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The evolution of duplicate gene expression in mammalian organs

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32 **ABSTRACT**

33 Gene duplications generate genomic raw material that allows the emergence of novel functions, likely
34 facilitating adaptive evolutionary innovations. However, global assessments of the functional and
35 evolutionary relevance of duplicate genes in mammals were until recently limited by the lack of
36 appropriate comparative data. Here we report a large-scale study of the expression evolution of DNA-
37 based functional gene duplicates in three major mammalian lineages (placental mammals, marsupials,
38 egg-laying monotremes) and birds, on the basis of RNA sequencing (RNA-seq) data from nine species and
39 eight organs. We observe dynamic changes in tissue expression preference of paralogs with different
40 duplication ages, suggesting differential contribution of paralogs to specific organ functions during
41 vertebrate evolution. Specifically, we show that paralogs that emerged in the common ancestor of bony
42 vertebrates are enriched for genes with brain-specific expression and provide evidence for differential
43 forces underlying the preferential emergence of young testis- and liver-specific expressed genes. Further
44 analyses uncovered that the overall spatial expression profiles of gene families tend to be conserved,
45 with several exceptions of pronounced tissue specificity shifts among lineage-specific gene family
46 expansions. Finally, we trace new lineage-specific genes that may have contributed to the specific
47 biology of mammalian organs, including the little-studied placenta. Overall, our study provides novel and
48 taxonomically broad evidence for the differential contribution of duplicate genes to tissue-specific
49 transcriptomes and for their importance for the phenotypic evolution of vertebrates.

50 INTRODUCTION

51 The process of gene duplication is widely recognized as an important contributor to the phenotypic
52 diversity of living organisms (Ohno 1970; Kaessmann 2010; Chen et al. 2013). It generates novel genomic
53 material that can be molded through selective and neutral evolutionary processes. Upon duplication, one
54 paralog may diverge in function, or both paralogs partition the ancestral function among them, in the
55 process of neo- and subfunctionalization, respectively (Lynch and Force 2000; Kaessmann 2010). Duplicate
56 genes may also be preserved by natural selection for gene dosage, enabling increased production of the
57 ancestral gene product (Ohno 1970; Kaessmann 2010). Although a number of individual examples of gene
58 duplicates with important novel functions have been described (reviewed in Kaessmann 2010; Long et al.
59 2013), we still know relatively little about the dynamics of functional evolution mediated through gene
60 duplications in mammals. Novel gene functions and associated phenotypes may arise through mutations
61 that alter the sequence of the gene product, and/or through regulatory mutations, which may affect gene
62 expression (Necsulea and Kaessmann 2014). Notably, regulatory mutations are thought to underlie much of
63 phenotypic evolution (King and Wilson 1975; Carroll 2008; Necsulea and Kaessmann 2014). Therefore,
64 comparative gene expression studies may provide unique insights into the functional evolution of both old
65 and new (duplicate) genes.

66

67 High-throughput RNA sequencing (RNA-seq) enables detailed cross-species transcriptome comparisons
68 (Necsulea and Kaessmann 2014). However, while mammalian RNA-seq data have been used to study the
69 evolution of 1:1 orthologous (single-copy) genes (Brawand et al. 2011; Warnefors and Kaessmann 2013;
70 Necsulea and Kaessmann 2014) and some specific aspects of paralogs (Chen and Zhang 2012; Rogozin et al.
71 2014), the evolutionary and functional relevance of gene duplication still remains little explored globally,
72 although two previous studies assessed patterns of expression evolution for subsets of duplicate gene pairs
73 (Assis and Bachtrog 2015; Lan and Pritchard 2016). Here we close this gap and, using an extensive RNA-seq
74 dataset, perform large-scale comparative analyses to assess short- and long-term dynamics of duplicate
75 gene expression evolution across eight mammals and one bird. We focus on DNA-based duplications, which
76 may arise through misguided recombination and replication processes in the germline, or through meiotic
77 non-disjunction, in the case of whole-genome duplication (Hastings et al. 2009; Marques-Bonet et al. 2009).
78 DNA-based duplicates constitute a major subset of duplicated genes in the genome, and we recently
79 described expression evolution of the other major type, RNA-based duplicates, in a dedicated study (Carelli
80 et al. 2016).

81

82 We start by studying general patterns of expression evolution and then focus on lineage-specific processes.
83 We ask: i) How do expression levels, expression divergence, and tissue specificity globally change with
84 duplication age, and do these patterns differ by tissues or among studied species?; ii) What is the

85 significance and evolutionary role of particular tissues in the retention of duplicate genes?; iii) What is the
86 potential contribution of paralogs to lineage-specific phenotypic evolution?

87

88 **RESULTS**

89 **Re-annotation of duplicated gene families and expression level dynamics**

90 To establish a high-confidence gene duplication dataset, we downloaded protein-coding gene family trees
91 from Ensembl v64 (Vilella et al. 2009; Flicek et al. 2012) and employed a rigorous multi-step filtering
92 procedure that removed poorly supported duplications, misannotations and intronless genes
93 (Supplemental Fig S1, Supplemental Methods). Inference of duplication age was based on gene tree
94 topology and validated by measuring the rate of synonymous substitutions (d_s) – the best-suited approach
95 for duplications across large temporal scales including ancient and recent events (Huerta-Cepas and
96 Gabaldón 2011). The final dataset contained 7350 duplication events in 4187 gene families (Supplemental
97 Fig S1, Supplemental Table S1). For comparisons, we also obtained a non-overlapping set of 3379 amniote
98 single-copy orthologous genes (Brawand et al. 2011; Methods). Expression analyses were based on our
99 previous RNA-seq datasets (Brawand et al. 2011; Necșulea et al. 2014), which comprise eight different
100 organs (cortex or whole brain without cerebellum, cerebellum, heart, kidney, liver, testis, ovary, placenta)
101 for eight representatives of the three major mammalian lineages (placental mammals: human, chimpanzee,
102 gorilla, orangutan, rhesus macaque, and mouse; marsupials: grey short-tailed opossum; monotremes:
103 platypus) and a bird (non-domesticated chicken) (Supplemental Table S2). We employed a careful read
104 mapping and expression level estimation procedure that takes into account divergence levels of duplicate
105 gene copies and proportions of uniquely mapping reads to infer reliable expression profiles of paralogs
106 (Supplemental Methods). Our procedure effectively removes 91.4% of problematic human genes, for which
107 expression levels cannot be reliably determined (Supplemental Methods, (Robert and Watson 2015)). The
108 few remaining genes have only been used in global analyses and therefore are unlikely to bias our results.

109

110 **Global patterns of expression divergence**

111 The neo- and subfunctionalization models of gene duplication postulate that paralogs diverge in function or
112 partition the ancestral functions among the copies (Kaessmann 2010; Long et al. 2013), resulting in greater
113 expression divergence and potentially higher tissue specificity in gene duplicates compared to single-copy
114 genes. To gauge how new expression patterns emerge following gene duplication, we assessed expression
115 divergence of paralogs of different duplication ages.

116

117 We observed an arch-shaped relationship between expression divergence (measured as Euclidean distance;
118 Supplemental Methods) and duplication age in all studied species (Fig. 1A, Supplemental Fig S2). Because
119 measures of expression divergence can be affected by the presence of tissue-specific expressed genes

120 (Chen and Zhang 2012), we confirmed that the observed pattern was robust to the removal of tissue-
121 specific expressed, non-expressed, or lowly expressed genes with the sum of expression across all tissues
122 <1 FPKM (Supplemental Fig S3). A similar, albeit considerably less pronounced pattern was observed with
123 an alternative measure of expression divergence, Pearson's correlation coefficient (r) (Fig. 1B,
124 Supplemental Fig S4). As expected, the youngest paralogs showed low expression divergence, likely
125 because their short time of independent evolution was not sufficient for them to diverge in expression.
126 However, while genes from the younger age classes showed progressively higher expression divergence
127 with age, as previously observed for a subset of these data (Assis and Bachtrog 2015), the pattern was
128 reversed for paralogs that predate the emergence of tetrapods. In these older genes, expression
129 divergence decreased with age, so that the oldest and the youngest paralogs showed similar degree of
130 expression divergence. High expression levels of old duplicates may partly explain their low expression
131 divergence: expression divergence is negatively correlated with expression levels in paralogs and single-
132 copy genes (Supplemental Fig S5, $\rho = -0.35$ and -0.47 , $P < 10^{-15}$, for human-mouse single-copy genes and
133 all studied paralogs, respectively) and old paralogs are highly expressed (Fig. 2A). Generally, expression
134 levels and duplication age are correlated in paralogs ($\rho = 0.18 - 0.35$ in all studied species, $P < 10^{-15}$, Fig.
135 2A). This relationship persists after removing lowly expressed genes with the sum of expression <1 FPKM
136 across all tissues ($\rho = 0.16 - 0.31$, $P < 10^{-15}$) and even within individual gene families ($\rho = 0.22$, $P < 10^{-15}$).

137
138 Estimates of expression divergence with Euclidean distances take into account expression levels (Pereira et
139 al. 2009). Recent studies in *Paramecium* and mammals highlighted the importance of considering
140 expression levels by demonstrating rapid emergence of significantly asymmetric expression levels between
141 paralogs (Gout and Lynch 2015; Lan and Pritchard 2016). In contrast, Pearson's r estimates reflect spatial
142 patterns of expression divergence without accounting for differences in expression levels. Pearson's r also
143 tends to overestimate expression divergence for uniformly expressed genes, whereas Euclidean distances
144 are robust to such expression patterns (Pereira et al. 2009). It is therefore possible that expression
145 divergence for ancient, more ubiquitously expressed paralogs has been overestimated with Pearson's r in
146 our dataset, masking the arch-shaped relationship between expression divergence and duplication age (Fig.
147 1B).

148
149 Expression divergence of paralogs of any duplication age was higher than that of human-mouse single-copy
150 genes (Fig. 1A), even if the paralogs were evolutionarily younger (Supplemental Fig S6, $P < 10^{-14}$), confirming
151 that paralogs diverge in expression soon after duplication (Chen and Zhang 2012; Rogozin et al. 2014; Assis
152 and Bachtrog 2015). However, paralogs are generally expressed at significantly lower levels than single-
153 copy genes in all studied species (Fig. 2A, $P < 10^{-13}$, Kruskal-Wallis test) and expression levels of young
154 paralogs are particularly low. To evaluate if higher expression divergence of paralogs is the result of

155 technical and/or biological noise typical for lowly expressed genes, we performed a global test for the
156 contribution of expression level, evolutionary time (duplication or speciation age) and duplication status
157 (duplicate or single-copy genes) on expression divergence. We used mouse and human data, for which the
158 presence of other rodent and primate genomes allowed more fine-grained duplication and speciation age
159 estimates. Because expression divergence was independent of the number of gene copies (including copies
160 stemming from subsequent rounds of duplication/speciation) that could be traced back to a given
161 duplication event (Spearman's $\rho = 0.019$, $P = 0.33$ and $\rho = -0.005$, $P = 0.81$ for mouse and human,
162 respectively), we did not include this factor in the model. We found that expression levels explained most
163 of the variance in expression divergence, followed by evolutionary time and duplication status
164 (Supplemental Table S3, Methods). Our results thus show that global differences in expression levels
165 explain much of the difference in expression divergence between duplicated and single-copy genes. Using
166 Pearson's r , the global model of expression divergence evolution had less explanatory power and the effect
167 of expression levels was smaller than the effect of evolutionary time, consistent with the insensitivity of
168 Pearson's r to differences in expression levels (see above, Supplemental Table S4).

169

170 **Expression divergence and protein sequence divergence are correlated in paralogs**

171 Two types of mutation underlie the evolution of novel gene functions: changes to the protein sequences
172 and changes to gene expression (Necsulea and Kaessmann 2014). Expression divergence is believed to be
173 acquired more rapidly than sequence divergence (Wapinski et al. 2007), but both were found to be
174 correlated in 1:1 orthologs (Khaitovich et al. 2005; Warnefors and Kaessmann 2013) and in paralogs that
175 have diverged little on sequence level (Gu et al. 2002; Makova and Li 2003; Nehrt et al. 2011; Liao et al.
176 2014), suggesting considerable coupling of these processes. Consistently, we observe weak, but significant
177 global correlation between expression divergence (Euclidean distances) and protein sequence divergence
178 (nonsynonymous substitution rates, d_N) in paralogs of two species (human, as the representative of
179 placental mammals, and opossum, as the representative of marsupials) (Fig. 3A). The correlation was
180 strongest for young paralogs and systematically decreased with duplication age. Expression divergence
181 levels off during amniote evolution and decreases for evolutionary older genes (Fig. 1A), whereas protein
182 sequence divergence continues to increase (Brawand et al. 2011; Warnefors and Kaessmann 2013).
183 Therefore, reduced correlation between expression and protein sequence divergence for ancient and old
184 paralogs can be expected, although it is still detectable even across large evolutionary scales.

185

186 Some genes are likely to diverge more in terms of expression than in terms of sequence, and *vice versa*.
187 Among 1:1 orthologs, genes escaping the global correlation were found to fulfill different functions
188 (Warnefors and Kaessmann 2013). To investigate if similar functional variation can be found in paralogs, we
189 performed Gene Ontology (GO) enrichment analyses, dividing human paralogs into those that diverged

190 more on the protein sequence level relative to their expression divergence (termed “ d_N -biased”) and those
191 that diverged more in expression profiles relative to protein sequence [“ED-biased”, Fig. 3B, (Warnefors
192 and Kaessmann 2013), Methods]. Because the correlation between expression and sequence divergence
193 changes with evolutionary time (Fig. 3A), we grouped the paralogs into two duplication age categories
194 (Methods). Similar to findings in 1:1 orthologs, we observed clear functional differences between d_N - and
195 ED-biased paralogs, with a considerable effect of duplication age. Young d_N -biased paralogs that duplicated
196 in the common amniote ancestor or more recently, shared with d_N -biased 1:1 orthologs enrichment for
197 genes implicated in transcription and regulation of gene expression (Supplemental Table S5, Fig. 3C,
198 Warnefors and Kaessmann 2013). They shared involvement in metabolic and biosynthetic processes with
199 old d_N -biased genes that duplicated in the common tetrapod ancestor or earlier (Supplemental Table S5, Fig.
200 3C). We also detected considerable functional agreement between old ED-biased paralogs and ED-biased
201 1:1 orthologs: they were involved in synaptic transmission, cell signaling and communication, ion transport,
202 and anatomical structure development (Supplemental Table S5, Fig. 3C). However, young ED-biased
203 paralogs were implicated in functions not observed among biased 1:1 amniote orthologs, such as metabolic
204 and cellular processes, with “xenobiotic metabolism” being the most significant term (adjusted $P = 6 \times 10^{-10}$
205 after correction for multiple testing, enrichment score = 1.92, Supplemental Table S5, Fig. 3C). This finding
206 is consistent with the high proportion of liver-specific expressed genes among young paralogs (see below),
207 strengthening the role of gene duplicates as sources of evolutionary novelty and emphasizing their
208 contribution to phenotypic evolution. Liver-expressed genes involved in detoxification and waste removal
209 were found to show lineage-specific expression changes among amniote orthologs (Brawand et al. 2011)
210 and pronounced inter-individual variation in expression levels (Khaitovich et al. 2005), possibly reflecting
211 regulatory plasticity, which may allow for more rapid expression evolution (Romero et al. 2012).

212

213 **Tissue-specific functional contributions of paralogs of different ages**

214 The expression patterns of paralogs have generally been found to be more tissue-specific than those of
215 single-copy genes (Huminiacki and Wolfe 2004; Huerta-Cepas and Gabaldón 2011), in line with the neo-
216 and subfunctionalization models of gene duplication. However, to understand why tissue specificity is
217 elevated in paralogs, it is necessary to study the contribution of expression levels, evolutionary time, and
218 duplication status in a common framework. We thus ran linear models using two alternative measures of
219 tissue specificity: 1) tau (Yanai et al. 2005) and, 2) calculating tissue specificity as the relative expression of
220 the gene in the tissue with highest expression (Methods). Expression levels explained most of the variance
221 in tissue specificity, whereas evolutionary time was not significant (Supplemental Table S6, S7). Paralogs
222 showed more pronounced tissue-specific expression than single-copy genes in the model based on tau
223 (Supplemental Table S7), in concordance with previous studies (e.g. Huminiacki and Wolfe 2004; Huerta-
224 Cepas and Gabaldón 2011). As we detail below, high tissue specificity of lowly expressed genes bears

225 biological relevance and cannot be explained by technical/biological noise alone. However, the overall low
226 fit of these models suggests that patterns of tissue expression specificity might be best explained by other
227 factors (e.g. tissue complexity). Nevertheless, combined with our analyses of expression divergence, this
228 result supports the notion that the expression level of a gene represents a strong predictor for explaining
229 the dynamics of evolutionary expression pattern divergence.

230

231 We speculated that the contributions of duplicated genes to the emergence of novel functions might be
232 reflected in the tissue-specific expression of paralogs that originated at different evolutionary times. Indeed,
233 we observed consistent, substantial, and statistically significant differences in the distribution of tissue
234 preferences by duplication age in all studied species (Fig. 4, Supplemental Fig S7). They were not affected
235 by our definition of tissue specificity (tau, “stringent”, or “twice uniform expression”, Methods), expression
236 levels, separate or combined treatment of neural tissues, or whether the analyses were carried out at gene
237 or gene family level (Supplemental Fig S8).

238

239 The proportions of lineage-specific and young heart- and particularly brain-specific expressed paralogs
240 were significantly lower than expected (Figs. 4B, C, Supplemental Figs S7-8), suggesting that young
241 duplicates have contributed proportionally less to the functional evolution of heart and brain tissues.
242 Across all expression levels, young paralogs showed low relative expression in heart and neural tissues
243 compared to older paralogs (Supplemental Methods, Supplemental Figs S9-10). Notably, the highest
244 proportion of brain-specific expressed genes was among old paralogs that duplicated in the common bony
245 vertebrate ancestor (Fig. 4B, Supplemental Figs S7-8) – an evolutionary time period marked by substantial
246 elaborations in the morphology and cyto-architectural complexity of the telencephalon and an overall
247 increase in brain-to-body size ratio (Butler 2010). Thus, our large-scale survey suggests that gene
248 duplications may have facilitated these phenotypic vertebrate brain innovations, consistent with a previous
249 analyses of individual gene families (Chen et al. 2011).

250

251 The proportion of testis-specific expressed paralogs was significantly higher than expected among lineage-
252 specific and young duplicates (Figs. 4B, C, Supplemental Figs S7-8). Testis-specific expressed single-copy and
253 duplicate genes of all ages tend to be overall lowly expressed (Supplemental Fig S11), consistent with the
254 idea of widespread spurious transcription in this tissue (Soumillon et al. 2013). However, despite low
255 expression, lineage-specific and young testis-expressed paralogs are significantly enriched for genes
256 functional in gamete generation, reproductive processes, and spermatogenesis (retinoic acid pathway)
257 compared to the background of all testis-expressed genes (Benjamini-Hochberg corrected $P < 10^{-3}$,
258 Supplemental Table S8). Thus, they are not merely a product of the permissive transcriptional environment

259 (Soumillon et al. 2013), but may have contributed important testis-specific functions during more recent
260 mammalian evolution.

261

262 Lineage-specific and young duplications also contained higher than expected proportions of genes
263 specifically expressed in the liver (Figs. 4B, C, Supplemental Figs S7-8). However, in contrast to the testis,
264 liver-specific genes tend to be highly expressed, so that relative liver expression increased with expression
265 levels in single-copy and duplicate genes (Supplemental Fig S12). Young liver-specific expressed paralogs
266 showed functional enrichments for metabolic and catabolic processes related to digestion and
267 detoxification (Supplemental Table S8, Benjamini-Hochberg corrected $P < 10^{-3}$), signifying their contribution
268 to the typical liver-associated functions over and above other liver-expressed genes.

269

270 **Evolutionary forces underlying the preferential emergence of testis- and liver-expressed genes**

271 The high proportion of young testis- and liver-specific expressed genes could be the result of two not
272 mutually exclusive processes: rapid gene turnover (i.e. increased duplication fixation rate in young paralogs,
273 followed by gene loss later in evolution) and/or changes in expression profiles with evolutionary time, so
274 that young genes become more broadly expressed with time or shift their expression to another tissue. To
275 study the contribution of these processes, we first quantified the rate of gene gain and gene loss in human
276 and mouse paralogs following a duplication in the common human-mouse ancestor (within the young age
277 class, Methods). We chose these two species as they have the highest-quality genomes among our study
278 species, which reduces biases from wrongly inferred gene losses or spurious duplicates. Gene families that
279 have experienced gene losses were identified as having fewer than four paralogs in both species together,
280 whereas gene families that have experienced gene gains had more than four mouse and human paralogs in
281 total. We established the major tissue of expression for each gene family by determining the tissue with
282 highest median expression across all paralogs. A global test showed significant differences in the numbers
283 of gene families that were dominated by gene losses and gene gains among gene families with
284 predominant expression in liver, testis, and all other tissues combined (chi-square = 9.77, df = 2, $P = 0.008$).
285 A post hoc procedure (Methods) revealed that gene families with highest expression in the liver had
286 significantly more gains and fewer losses following a duplication in the common human-mouse ancestor
287 (Fig. 4D). This finding suggests that new gene copies from liver-expressed gene families tend to be fixed
288 more often than gene copies from families expressed in other tissues, thus contributing to the high
289 proportion of young and lineage-specific liver-expressed paralogs. The proportions of gains and losses did
290 not differ between testis-expressed gene families and gene families expressed in other tissues (Fig. 4D),
291 suggesting that a different process leads to increased proportions of young testis-specific expressed genes.

292

293 Thus, we next tested for changes in expression profiles in testis- and liver-expressed young paralogs,
294 focusing on a subset of duplication events in mammals, for which a single-copy chicken gene could be
295 determined as an outgroup (n=821, containing 2662 paralogs in all mammalian species included in our
296 study). We hypothesized that if expression profiles are preserved following recent duplication, the
297 proportion of single-copy outgroup chicken genes with highest expression in liver and testis should be the
298 same as the proportion of mammalian paralogs with highest expression in these tissues. Indeed, we
299 observed no difference in the proportion of single-copy outgroup chicken genes and mammalian paralogs
300 with highest expression in liver (chi-square = 0.08, df = 1, p-value = 0.78, Fig. 4E). However, we found a
301 greater proportion of mammalian paralogs with highest expression in testis compared to chicken outgroup
302 genes (chi-square = 6.45, df = 1, p-value = 0.01, Fig. 4E), indicating that young paralogs tend to be testis-
303 expressed but change their expression pattern with evolutionary time.

304

305 Taken together, our results suggest that different processes are responsible for the high proportion of
306 young and lineage-specific testis- and liver-specific expressed paralogs. Fast gene turnover in liver-
307 expressed gene families could provide an important mechanism for rapid, lineage-specific dietary
308 adaptations, together with species-specific changes in expression of liver-specific genes, which were
309 suggested to be linked to ecological adaptations in primates (Perry et al. 2012). In contrast, the evolution of
310 testis-expressed gene families predominantly involves shifts and/or broadening of expression profiles with
311 evolutionary time. This observation is in agreement with the “out of the testis” hypothesis of gene
312 origination (Kaessmann 2010), making it a shared mechanism for DNA- and RNA-based gene duplications
313 (Carelli et al. 2016, see also Assis and Bachtrog 2015). Young testis-expressed paralogs may directly
314 contribute to lineage-specific biology as evidenced by their involvement in reproductive functions despite
315 low expression levels (see above; Supplemental Table S8). Reproductive proteins show rapid sequence
316 evolution in animals (Swanson and Vacquier 2002) and our findings support the notion that gene
317 duplications contribute to species-specific reproductive characteristics (Clark et al. 2007; Almeida and
318 Desalle 2008; Kelleher and Markow 2009; Kaessmann 2010; Betrán 2015). Overall, our results illustrate
319 how gene duplications from different evolutionary time periods have contributed differentially to the
320 transcriptomes and functions of various mammalian/amniote organs.

321

322 **Gene duplications and the emergence of the placenta**

323 The placenta is an evolutionarily young tissue and the most varied mammalian organ, with physiological
324 and anatomical homologies among marsupials and eutherians (Renfree 2010; Wildman 2016). We
325 hypothesized that new duplicate genes might have been recruited into the placenta during the
326 establishment of this organ, thus providing raw material for the evolution of placenta-specific functions.
327 However, instead we found that older paralogs with duplication ages in mammals and amniotes contained

328 significantly more genes with placenta-specific expression than expected, whereas paralogs that duplicated
329 in the common therian and eutherian ancestors did not show increased numbers of placenta-specific
330 expressed genes (Fig. 5). Thus, paralogs that predate the placenta emergence appear to have been coopted
331 for functions in this tissue, in line with previous work (Knox and Baker 2008). Placental morphology and
332 physiology is highly varied in therian mammals (Renfree 2010; Wildman 2016) and this organ has
333 repeatedly recruited genes with similar functions, but evolutionary independent origins, e.g. the syncytin-
334 like genes in primates, rodents, and lagomorphs (Mi et al. 2000; Dupressoir et al. 2009; Heidmann et al.
335 2009). It is thus conceivable that new and old genes acquired functional roles ever since the emergence of
336 the placenta in a lineage-specific manner.

337

338 We identify well-known genes that have acquired placenta-specific expression following gene duplication
339 (e.g. *IGF2* [Supplemental Table S1, *genefam_3056*], *HBG1* and *HBG2* [Supplemental Table S1,
340 *genefam_1850*]). However, the most extreme example of placenta-specific expression gain following
341 duplication is *PAGE4* (P antigen family, member 4), a member of the *GAGE/PAGE* gene family (Fig. 6,
342 Supplemental Table S1, *genefam_2135*). Homologs of *PAGE4* show moderate expression levels (1-95 FPKM)
343 specifically in the testis and have experienced multiple rounds of duplication. In contrast, *PAGE4* acquired
344 high levels (>2600 FPKM) of placenta-specific expression in the human lineage, following a duplication
345 event in the common eutherian ancestor (Fig. 6). The entire gene family appears to be missing in the
346 mouse, but *PAGE4* is expressed in the elephant placenta (Hou et al. 2012). Previous studies showed that
347 *PAGE4* is also expressed in other reproductive organs and cancer cell lines (Iavarone et al. 2002) and thus
348 seems to be associated with fast-proliferating cells. It has received attention as a cancer/testis antigen that
349 is upregulated in prostate cancer and is involved in stress-response pathway linked to prostatic
350 development and disease (Mooney et al. 2014). However, its expression patterns suggest that it may fulfill
351 an important placenta-specific function in primates and possibly other eutherians. In mice, its role may be
352 taken by a different gene or set of genes. Altogether, we can pinpoint interesting candidate paralogs with
353 extreme shifts in expression profiles, which potentially signal their functional relevance in a newly emerged
354 tissue.

355

356 **Spatial expression dynamics and lineage-specific expansions of amniote gene families**

357 To better understand the contribution of duplicated genes to phenotypic evolution, we set out to assess
358 how tissue-specific expression profiles of amniote gene families evolve following repeated duplications. We
359 characterized expanded amniote gene families into those containing only broadly expressed paralogs and
360 those containing primarily tissue-specific expressed paralogs. Among tissue-specific expressed gene
361 families, we investigated how often specificity for the same tissue is preserved in repeated, independent
362 duplications along individual amniote lineages.

363

364 We used twice uniform measure of tissue specificity to classify gene families in each of our study species
365 into: i) “broad” gene families, in which all members are expressed at comparable levels in all tissues (0-
366 8.9% of gene families by species); ii) “diverse” gene families, which contain paralogs specific for different
367 tissues (26.0-51.2%); and iii) “specific” gene families, in which the majority of paralogs are specific for the
368 same tissue (37.7-58.6%, Table 1, Methods). Using tau as the alternative measure of tissue specificity, the
369 results were similar, although fewer gene families were classified as “specific” and “diverse” and more as
370 “broad” (Supplemental Table S9).

371

372 We found that “broad” gene families frequently contain zinc-finger genes (43-100%, Table 1, Supplemental
373 Table S9), in accordance with the universal role of these genes in regulating fundamental processes, such as
374 transcription. Species-specific expansions of KRAB zinc-finger gene families are well characterized in
375 eutherians (Emerson and Thomas 2009) and marsupials (Goodstadt et al. 2007). These genes are implicated
376 in expression control of mobile genetic elements during development (Rowe and Trono 2011) and in adult
377 tissues (Ecco et al. 2016). However, assessing the individual expression patterns of many young zinc-finger
378 paralogs is hampered by the difficulty of distinguishing transcribed copies from a pool of closely related
379 paralogs.

380

381 Next, we asked how often gene families preserved their expression profile after experiencing multiple
382 independent duplications along individual lineages (Fig. 7, Supplemental Table S10, Methods). Considering
383 human, mouse, opossum, platypus, and chicken, we identified a total of 25 gene families that expanded
384 independently in two or more species (Fig. 7). Only two gene families expanded independently in more
385 than two species. One of them duplicated in mouse, opossum, and platypus and contained butyrophilin
386 and its paralogs that are involved in adaptive immune response and lipid, fatty acid, and sterol metabolism,
387 including variants linked to metabolic syndrome. It showed specific expression in mouse testis and
388 opossum liver, but was “diverse” in platypus. The other gene family duplicated independently in mouse,
389 opossum, platypus, and chicken and contained guanylate binding peptides that are involved in immune
390 response against different classes of pathogens. Opossum and chicken paralogs were specifically expressed
391 in ovary, whereas mouse and platypus paralogs showed a wide range of tissue specificities (Fig. 8A).

392

393 Among the 23 gene families that expanded independently in only two species, none were “broad”, seven
394 were “diverse” in both species, eight were “diverse” in one but “specific” in the other species, and eight
395 were “specific” in both species (Fig. 7B). There was a tendency to preserve specificity for the same tissue
396 (six of the eight events, Fig. 7B). In one of the “specific” gene families that differed in tissue specificity
397 between the two species, expansions along the primate lineage produced brain-specific expressed paralogs,

398 whereas paralogs on the marsupial lineage showed preference for liver expression (Fig. 8B). Human
399 paralogs of the aforementioned gene family are known to be functional in brain development and possibly
400 synaptogenesis (e.g. *SIRPA*) (UniProt Consortium 2015), supporting the idea that young duplicates have an
401 important role in species-specific phenotypes. Our analyses thus indicate that lineage-specific expansions
402 of the same gene family can evolve unique expression patterns in different lineages, highlighting the
403 dynamic nature and functional flexibility of gene duplicates.

404

405 **DISCUSSION**

406 Gene duplications are unequivocally recognized as an important source of evolutionary novelty (Kaessmann
407 2010; Chen et al. 2013). Here, using comparative RNA-seq data from a comprehensive set of nine amniotes,
408 we conducted an in-depth study of evolutionary dynamics of gene expression changes following DNA-based
409 duplications.

410

411 Divergent expression profiles and increased tissue specificity are frequently considered to be the hallmarks
412 of gene duplication (Conant and Wolfe 2008; Huerta-Cepas et al. 2011). In this study, we confirm that
413 paralogs are generally more divergent in expression profiles and more tissue-specific than single-copy
414 genes and that expression divergence is acquired quickly after gene duplication. We also highlight the
415 overall pronounced difference in expression levels between single-copy and duplicated genes and that the
416 expression level of a gene represents a strong predictor of evolutionary expression pattern divergence. A
417 combination of various factors may explain these observations. Gene duplications can directly lead to
418 reduced expression levels of resulting copies, either through incomplete duplication of regulatory elements
419 or due to a special form of subfunctionalization, in which expression reduction facilitates paralog retention
420 (Qian et al. 2010). Indeed, rapid reduction in expression levels was demonstrated in human paralogs that
421 emerged since the human-macaque split (Lan and Pritchard 2016). Also, lowly expressed non-essential
422 gene families may duplicate and be retained after duplication more readily, and young paralogs were
423 shown to be enriched for non-essential genes (Woods et al. 2013; Grishkevich and Yanai 2014), providing a
424 link between gene age and expression levels. The overall rapid expression divergence of duplicate gene
425 copies is likely explained by the frequent change in the regulatory landscape following gene duplication (i.e.,
426 the loss and gain of regulatory elements; see also above), the reduced selective constraint afforded by the
427 availability of an extra gene copy (Ohno 1970), and the reduced selective constraint due to the generally
428 lower expression levels of duplicates (COSTEX model, (Gout et al. 2010)). The latter likely also explains the
429 rapid divergence of lowly expressed single-copy genes. In summary, gene expression levels are an
430 important factor for understanding the dynamics of functional divergence and emergence of evolutionary
431 novelty.

432

433 Despite generally low expression levels of young paralogs, our analyses indicate that they can be
434 functionally relevant (Supplemental Table S8). We further show that expression levels systematically
435 increase with evolutionary time, so that old paralogs are expressed at higher levels than young paralogs
436 (Fig. 2). It is worthwhile to note the large variance in expression levels of young and lineage-specific
437 paralogs, in agreement with increased expression asymmetry of genes with these evolutionary ages (Gout
438 and Lynch 2015; Lan and Pritchard 2016). However, contrary to our findings, no differences in expression
439 levels were found in yeast paralogs of different evolutionary ages (Qian et al. 2010). The yeast study
440 considered older evolutionary branches than the duplication ages analyzed here, and it is possible that old
441 paralogs reach a plateau, beyond which the increase in expression levels is only marginal. Indeed, we
442 observe little change in expression level in duplications older than the common vertebrate ancestor (535
443 Mya, Fig. 2A). Furthermore, inherent biological factors (e.g. unicellularity) may prevent changes in
444 expression levels of yeast paralogs, or these changes may have remained undetected due to low sample
445 size of analyzed yeast paralogs of any given evolutionary age (Qian et al. 2010).

446

447 The pattern of increasing expression divergence with duplication age for young paralogs (Fig. 1) is highly
448 consistent with the recent study by Lan and Pritchard (2016), who demonstrated that chromosomal
449 separation of paralogs is crucial for acquisition of independent expression profiles and that this separation
450 is achieved gradually with evolutionary time. Hence, as chromosomal rearrangements decouple gene
451 expression regulation of initially tandem duplicates, their expression profiles diverge. In contrast, for old,
452 highly expressed paralogs, reduced expression divergence is expected under the COSTEX model (Gout et al.
453 2010). In addition, all ancient genes have experienced two rounds of whole-genome duplications in the
454 common vertebrate ancestor (Dehal and Boore 2005). This process, by duplicating the entire genomic
455 content, preserves stoichiometric relationships between gene products and the resulting paralogs
456 experience increased selection against changes in expression and copy number, in the case of dosage
457 sensitive genes (Makino and McLysaght 2010; McLysaght et al. 2014). These two processes, one specific to
458 young paralogs and the other dominant in old paralogs, reconcile the observed arch-shaped distribution of
459 expression divergence observed in our data (Fig. 1).

460

461 Our set of study tissues includes representatives of all three germ layers and covers major internal organs.
462 However, different tissues are affected by different biological and evolutionary processes, as exemplified
463 by our finding of dynamic changes in the proportions of tissue-specific expressed genes through time.
464 Therefore, future work that includes a larger tissue collection is needed for a thorough exploration of the
465 anatomic complexity of amniotes. For instance, additional organs involved in digestion (e.g. stomach, and
466 pancreas) could help refine the suggested role of new genes in dietary adaptations. Our tissue selection
467 was inadequate to study single coding exon olfactory genes that are enriched for young duplicates and

468 contained within heavily expanded gene families (Young and Trask 2002; Nei et al. 2008). This task will
469 require dedicated RNA expression profiling of olfactory (sensory) tissues.

470

471 Our analyses consistently point to the importance of taking into account tissue complexity when studying
472 the contributions of genes to organismal diversity. For instance, we found many old paralogs that show low
473 levels of brain-specific expression but are enriched for functions related to synaptic transmission, cognition,
474 learning and memory (Supplemental Table S11), which suggests that their preferential expression in the
475 brain is genuine and biologically relevant. Because we analyzed bulk tissues, genes that are specifically
476 expressed in certain regions or cell types will show high tissue specificity but low expression. In the future it
477 will thus be important to systematically study transcriptional differences between cell types and tissue
478 regions (Hawrylycz et al. 2012).

479

480 Another important aspect to be considered in future studies is the changing dynamic of duplicate gene
481 expression during development. For instance, our analyses of adult brain suggest that young paralogs
482 contribute little to the brain-specific transcriptome, whereas Zhang et al. (2011) found young paralogs to
483 be specifically expressed in the developing human brain. A study that tracked changes in gene expression
484 during placentation showed how paralogs of different duplication ages are expressed at different stages of
485 pregnancy (Knox and Baker 2008). It is likely that such dynamics will be observed in many, if not most
486 organs.

487

488 Taken together, our study provides a comprehensive evolutionary analysis of amniote gene families in a
489 comparative manner, spanning a large evolutionary time scale. We describe general features of duplicate
490 evolution, which allow pinpointing cases with unique trajectories and therefore potentially lineage-specific
491 adaptations. The stringently filtered database of gene duplications and associated expression values allows
492 exploring lineage-specific shifts in expression profiles that might be indicative of evolutionary innovations
493 and identifying interesting candidate genes with specific characteristics that merit experimental evaluation.

494 METHODS**495 Duplication and single-copy genes datasets**

496 From the initial set of protein-coding gene family trees that were obtained from Ensembl v64 (Flicek et al.
497 2012; Vilella et al. 2009) we retained only those gene family trees ($n = 12,452$, number of duplication
498 events = 18,859) that contained at least one of the species for which transcriptome data were available
499 (Supplemental Fig S1). To retrieve and annotate duplication events we relied on ETE v2 (Huerta-Cepas et al.
500 2010). Each gene family tree was parsed from the root to the leaves. Upon encountering a duplication
501 event, the daughter clades were analyzed and a number of filtering steps employed to remove poorly
502 supported duplication nodes, duplications with incorrectly inferred duplication ages, erroneously inferred
503 duplications (stemming from split genes or based on transcriptional evidence with overlapping coordinates),
504 and intronless gene copies (Supplemental Methods).

505

506

507

508 A dataset of 1:1 amniote orthologs was taken from (Brawand et al. 2011). However, because we also
509 considered duplications with ages older than the common amniote ancestor, we removed genes that were
510 present in our duplication dataset from the single-copy dataset. In total, we retained 3379 single-copy
511 genes.

512

513 Expression data

514 RNA-seq expression data was available for nine species, belonging to the three main mammalian lineages
515 (placental mammals: human, chimpanzee, gorilla, orangutan, rhesus macaque, mouse; marsupials: grey
516 short-tailed opossum; monotremes: platypus) and a bird (non-domesticated chicken) from five somatic
517 (cortex or whole brain without cerebellum, cerebellum, hear, kidney, liver) and three reproductive tissues
518 (testis, ovary, placenta, Supplemental Table S2, Gene Expression Omnibus accession numbers [GSE30352](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE30352)
519 ([Brawand et al. 2011](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE30352)) and [GSE43520](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE43520) ([Necsulea et al. 2014](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE43520))). Adapter-trimmed RNA-seq reads were aligned
520 on the reference genomes with TopHat (Trapnell et al. 2009) and gene expression was estimated as FPKM
521 (fragments per kilobase of exon per million of mapped reads) with Cufflinks v2.0.0 (Trapnell et al. 2010).
522 The procedure was repeated for unambiguously mapped reads only and for all mapped reads, using multi-
523 read and fragment-bias correction methods as implemented in Cufflinks, where applicable. We used these
524 two estimates of expression levels to identify gene copies for which expression levels can be determined
525 with certainty, and flag those for which reliable expression values cannot be estimated (Supplemental
526 Methods). We checked our method against a dataset of “problematic” human genes, for which expression
527 levels cannot be reliably estimated (Robert and Watson 2015). Our approach of filtering and flagging
528 effectively removes problematic genes (Supplemental Methods). Importantly, flagged genes and gene

529 families in all species were excluded from any analysis that required gene-specific expression levels. The
530 final expression levels were calculated for each gene using all reads (even if no unique reads were present)
531 and employing the `-multi-read-correct` option.

532

533 Because expression profiles of the two neural tissues, cortex and cerebellum, are highly correlated, we
534 computed the mean of their expression for each gene and used this value in all subsequent analyses. We
535 validated the results treating brain and cerebellum as separate tissues (Supplemental Methods). We
536 normalized the expression levels among samples with a median scaling procedure (Brawand et al. 2011)
537 and calculated species median expression levels for each tissue. All expression levels were \log_2 -transformed.
538 To be able to take the logarithm of all values, we set the smallest value to 10^{-6} and replaced all values
539 smaller than 10^{-6} with it.

540

541 **Statistical analysis**

542 All statistical analyses were performed in R 2.15.3 (R Core Team 2012). Significance levels were adjusted
543 with Benjamini-Hochberg correction (Benjamini and Hochberg 1995). Significant chi-square tests were
544 followed by a posthoc procedure as implemented in the R package *polytomous* (Arppe 2013). We used
545 standardized Pearson residuals to assess if individual observed values differ significantly from an overall
546 hypothetical homogeneous distribution and to identify the direction of these differences (over- or under-
547 representation) by tissue or by duplication age. Wilcoxon rank sum test was used to study differences in
548 expression divergence between species-specific paralogs and 1:1 orthologs.

549

550 To evaluate the contribution of different factors (expression levels, duplication status [i.e. paralog or single-
551 copy gene], evolutionary time [duplication or speciation age]) to expression divergence and tissue
552 specificity we constructed linear models in R that included all orthologous relationships between human
553 and other species as well human paralogs of different duplication ages. Human was chosen as, given our
554 collection of species, it provides the most detailed data on duplication and speciation ages. Qualitatively
555 similar results were obtained with other focal species. Because most paralogs in our dataset have much
556 older duplication ages than the oldest speciation age of the single-copy genes (human-chicken divergence)
557 and this can affect the linear model, we repeated the analyses by removing all paralogs with duplication
558 ages older than amniotes. We also repeatedly (100 times) subsampled as many single-copy genes as there
559 are paralogs to match the sample sizes of both gene types.

560

561 **Gene ontology analysis**

562 Overrepresentation of gene ontology (GO) terms (The Gene Ontology Consortium 2000) in human and
563 mouse genomes were identified in GOrilla (Eden et al. 2009). This tool allows to either find enrichments in

564 a ranked gene list or to evaluate functional overrepresentation in a candidate dataset against a specified
565 list of background genes. We set the false discovery rate (FDR) of 0.1% as our cutoff value and employed
566 the Benjamini-Hochberg correction for multiple testing within each dataset.

567

568 **Expression divergence and protein sequence divergence**

569 Expression divergence was calculated by species for normalized, log-transformed expression values across
570 all available tissues in a pairwise manner across sister clades resulting from a given duplication event
571 (Supplemental Methods). Protein sequence divergence (d_N) was calculated on the basis of pairwise
572 alignments between paralogs using the longest transcript of each paralog. Spearman's correlation
573 coefficients were calculated between expression divergence and protein sequence divergence for human
574 and opossum paralogs by duplication age. To avoid the potential bias introduced by a small number of
575 highly expanded gene families, we randomly sampled a single gene pair per gene family and calculated the
576 correlation between expression and protein sequence divergence on this dataset.

577

578 To assess in how far genes that differ in their extent of protein sequence divergence compared to
579 expression divergence and *vice versa* fulfill different biological functions, we ranked paralog pairs in two
580 different ways, following (Warnefors and Kaessmann 2013): i) genes with higher expression divergence
581 rank relative to their protein sequence divergence rank were classified as ED-biased, ii) genes with higher
582 protein sequence divergence rank relative to their expression divergence rank were classified as d_N -biased.
583 We also grouped genes into two age classes: lineage-specific and young paralogs that have duplicated in
584 the common amniote ancestor or more recently, and old and ancient paralogs that have duplicated in the
585 tetrapod ancestor and earlier (see below for more details). We performed GO enrichment analyses
586 separately for these groups as described above.

587

588 **Duplication age groups**

589 For our analyses of evolutionary dynamics of tissue specificity we defined four groups of duplication ages: i)
590 lineage-specific paralogs, e.g. primate-specific, rodent-specific, marsupial-specific, etc; ii) "young" paralogs
591 that duplicated along the branch leading to the amniote ancestor and are older than lineage-specific
592 duplications; iii) "old" paralogs with duplication ages in the tetrapod and bony vertebrate (Euteleostomi)
593 ancestors; iv) and "ancient" paralogs with duplication ages in the ancestors of vertebrates, chordates,
594 coelomates, and bilaterals. For the analysis of placenta-specific expressed paralogs we subdivided the
595 "young" category into genes that emerged in the common eutherian ancestor or before that, on the branch
596 leading to the ancestor of therians and amniotes.

597

598 **Tissue specificity**

599 We used two measures of tissue specificity: tau (Yanai et al. 2005) and relative gene expression. For the
600 second measure, we defined tissue specificity as relative expression of the gene in the tissue with highest
601 expression ($\text{exp}_{\text{MaxTissue}}/\text{sum}(\text{exp}_{\text{AllTissues}})$). This calculation was performed on normalized, but not
602 transformed expression values. For a set of n tissues, uniformly expressed genes show tissue specificity of
603 $1/n$, whereas genes expressed in a single tissue show tissue specificity of 1, independent of n . Relative
604 tissue expression was calculated as the expression of a gene in the target tissue divided by the sum of its
605 expression in all tissues. Both measures of tissue specificity (tau and relative gene expression) were
606 strongly correlated with each other in our dataset ($\rho = 0.968, P < 10^{-15}$).

607

608 Genes with $\tau \geq 0.8$ were defined as tissue-specific expressed. Using relative gene expression, tissue-
609 specific expressed genes were defined in two different ways. For most of our analyses genes were
610 considered tissue-specific expressed if they showed at least two-fold higher expression in the tissue of
611 highest expression than under uniform expectation: $2/n$ (“twice uniform expression”). In the alternative,
612 more stringent definition of tissue specificity, we required the tissue-specific expressed gene to show at
613 least two-fold higher expression in tissue with highest expression than in any other tissue (“stringent”). To
614 evaluate the correlation between expression levels and tissue specificity we assessed expression levels as
615 means across all tissues.

616

617 To test for changes in the proportion of genes with the highest expression in a given tissue, we calculated
618 for each tissue and in each species the number of genes with the highest expression in this tissue for each
619 age category (as shown in Fig. 4C). We then applied a chi-square test, followed by a posthoc procedure (as
620 described above) to identify age categories with significantly more or fewer genes.

621

622 Lineage-specific expansions of amniote gene families

623 Only gene families with at least three paralogs that emerged in the common amniote ancestor or more
624 recently for any given species were considered in this analysis, as we were interested in studying how
625 repeated duplications influence lineage-specific repertoire of paralogs. We used twice uniform expression
626 (see previous section) to define tissue-specific expressed genes and only retained paralogs, for which gene-
627 specific expression patterns could be unambiguously inferred. We also evaluated duplications along
628 individual amniote lineages that were not present in their common ancestor (human paralogs that have
629 duplicated along the primate lineage, mouse paralogs that have duplicated along the rodent lineage, etc.)
630 with the aim of identifying gene families that have repeatedly and independently produced lineage-specific
631 expansions (Fig. 7, Supplemental Table S10). We treated them in the same way as above, requesting at
632 least three paralogs per species for which expression patterns could be unambiguously determined.

633 Including genes for which the expression patterns could be confounded by closely related paralogs did not
634 change the results (Supplemental Table S14).

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644 **FIGURE LEGENDS**

645 **Figure 1: Expression divergence of single-copy and duplicate genes in the mouse genome.** Expression
 646 divergence of single-copy human-mouse orthologs (yellow) and age-grouped mouse paralogs (blue) based
 647 (A) on Euclidean distances and (B) on Pearson's correlation coefficient r (displayed as $1-r$). Paralogs are
 648 grouped into age classes according to gene tree topology. C) The species tree shows divergence times in
 649 million years, with highlighted branches corresponding to the evolutionary groups for which divergence
 650 was inferred.

651

652 **Figure 2: Expression levels of single-copy and duplicate genes.** Median expression levels for single-copy
 653 (yellow) and duplicate genes (shades of blue) across all organs in the mouse genome. Duplicate genes are
 654 grouped into age classes.

655

656 **Figure 3: Expression divergence and protein sequence divergence.** A) Correlation between expression
 657 divergence and protein sequence divergence for human and opossum paralogs of different duplication ages
 658 and for 1:1 amniote and primate orthologs (Warnefors and Kaessmann 2013). Numbers of gene families
 659 within each group are shown above bars. A single gene pair was sampled for each gene family and
 660 expression divergence was measured as Euclidean distances. * $p < 0.02$, ** $p < 0.001$, *** $p < 0.00001$, NS=not
 661 significant. B) Expression divergence (measured as Euclidean distances across all organs) and d_N values for
 662 human duplicate genes of all duplication ages. Increasing bias toward expression divergence is indicated in
 663 shades of yellow and increasing bias toward protein divergence in blue. C) Relationships among the five
 664 most overrepresented GO terms for ED-biased (orange) and d_N -biased (blue) genes in each age category.
 665 Brighter colors correspond to young and lineage-specific duplication ages, darker colors to old and ancient
 666 duplication ages. Some intermediate terms were omitted for clarity.

667

668 **Figure 4: Evolutionary dynamic expression profiles of tissue-specific expressed genes.** A) Schematic
 669 representation of duplication age categories using opossum as example. B) Proportion of genes specific to
 670 any given tissue is plotted by gene type and duplication age. Tissue specificity of opossum genes was
 671 assessed using the "stringent" definition (see Methods). Single-copy genes and paralogs, grouped in four
 672 age classes, are shown for each tissue. Bars represent 95% confidence intervals. Analyses carried out at
 673 gene level. C) Significant differences in evolutionary dynamics of tissue preferences of duplicated genes.
 674 Duplication ages are given in the upper row. Bars above group the fine-grained duplication ages into the
 675 same four age categories as in A (Methods). Significant over- (blue) or under-representation (orange) of
 676 genes with highest expression in a given tissue was tested for paralogs in each species and for each
 677 duplication age with chi-square test, followed by a post-hoc procedure (Methods). Grey cells signify no
 678 statistical difference. D) Analysis of differential gene loss/gain in gene families with highest expression in

679 liver, testis, and all other tissues combined. E) Expression shift analysis: Proportions of chicken outgroup
 680 genes with highest expression in liver and testis compared to the proportion of resulting mammalian
 681 paralogs with highest expression in these tissues. Significance was assessed with chi-square test.

682

683 **Figure 5: Placenta-enrichment by duplication age in three therian species: human, mouse, and opossum.**

684 Significant global chi-square tests indicate differences in the number of placenta-specific expressed genes
 685 by age group in each of the studied species (placenta-specific expressed genes are defined as having more
 686 than twice uniform expression level in the placenta), after which a post-hoc procedure was applied, as
 687 described in Methods. + significant over-representation of placenta-specific expressed genes; – significant
 688 under-representation of placenta-specific expressed genes; NS not different from expectation. Overall,
 689 results obtained with the alternative definition of tissue specificity (τ) were the same, with the exception
 690 of mouse and human, where lineage-specific paralogs showed more placenta-specific expressed genes than
 691 expected.

692

693 **Figure 6: *PAGE4* gene family tree.** Duplication events are marked with blue squares. Gene names, Ensembl

694 gene ID, and the species are given at the tips of the tree. Circles represent expression patterns, with circle
 695 color corresponding to the tissue of highest expression of the respective gene and circle size approximating
 696 the expression level. It is likely that homologs of *PAGE4* are placenta-specific expressed in other eutherian
 697 species, as suggested by the study in the elephant (Hou et al. 2012), however, we could not assess this
 698 pattern, as placenta samples from other primates were not available to our study. The absence of
 699 expression of *PAGE3* and *PAGE5* in orangutan is likely explained by the unavailability of testis samples from
 700 this species to our study.

701

702 **Figure 7: Lineage-specific and shared gene family expansion in amniotes.** A) Gene family expansions along

703 primate, rodent, marsupial, monotreme, and bird lineages are depicted along the tree branches as bar-
 704 plots (number of gene families with at least three paralogs, number of “specific”, number of “diverse”, and
 705 number of “broad” gene families). B) Gene families that expanded independently in more than a single
 706 lineage, with gene family identifiers depicted above (as in Supplemental Table S1). Color of the cells
 707 corresponds to the predominant tissue of expression of each gene family in the given species for “specific”
 708 gene families. White cells correspond to “diverse” gene families. Gene family highlighted in red above
 709 expanded independently in mouse, opossum, platypus, and chicken; the blue gene family expanded
 710 independently in mouse, opossum, and platypus.

711

712 **Figure 8: Lineage-specific expansion and expression changes in amniotes.** Duplication events are marked

713 with blue squares. Ensembl gene IDs and species are shown at the tips of the trees. Circles represent

714 expression patterns, with circle color corresponding to the tissue of the highest expression of the
715 respective gene. A) Gene family (Fam612) containing guanylate binding peptides has expanded
716 independently in mouse, opossum, platypus, and chicken. Note multiple lineage-specific changes in tissue
717 preference. B) Independent lineage-specific duplications in primates and marsupials. Expansions along the
718 primate lineage produced paralogs with high brain-specific expression, whereas the majority of opossum
719 genes show low liver-specific expression.
720

721 **TABLES**722 **Table 1:** Expression dynamics of amniote gene families.

Species	“Diverse”	“Specific”	“Broad” (total number of gene families)	Percentage (number) of gene families containing ZNF
Human	37.7 (63)	37.7 (63)	4.2 (7)	100.0 (7)
Chimpanzee	28.7 (51)	44.9 (80)	5.6 (10)	70.0 (7)
Gorilla	26.0 (44)	43.2 (73)	8.9 (15)	66.7 (10)
Orangutan	21.7 (26)	35.0 (42)	15.0 (18)	77.8 (14)
Macaque	27.4 (46)	41.7 (70)	8.3 (14)	42.9 (6)
Mouse	51.2 (64)	42.4 (53)	0.0 (0)	0.0 (0)
Opossum	34.9 (44)	44.4 (56)	0.8 (1)	0.0 (0)
Platypus	40.8 (29)	45.1 (32)	4.2 (3)	66.7 (2)
Chicken	41.4 (12)	58.6 (17)	0.0 (0)	0.0 (0)

723 Proportion of diverse, specific and broad gene families among all gene families containing at least three
724 paralogs that duplicated in the common amniote ancestor or more recently (see text for more detail,
725 Methods). Orangutan is lighter colored as only 5 organs were studied in this species (testis is missing from
726 the dataset, Supplemental Table S2), which results in increased percentage of broadly expressed genes.

727

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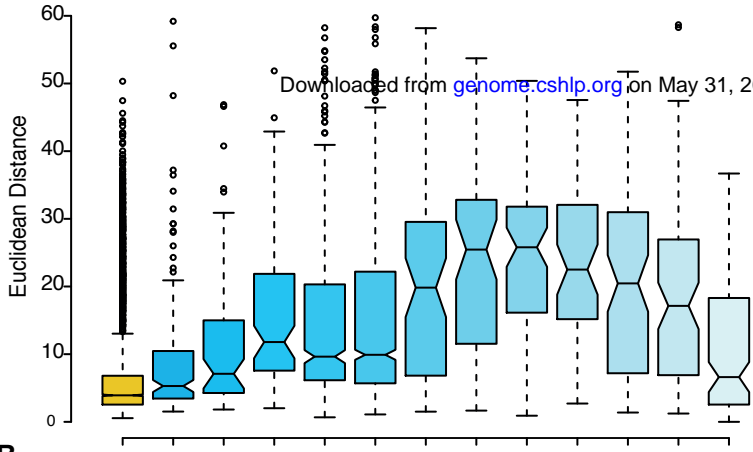
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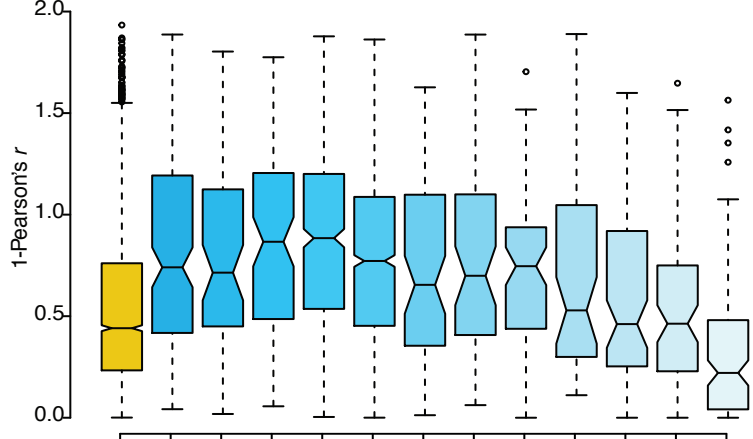
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Mouse genes

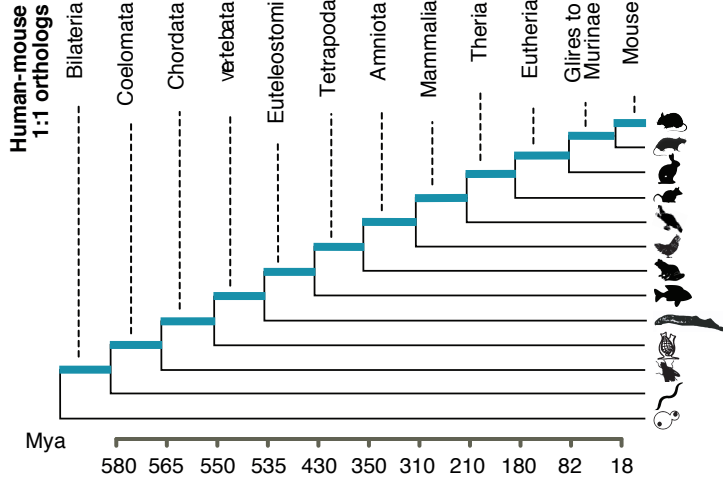
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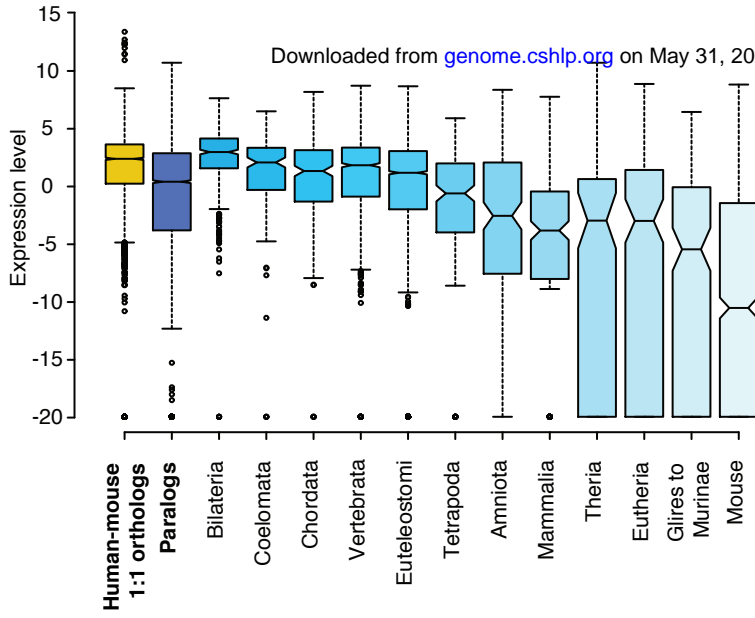


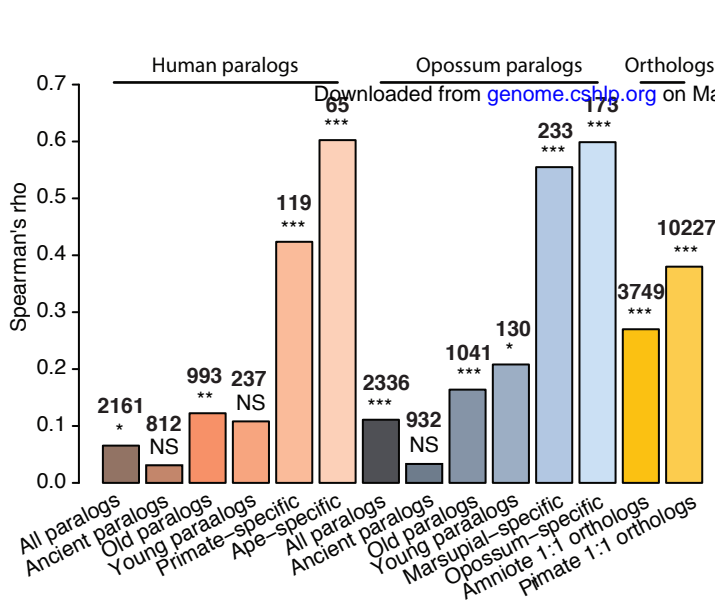
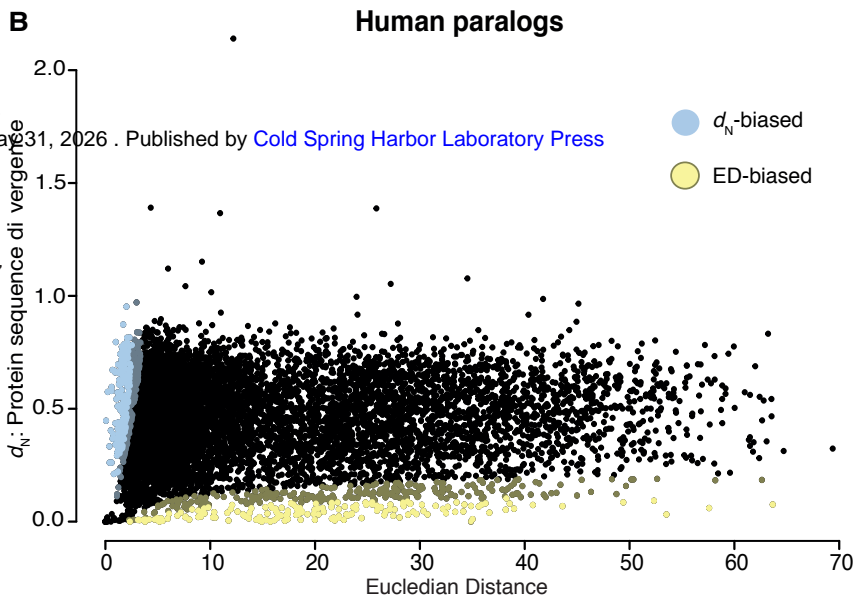
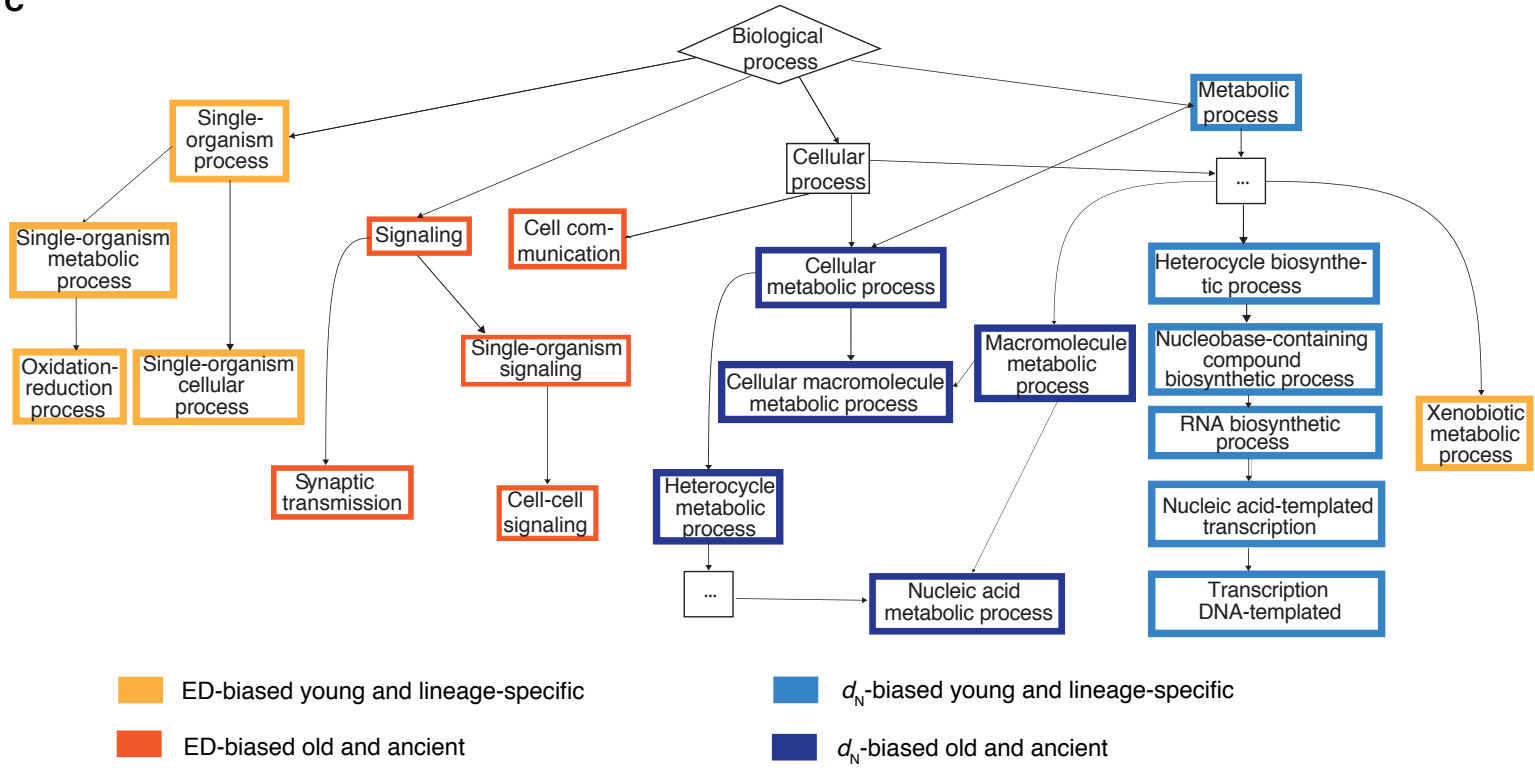
C



Mouse genes

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A**B****C**

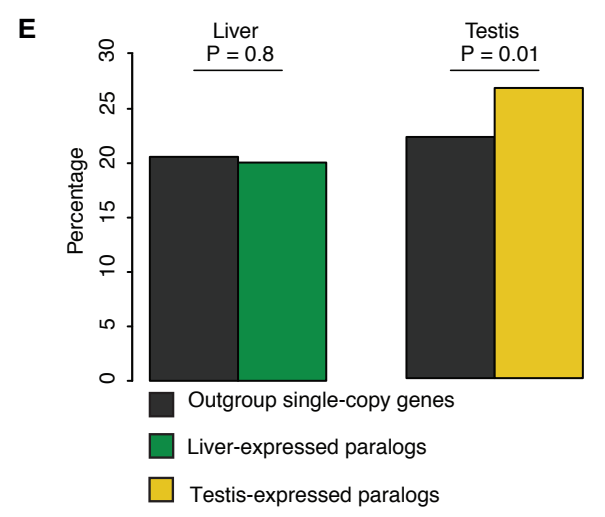
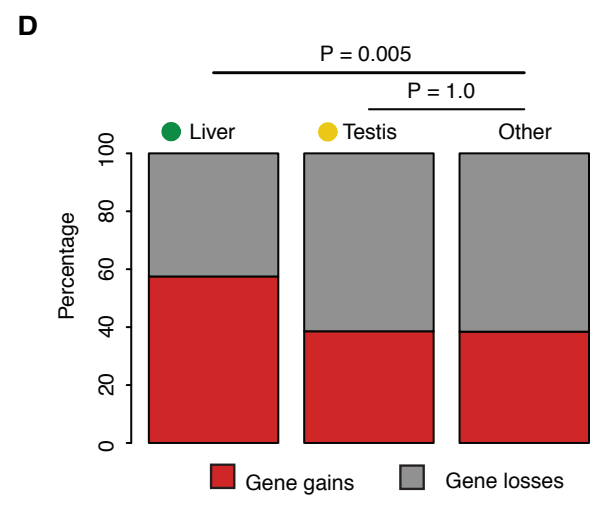
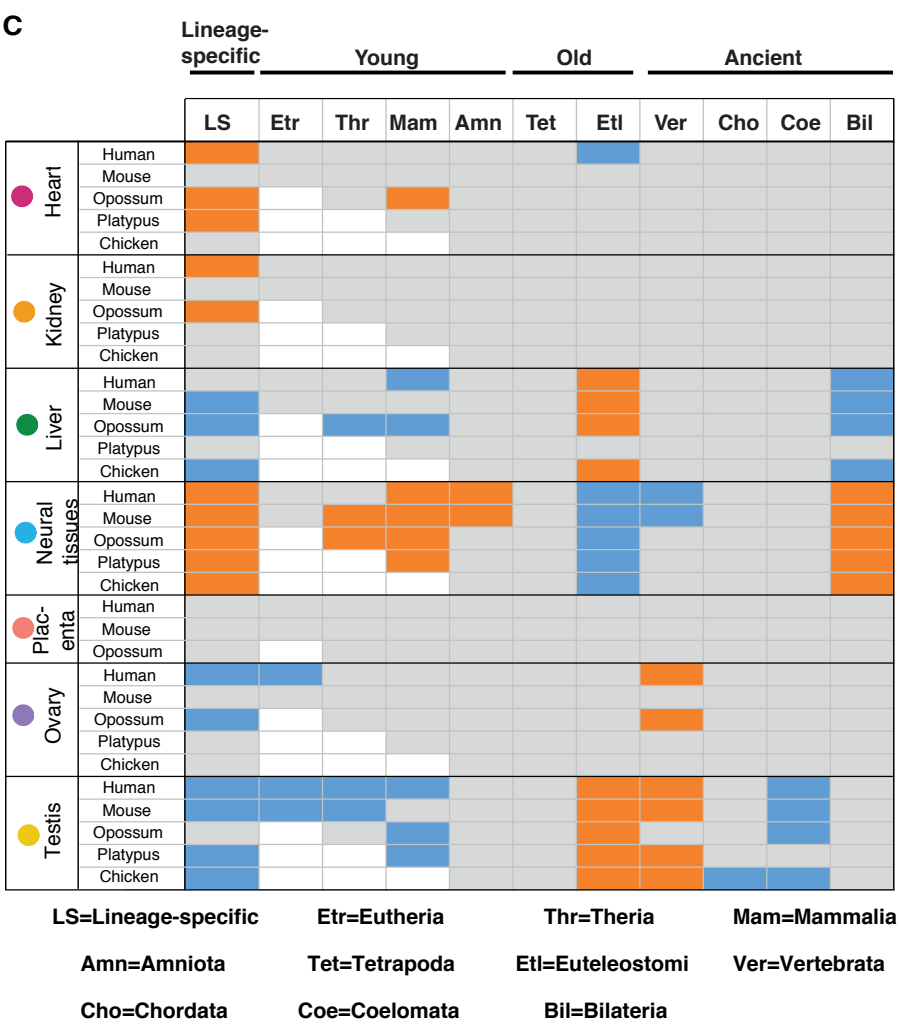
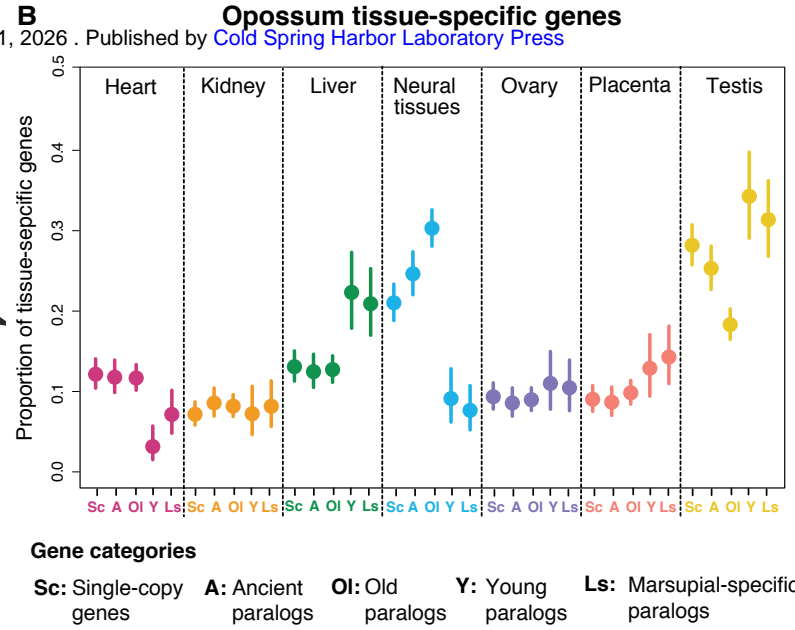
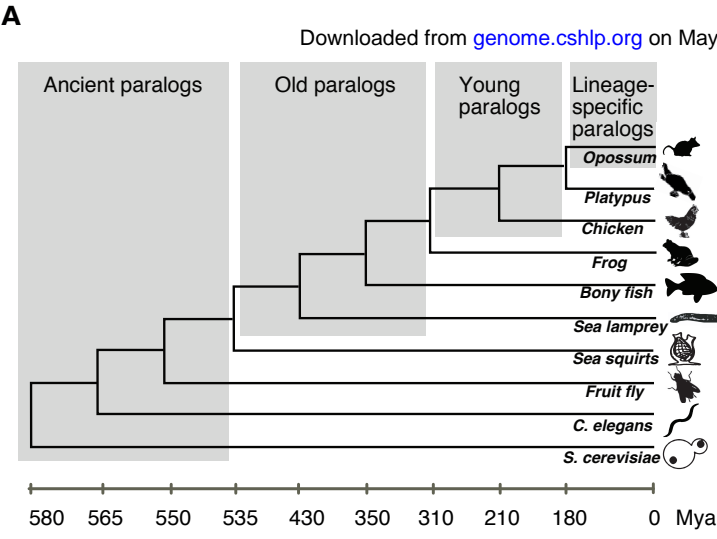
■ ED-biased young and lineage-specific

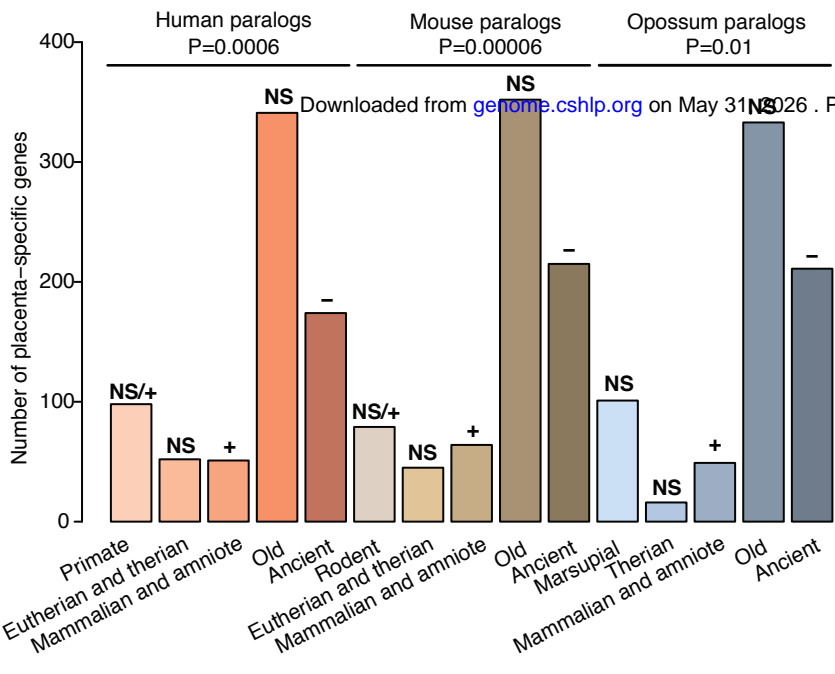
■ ED-biased old and ancient

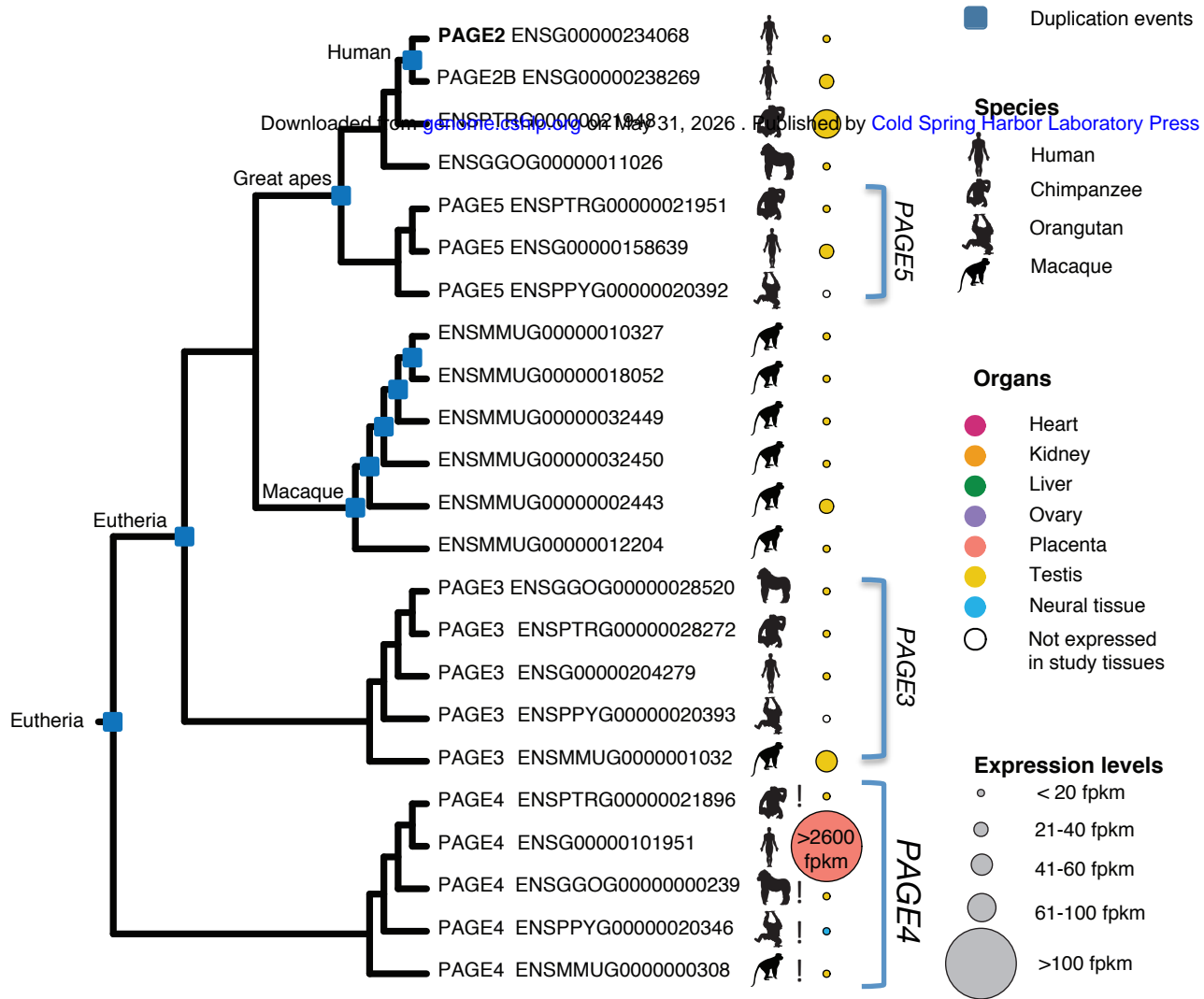
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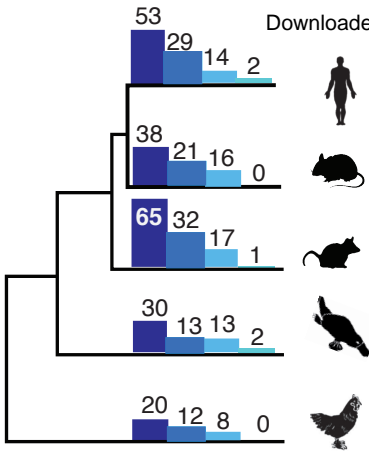
■ d_N -biased old and ancient

Opossum tissue-specific genes

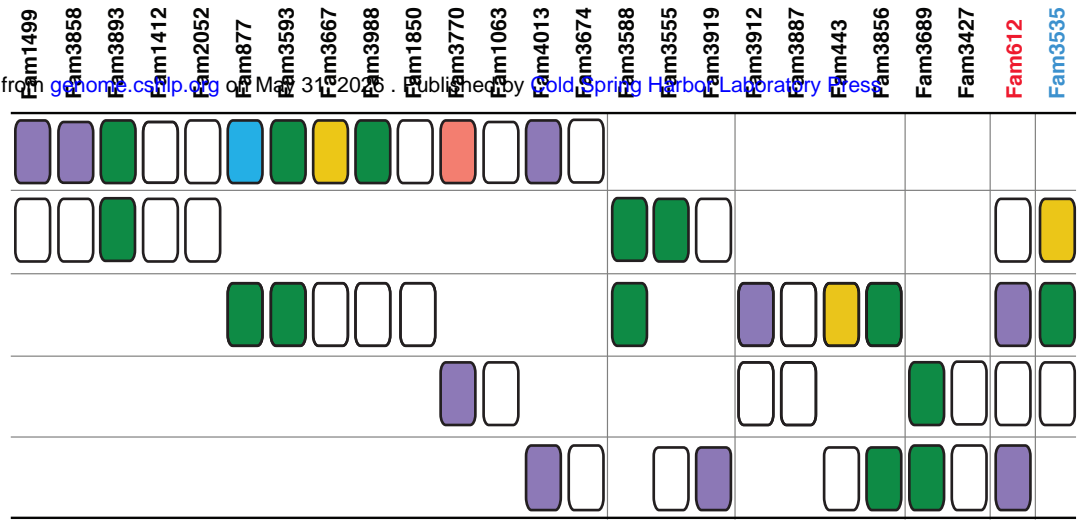






A**B**

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Predominant tissue of expression within gene family



