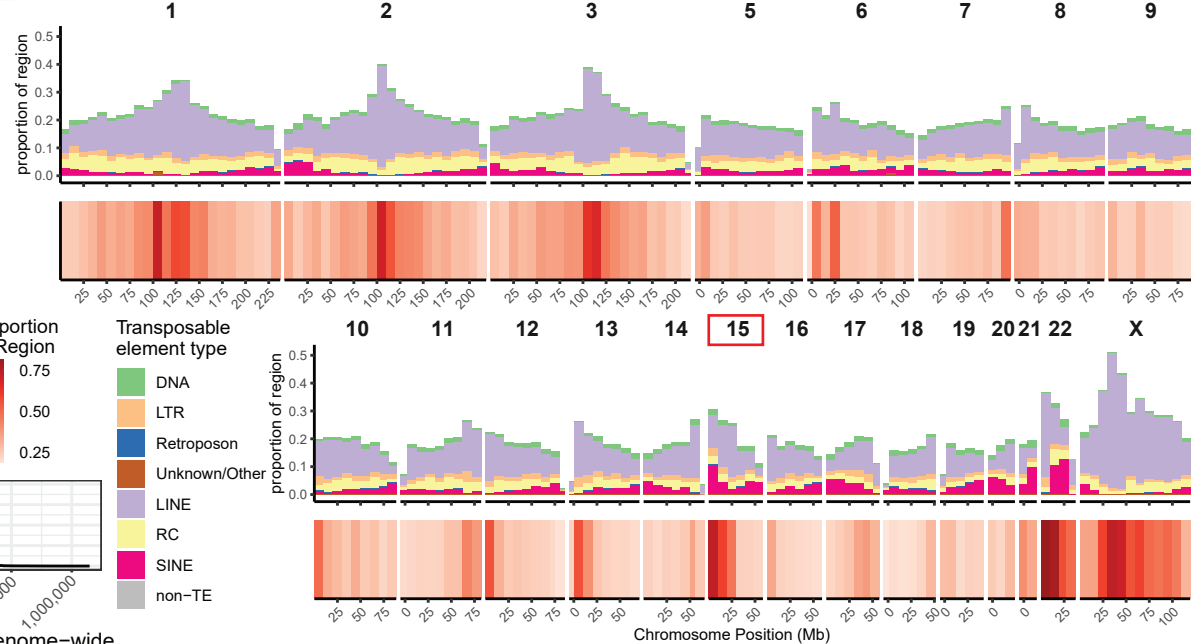
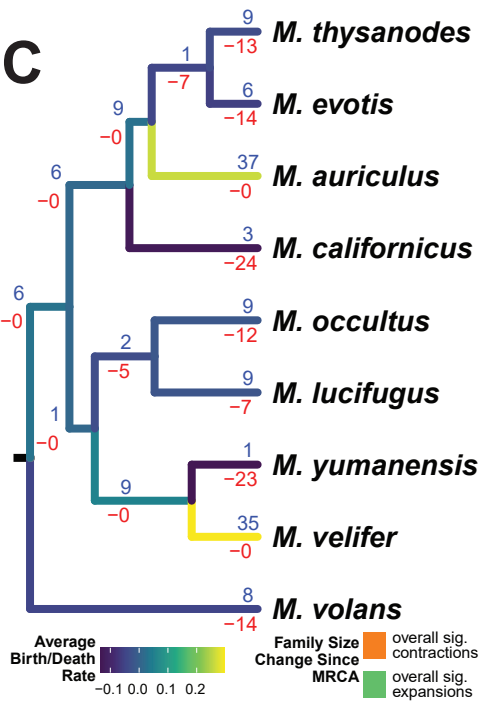
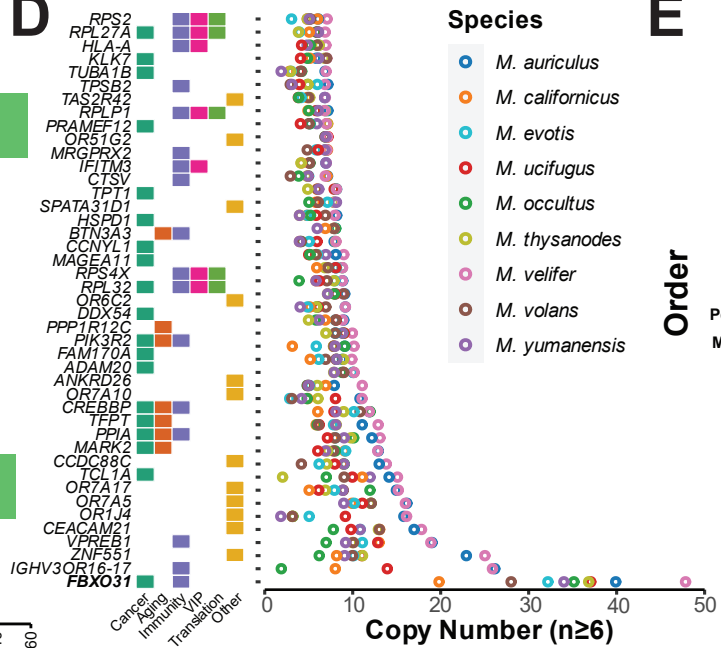
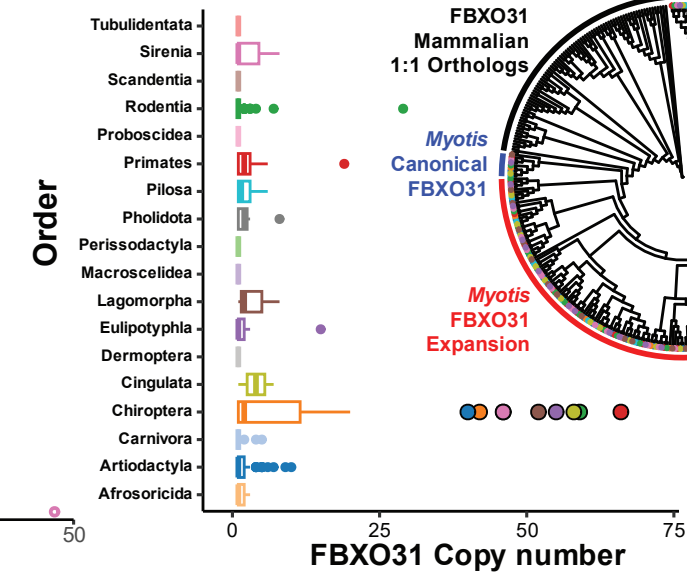
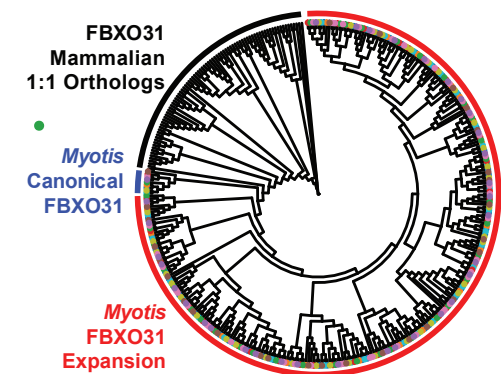


1906 **Figure 2: Evolution of body size, lifespan, and cancer risk in bats and mammals. A, B)** Cophylo plot  
1907 of the evolution of body size (**A**) and lifespan (**B**) across *Eutheria*. **C, D)** Cophylo plot of the evolution of  
1908 body size (**C**) and lifespan (**D**) in bats. Branch lengths in **A-D** are scaled proportional to the rate of change  
1909 of the trait over time, and tree scales are shown below their respective phylogenies. **E)** Diagram illustrating  
1910 the relationship between changes in body size and lifespan with changes in cancer risk and resistance. **F)**  
1911 Reduced Intrinsic Cancer Risk (RICR) for every node in *Eutheria*, ranked from greatest reduction in cancer  
1912 risk to greatest increase in cancer risk. RICR relative to the most recent ancestor of select nodes are  
1913 highlighted, as well as the average RICR across for all nodes within select clades.

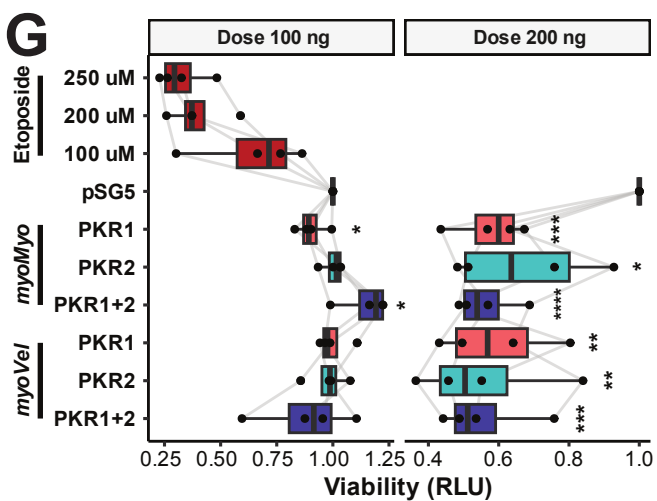
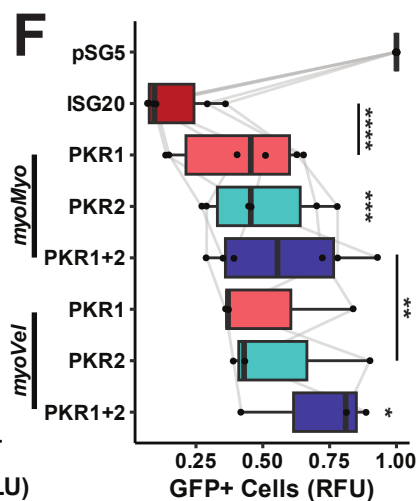
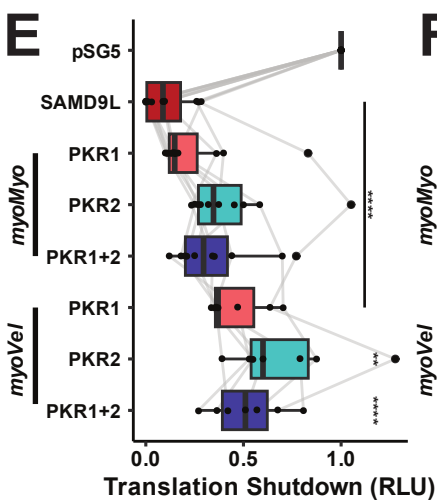
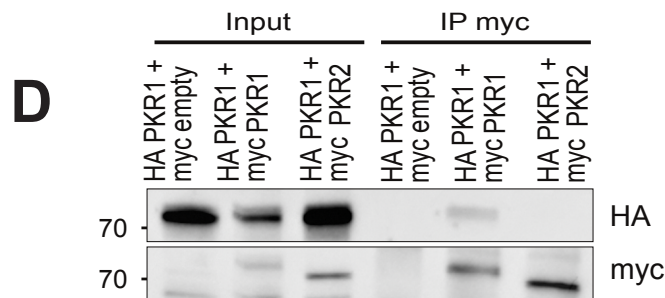
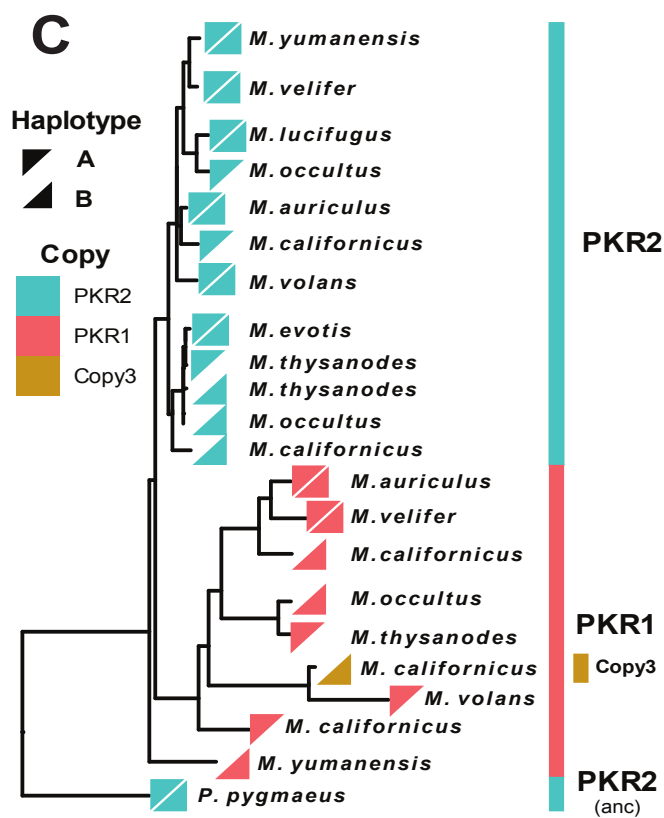
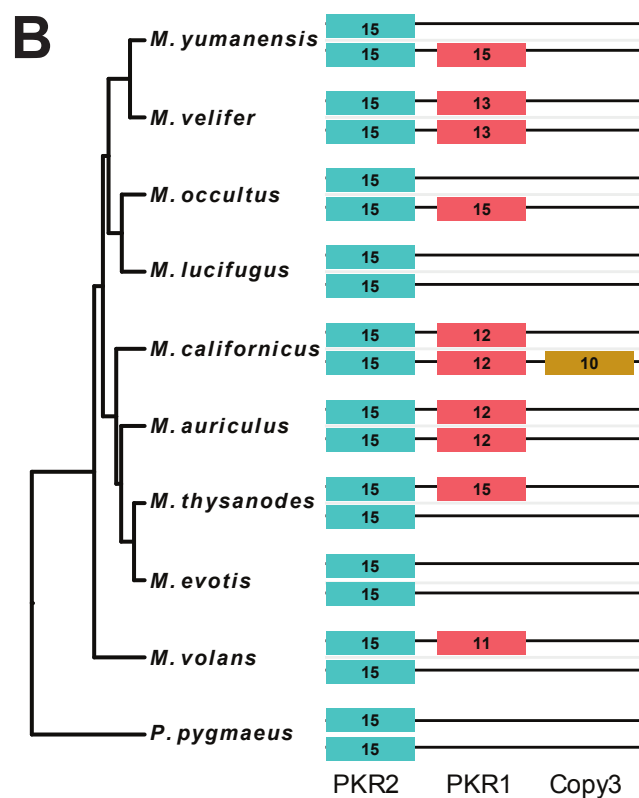
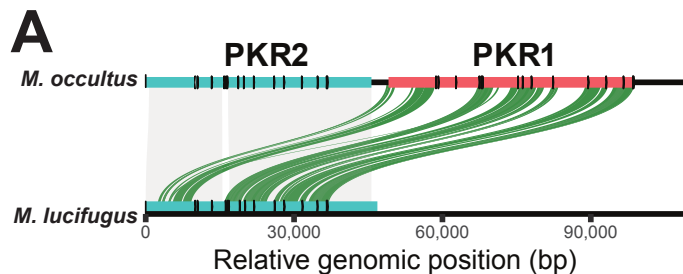




1914 **Figure 3: Selection in Nearctic *Myotis* is enriched for pleiotropic cancer resistance pathways. A)**  
1915 **Left:** phylogeny of Nearctic *Myotis*; **Right:** raincloud plot of omega values for all genes in each species  
1916 since its most recent ancestor. The distribution of omega ( $\omega$ ) values for significant ( $p \leq 0.05$  after multiple  
1917 testing correction) genes and all genes is shown in color above the line. The 95% confidence interval and  
1918 median for significant  $\omega$ 's are represented by the black bar and circle, respectively; the overall 95%  
1919 confidence interval and median are shown in grey below. Individual genes'  $\omega$ 's are represented by colored  
1920 points. Individual genes' omega values and grey, respectively. **Left inset:** Proportion of cancer-associated  
1921 Reactome pathways among the top 100 pathways overrepresented among genes under selection at each  
1922 node. Below, pie chart indicates expected proportion of pathways out of 100 that are cancer-associated  
1923 after 1000 random samples. Nodes with proportions greater than the expected value with  $p \leq 0.05$  using  
1924 Fisher's exact test are indicated with an asterisk. **B)** Proportion of cancer-associated Reactome pathways  
1925 among the top 100 pathways overrepresented among genes under selection across all nodes in a species'  
1926 evolutionary history. **C)** Volcano plot of overrepresented pathways in Reactome among the union set of  
1927 genes under selection across all nodes in the evolutionary history for *M. lucifugus*. **D)** Viability and  
1928 Apoptosis fold-change in 5 bat species in response to different doses of neocarzinostatin, a potent inducer  
1929 of DNA double-strand breaks. Points represent individual replicates normalized to each species' control,  
1930 while bars represent mean  $\pm$  95% confidence intervals.

**A****Chr V15****B****C****D****E****F**

1931 **Figure 4: Adaptation to DNA viruses, but not RNA viruses, is enriched in *Myotis* and other bats. A)**  
1932 Diagram of an example VIP, CD45: a host cell transmembrane receptor that interacts with the human  
1933 adenovirus protein sec49K. Previous work has shown that the amino acids of CD45 that participate in this  
1934 direct interaction are under strong positive selection, as indicated in the graph above the cartoon. **B-F)**  
1935 Enrichment plots showing the ratio of positive selection in VIPs versus matched sets of control genes at  
1936 different p-value thresholds. The solid line shows the median ratio; the color of the line, and the number  
1937 above each point, represents the number of VIPs with significant BUSTED-MH p-values at the given  
1938 threshold; the grey band represents the 95% confidence interval generated by bootstrapping sets of  
1939 matched control genes. Inset plots show the same for all bats in this study excluding *Myotis*.



1940 **Figure 5: A varied structural variation landscape across 9 nearctic *Myotis* species. A)** Synteny  
1941 between *Myotis* species on chromosome V15, showing syntenic regions (grey), inversions (orange),  
1942 translocations (green), and duplications (blue). Regions with high proportions of telomeric repeats were  
1943 masked prior to alignment. **B)** Distribution of transposable elements and segmental duplications (red  
1944 heatmap) in mMyoVel1. Pie chart indicates overall genome proportions of TEs; histogram represents the  
1945 size distribution of segmental duplications genome-wide. **C)** CAFE results among our Nearctic *Myotis*  
1946 relative to single-copy human orthologs. Phylogeny is colored by the estimated birth/death rate ( $\lambda$ ) for all  
1947 genes examined. Bar plot indicates the cumulative number of significant gain and loss events for each  
1948 species. **D)** Per-genome copy numbers of all genes with over 6 copies in any Nearctic *Myotis* genome.  
1949 Genes are classified into 5 categories (cancer, aging, immunity, VIP, translation, and “Other”) based on  
1950 literature reviews on PubMed. **E)** Copy number estimates of *FBXO31* across 536 mammalian genomes  
1951 identified using Reciprocal Best-Hit BLAT. **F)** Gene-tree reconciliation of *FBXO31* across mammals  
1952 generated using GeneRax.

1953 **Figure 6: Evolutionary history and function of an actively segregating copy number polymorphism**  
1954 **of PKR in *Myotis*.** **A)** Structural comparison of the main PKR haplotypes in two species. Orthologous  
1955 regions between the two haplotypes are indicated by grey bands, while syntenic duplications are indicated  
1956 in green. Exons are annotated with black marks. **B)** Cartoon of the PKR locus in the two phased haplotype  
1957 assemblies of each species. While *PKR2* is present across all haplotypes, *PKR1* and *PKR copy 3* are  
1958 polymorphic within and across species. Each number indicates the number of exons per gene. **C)**  
1959 Reconciled gene tree for *PKRs* across all haplotypes and species shown in **B**. Haplotype corresponding to  
1960 the reference (A) and alternate (B) haplotype for each species are represented by upper- and lower-  
1961 diagonal triangles, respectively. **D)** Co-immunoprecipitation (IP) of PKR-KO HeLa cells transfected with *M.*  
1962 *myotis* HA-PKR1 and either *M. myotis* myc-PKR1, *M. myotis* myc-PKR2 or a myc-empty vector control.  
1963 Proteins were pulled down with anti-myc beads and lysates from 5% input or IP samples were run on a  
1964 western blot and stained for HA and myc. Representative of 3 independent experiments. **E-G)** Effect of  
1965 *Myotis* PKR copy numbers: **E)** On luciferase translation, measured in Relative Light Units (RLU) and  
1966 normalized to the empty pSG5 control; co-expression of PKR1 and PKR2 has an additive effect on cell  
1967 translation shutdown (no synergy or dominant negative effects). Human SAMD9L-GoF is a positive control  
1968 of translation inhibition<sup>249</sup>. **F)** On viral VSV infectivity, measured via flow cytometry as VSV-GFP-positive  
1969 cells normalized to the control. Although all conditions restrict VSV, the expression of both PKR1 and PKR2  
1970 is not beneficial against VSV. ISG20 is a positive control of VSV-GFP restriction<sup>241</sup>. **G)** On cell viability,  
1971 normalized to the control. While no effect was observed at a low total dose of PKRs, at higher doses PKRs  
1972 significantly reduce cell viability. Etoposide treatments are positive controls of cell death. For **E-G**, error  
1973 bars indicate the means  $\pm$  SEM for at least three independent experiments. Statistics, unpaired t-test of  
1974 each condition versus control.