New genes and functional innovation in mammals

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ABSTRACT

The birth of genes that encode new proteins is a major source of evolutionary innovation. However, we still understand relatively little about how these genes come into being and which functions they are selected for. Here we address this question by generating a comprehensive list of mammalian-specific gene families originated at different times during mammalian evolution. We combine gene annotations and *de novo* transcript assemblies from 30 mammalian species, obtaining about 6,000 families with different species composition. We show that the families which arose early in mammalian evolution (basal) are relatively well-characterized. They are enriched in secreted proteins and include milk and skin polypeptides, immune response components and, proteins involved in spermatogenesis. In contrast, there is a severe lack of knowledge about the functions of proteins which have a more recent origin in certain mammalian groups (young), despite the fact that they have extensive proteomics support. Interestingly, we find that both young and basal mammalian-specific gene families show similar gene expression biases, with a marked enrichment in testis. Proteins from both groups tend to be short and depleted in aromatic and negatively charged residues. This indicates shared mechanisms of formation and suggests that the youngest proteins may have been retained for similar kinds of functions as the oldest ones. We identify several previously described cases of genes originated *de novo* from non-coding genomic regions, supporting the idea that this mechanism frequently underlies the evolution of new protein-coding genes. The catalogue of gene families generated here provides a unique resource for studies on the role of new genes in mammalian-specific adaptations.

INTRODUCTION

The genome and mRNA sequencing efforts of the last two decades have resulted in gene catalogues for a large number of organisms [1–3]. This has spurred the comparison of genes across species and the identification of a surprisingly large number of proteins that appear to be species- or lineage-specific [4–14]. Although these proteins probably hold the key to many recent adaptations, they remain, for the most part, still uncharacterized [15].

Species- or lineage-specific genes, sometimes called orphan genes, are defined by their lack of homologues in other species. They may arise by rearrangements of already existing coding sequences, often including partially duplicated genes and/or transposable elements [16–18], or completely *de novo* from previously non-coding genomic regions [11,17,15,19–24]. The latter process is facilitated by the high transcriptional turnover of the genome, which continuously produces transcripts that can potentially acquire new functions and become novel protein coding-genes [25–27].

Lineage-specific genes are expected to be major drivers of evolutionary innovation. A well-known example is the nematocyst in the Cnidaria (anemones, jellyfish, etc), which is used to inject toxin into their preys. Some of the major constituents of the nematocyst are Cnidaria-specific proteins, indicating that the birth of new genes in a Cnidaria ancestor was intimately linked to the emergence of a novel trait [10]. In mammals, diverse adaptations have also been related to new genes. The caseins, which transport calcium in the milk, have originated from duplications of sequences initially coding for teeth proteins which have undergone very rapid sequence diversification [28].

In mammals, there has been a number of systematic studies aimed at the identification of recently evolved genes, particularly in human and to a lesser extent in mouse [8,17,21,24,29,30]. These works have been very valuable in understanding the origin of new genes, providing some of the first examples of *de novo* gene evolution. However, our knowledge of how these

genes have impacted mammalian biology has lagged behind. To fill this gap we have generated a comprehensive list of gene families originated at different times during the evolution of mammals. By combining gene expression data, functional annotation, proteomics, and amino acid sequence properties we obtain a global picture of the biology underlying the birth of new genes in mammals.

RESULTS

Identification of mammalian-specific gene families

Our first goal was to build a comprehensive catalogue of mammalian-specific protein-coding gene families. To ensure consistency, our analysis was based on 30 mammalian species for which we had the genome sequence, a complete set of gene annotations and RNA sequencing data (RNA-Seq) (Table S1). The RNA-Seq data was used to assemble transcripts *de novo*, which provided a source of expressed sequences for each species that was independent of the gene annotations and which served to complement them.

First, in each of the 30 species, we selected the genes that did not have homologues in non-mammalian species using BLASTP sequence similarity searches against a panel of 34 species including vertebrates, insects, plants, fungi, and bacteria (Table S1). Subsequently, we eliminated any genes that, albeit initially classified as mammalian-specific, had indirect homology, through paralogues, to genes in more distant species. This was done to minimize the inclusion of very rapidly evolving duplicated genes [17].

Next, we used the remaining mammalian-specific genes in each species to perform all against all sequence similarity searches. We clustered the genes into families by iteratively inspecting the lists of sequence similarity hits until no more homologues could be added to a given family. Then we assigned each family to the most ancestral node that connected the different species in the family. This node was the point from which no further ancestors could be traced back and it represented the time of origin of the family.

A method that relies solely on gene annotations is likely to miss homologues in the species which are less well annotated. For this reason, we performed additional searches of all members of a family against the novel transcripts generated with the RNA-Seq data. In most families the use of RNA-Seq data expanded the range of species with evidence of expression of the gene (Figure S1). In some cases it also resulted in a deepening of the node of origin. For example, about 22% of the genes initially classified as species-specific were now found in multi-species families. To ensure robustness, in each node we only considered the gene families that contained sequences from at least half of the species derived from that node. The procedure resulted in 2,034 multi-species gene families, altogether containing 10,991 different proteins. We also obtained 3,972 species-specific families, containing 4,173 different proteins. The complete catalogue of gene families, protein sequences, and transcript assemblies is provided as supplementary material.

Figure 1 shows the distribution of gene families in the different nodes of the mammalian tree. About one fourth of the families (439) had a basal distribution, they had originated before the split of the major mammalian groups (class 'mam-basal' or 2; nodes 1, 2, 4, 5 and 6 in Figure S2). The rest of multi-species families corresponded to more recent nodes and were classified as 'mam-young (class 1). This included, for example, the 269 families which were specific of the Catarrhini (old World monkeys, node 21 in Figure S2). These genes were present in macaque and/or baboon, and in the great apes, but were absent from other primate or mammalian branches. Other examples of large sets of phylogenetically-restricted gene families corresponded to the primates (135 families, node 7), the muridae (173 families, node 23) or the felids (52 families, node 14).

Our dataset included several previously described mammalian-specific genes. One example was neuronatin, an imprinted gene involved in the regulation of ion channels during brain development [31]. We found members of this family in 26 placental species but not in marsupials or Monotremata. Another example was the antimicrobial peptide dermcidin [32]. This protein is secreted in the skin and has been previously been described as a primate

invention [17]. An illustrative list of genes, including these examples and other cases discussed in the next sections, is available from Table 1.

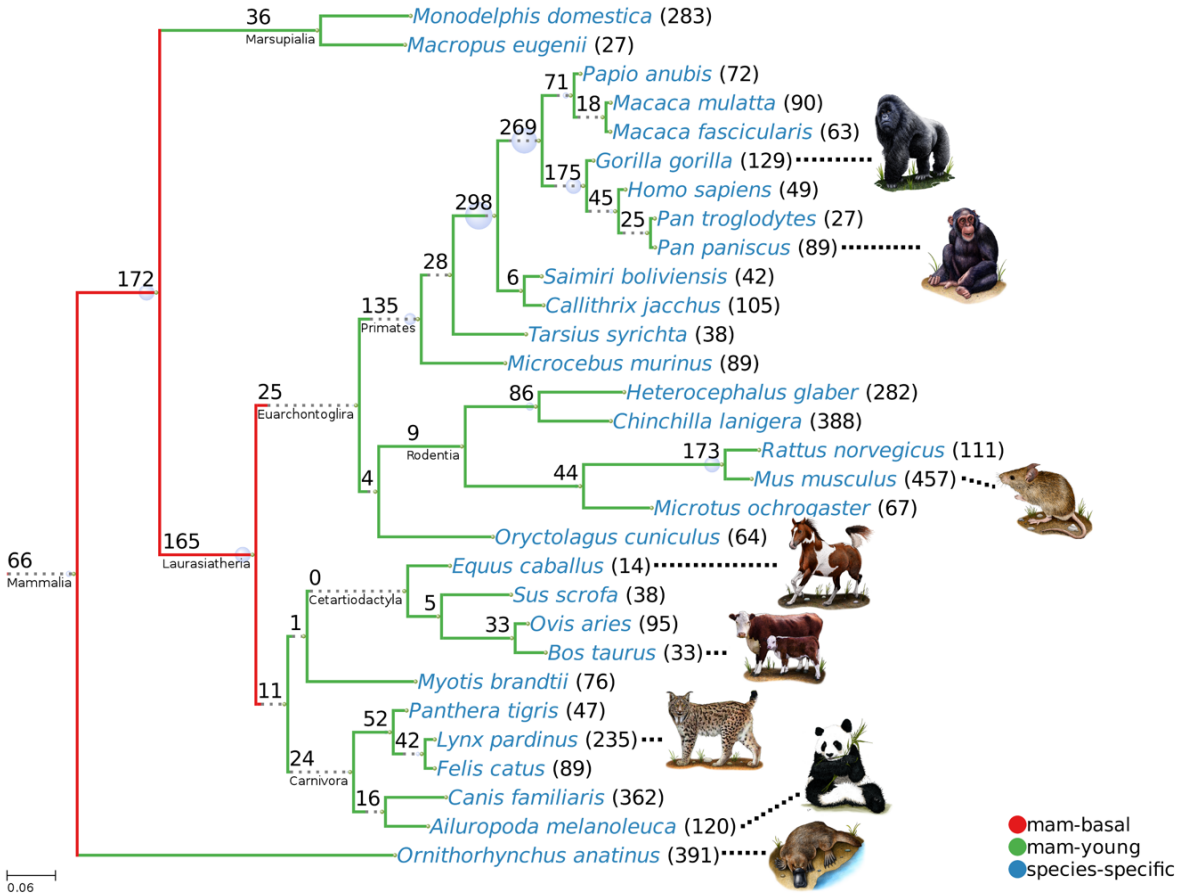

Figure 1. Mammalian tree and number of mammalian-specific gene families. The tree depicts the phylogenetic relationships between 30 mammalian species from different major groups. The values in each node indicate the number of families that were mapped to the branch ending in the node. We define three conservation levels: 'mam-basal' (class 2, most families older than 100 Million years, red), 'mam-young' (class 1, green) and 'species-specific (class 0, blue). The branch length represents the approximate number of substitutions per site as inferred from previous studies (see Methods). The scale bar on the bottom left corner represents 6 substitutions per 100 nucleotides. Dotted lines have been added to some branches to improve readability.

Gene Name	Description	Node	Features	Ref.
SCGB	secretoglobin	Mammalia	Gene family, modulation of inflammation	[93]
PRM3	protamine 3	Theria	Affects sperm motility	[46]
CSN1S1	casein alpha s1	Eutheria	Ca-sensitive milk protein, related to vertebrate calcium-binding protein SPARCL1	[28]
LCE6A	late cornified envelope 6A	Eutheria	Formation of the skin, part of the epidermal differentiation gene complex	[49]
IL2	interleukin 2	Eutheria	Cytokine, rapid sequence divergence	[94]
MUC7	mucin 7	Eutheria	Antimicrobial peptide, secreted in saliva	[42]
NNAT	neuronatin	Eutheria	Neural development.	[31]
IGIP	IgA-inducing protein	Eutheria	Activates the production of immunoglobulin A by B cells.	[66]
SMCP	sperm mitochondrial-associated cysteine-rich protein	Eutheria	Involved in sperm motility.	[47]
CLLU1	chronic lymphocytic leukemia up-regulated 1	Primates	Over-expressed in leukemia, *de novo* origin.	[21]
HMHB1	histocompatibility (minor) HB-1	Primates	Precursor of the histocomplatibility antigen HB-1, *de novo* origin.	[16]
DCD	dermcidin	Node 16	Antimicrobial peptide, secreted in the skin	[16,32]
MYEOV	myeloma over-expressed	Node 16	Over-expressed in myeloma, *de novo* origin.	[50]
RP11-429E11.3	uncharacterized protein	Node 26	*De novo* origin.	[30]
RP11-45H22.3	uncharacterized protein	Node 28	*De novo* origin.	[27]

Table 1. Examples of mammalian-specific genes families. Node 1: Mammalia; Node 2: Theria; Node 4: Eutheria; Node 7: Primates; Node 16: Haplorhini; Node 26: great apes; Node 28: human and chimpanzee.

Mammalian-specific genes are enriched in testis

We compared the gene expression patterns in different tissues using human data from GTEx [33] and mouse data from ENCODE [34] (Tables S2-S7). We generated two control gene sets: a set of randomly chosen genes that were not mammalian-specific ('random') and a collection of genes with homologues in all 34 non-mammalian species used in step 1 of our method ('ancestral'). We found that mammalian-specific genes were strongly enriched in testis. The number of mammalian-specific genes with maximum expression in this organ was 50% in human and 40% in mouse, compared to 20% and 13%, respectively, for randomly selected non mammalian-specific genes (Fisher test p-value $<10^{-5}$ for both species). The genital fatpad in mouse also showed a significant enrichment of mammalian-specific genes (Fisher test p-value <0.01), although this affected a much smaller percentage of genes (~ 5%).

We estimated the number of genes that were tissue-specific using a previously described metric [35]. The majority of mammalian-specific genes were tissue-specific, whereas this was not the case for the control gene sets (Figure 2A and S3A, for mouse and human, respectively; Fisher test p-value $< 10^{-5}$ for both species). Curiously, the 'mam-basal' (class 2) genes tended to be more tissue-specific than younger gene classes (Fisher test p-value < 0.005 for both human and mouse). Most mammalian-specific genes that were tissue-specific showed maximum expression in testis (Figure 2B and S3B).

To compare the gene expression levels between datasets we used the FPKM value (Fragments Per Kilobase per Million mapped reads) of the tissue with maximum expression of the gene. Interestingly, although the three classes of mammalian-specific genes showed a very similar enrichment in testis, 'mam-basal' genes were expressed at significantly higher levels than the other gene classes (Wilcoxon test p-value $< 10^{-4}$, in both human and mouse)(Figure 2C and S3C).

An enrichment of very young genes in testis was expected given previous observations that nascent transcripts in humans are highly enriched in this

organ [27]. However, we expected that the fraction would progressively decrease as we considered older classes. This would fit the 'out of testis' hypothesis, which proposes that new genes would be first expressed in testis, probably due to highly permissive transcription in the germ cells [36], and would later gain expression in other tissues [37]. However, we did not observe any difference between the groups. This suggests that not only new genes are born in testis but that many lineage-specific genes play important roles in this organ.

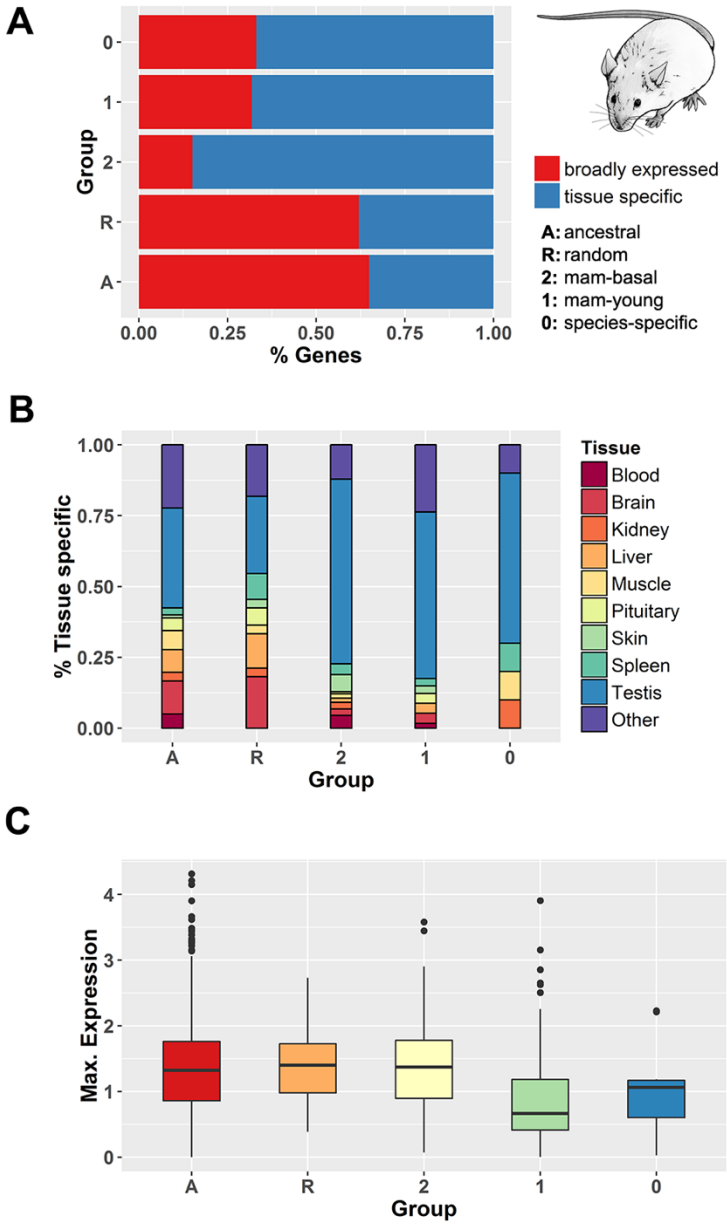

Figure 2. Gene expression patterns of genes from different conservation levels. A. Proportion of broadly-expressed and tissue-specific genes in different conservation classes. **B.** Number of genes with maximum expression in a given tissue for different conservation classes. **C.** Box-plot showing the distribution of FPKM gene expression values, at a logarithmic scale, in different conservation classes. Data in B and C is for tissue-specific genes. All data shown is for mouse genes. See Figure S3 for the same data for human genes.

Mammalian-specific proteins tend to be short

Different studies have found that proteins encoded by genes with a narrow phylogenetic distribution tend to be shorter than average [6,8,12,14,38,39]. We investigated whether this trend was also true in our dataset. We compared mammalian-specific genes from different conservation levels to the 'random' and 'ancestral' gene sets. We confirmed that the non-mammalian-specific proteins were significantly longer than the mammalian-specific ones (Figure 3, Wilcoxon test p-value $< 10^{-5}$). We also found that 'mam-basal' proteins were in general somewhat longer than 'mam-young' genes (Wilcoxon test p-value < 0.01). When we restricted the analysis to human or mouse proteins we obtained similar results (Figure S4).

Proteomics support

We used the PRIDE peptide database to search for matches to the sets of human and mouse mammalian-specific proteins. Despite the short size of these proteins, potentially hindering their detection by mass spectrometry [40], we could find proteomics evidence for a large number of them (Figure 4A, Table S8 and S9 for human and mouse, respectively). In the class 'mam-basal', which predates the separation of human and mouse, the number of genes encoding proteins with at least two unique peptides was 88% in mouse and 50% in human. The marked difference between the two species indicates that proteomics coverage in human is quite incomplete. In mouse, the number of proteins with proteomics evidence in the classes 'mam-young' and 'species-specific' was remarkably high (≥ 75%). Besides, in most cases this was supported by more than two PRIDE peptides (Figure 4B and 4C).

Biases in aromaticity and isoelectric point

In addition to sequence length, some studies have reported differences in the sequence composition of young proteins with respect to older ones [12,39]. Here we inspected the distribution of aromaticity and isoelectric point (IP) values in the different gene sets (Figure 3). We found that aromaticity values were significantly lower in mammalian-specific proteins than in proteins from the classes 'ancestral' and 'random' (Wilcoxon test p-value < 10^{-5}). This effect could be clearly appreciated at the different conservation levels. These findings were confirmed in the human and mouse protein subsets (Figure S5).

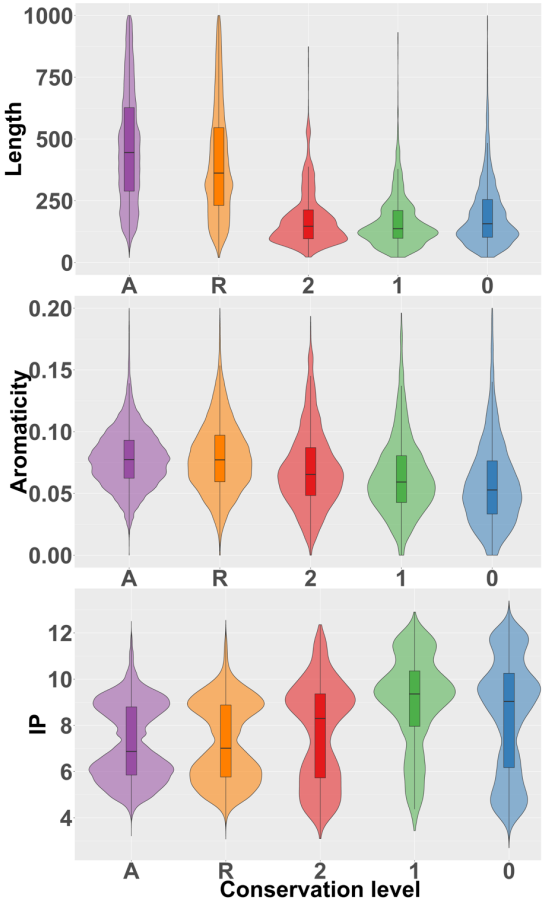

Figure 3. Sequence properties of mammalian-specific genes. Length: Protein sequence length. IP: Isoelectric point. Protein sequences were extracted from the complete gene families set. We used the following gene groups: A: ancestral; R: random; 2: 'mam-basal'; 1: 'mam-young'; 0: species-specific.

IP values were significantly higher in mammalian-specific proteins than in 'ancestral' and 'random' proteins (Wilcoxon test, p-value < 10^{-5}). Additionally, among mammalian-specific genes, the most recent families ('mam-young' and 'species-specific') had significantly higher IP values than the oldest ones ('mam-basal') (Wilcoxon test, p-value < 10^{-5}). This indicated a depletion of negatively charged amino acids in the youngest proteins. This trend was confirmed in the human and mammalian protein subsets (Figure S6).

Functions of mammalian-specific genes

We searched for information on gene function using Gene Ontology (GO) [41]. We focused on human and mouse because these species are relatively well-annotated and at the same time sufficiently distant as to contain completely different families in the class 'mam-young'. We found functional data for 38% of that 'mam-basal' families, a relatively low percentage when compared to that for more widely conserved genes ('random', ~90%)(Tables S10 and S11). But the most striking finding was the low percentage of 'mam-young' families with functional data, which was only of about 6% for both human and mouse containing families. This anecdotal level of annotation was consistent across the different 'mam-young' nodes in the phylogeny. Figure 4A shows the percentage of genes annotated with GO terms for the different mouse conservation groups. There is a striking contrast between the low level of functional annotation and the high level of proteomics support for young mammalian-specific gene families.

We performed a functional enrichment test with the software DAVID [42]. Table 2 shows a summary of the main functional groups for human mammalian-specific genes, very similar results were obtained for mouse (Tables S12 and S13). The 'mam-basal' class was significantly enriched in terms related to 'immune response', 'reproductive process' and 'extracellular region' (Fisher exact test, Benjamini-Hochberg multiple test correction p-value<0.01). The 'mam-young' class was only weakly enriched in the term 'extracellular region' (p-value = 0.04). The lack of results in this group was expected given the very low percentage of genes with known functions. We describe the main overrepresented functional classes below.

1. Immune response

Proteins involved in immune response encoded by mammalian-specific genes included several proteins modulating the activity of B or T cells (cytokines) as well as a number of known antimicrobial peptides (AMPs). The first group included a number of interleukins (IL2, IL3, I13, IL32 and IL33), the tumor necrosis factor superfamily 9 and the IgA-inducing protein (IGIP) (see Table 1). AMPs comprised dermcidin [32], mucin-7 [43] and C10orf99 [44].

AMPs are part of the innate immune system. They are small proteins that contain specific sequence patches, often enriched in basic amino acids, that directly interfere with the bacteria or fungus cell membrane. We used the software AMPA [45] to evaluate the AMP sequence propensity of mammalian-specific proteins. This software produces a score that is inversely related to the AMP potential of the protein, and which identifies protein stretches with putative antimicrobial activity. First, we confirmed that human proteins with known AMP activity had lower AMPA scores than proteins for which such activity has not been described (Table S14, Wilcoxon test p-value $< 10^{-5}$). Second, we compared mammalian-specific proteins to a set of proteins of similar size but conserved in other vertebrates (control set, Table S14). We found that the mammalian-specific proteins had significantly lower scores than the control set (Wilcoxon test p-value = 0.0009). We also discovered that the proteins classified as 'mam-basal' had an excess of proteins with two or more putative AMP sequence stretches (Fisher test p-value=0.016). One example was mucin-7, which contained a previously described stretch with anti-fungal activity [43]. The program predicted a second putative sequence with antimicrobial activity that we validated experimentally (Figure S7). Although it is difficult to predict which specific proteins may be AMPs based on this data alone, the general enrichment in AMP-like features may indicate that there are a number of uncharacterized AMPs among young proteins.

2. *Reproduction*

Genes with the term reproductive process had a varitey of functions related to spermatogenesis or sperm motility. The group included proteins involved

in the replacement of histones by protamines in the germ cells, such as transition protein 2. Other proteins had structural roles, such as the sylicins, which are an integral part of the complex cytoskeleton of sperm heads [46]. Proteins affecting sperm motility included protamine 3 [47] and the mitochondrion-associated cysteine-rich protein (SMCP) [48]. Mice in which the gene encoding SMCP has been disrupted exhibited reduced motility of the spermatozoa and decreased capability of the spermatozoa to penetrate oocytes [48]. Here we dated the SMCP gene to the common ancestor of mouse and rat. In general, many of the mammalian-specific genes showed maximum expression in testis, suggesting that a substantial fraction of them is likely to have reproduction-related functions.

3. *Secreted proteins*

A large group of mammalian-specific gene families were annotated as 'secreted protein' and/or 'extracellular space' (Table 2). This group included many proteins from the immune response system but also proteins secreted in other organs, including the mammary gland (caseins), skin (dermokine) or the lung (secretoglobins). This highlights the importance of secreted or extracellular molecules in recent mammalian adaptations.

Enriched function	**Representative terms**	**N genes**	**Corrected p-value**
1. Immune response	1.1 immune response (GO)	14	2.2E-3
	1.2 cytokine activity (GO)	13	1.6E-10
	1.3 Jak-STAT signaling pathway (KEGG)	6	5.5E-4
2. Reproduction	2.1 reproductive process in a multicellular organism (GO)	12	1.3E-3
	2.2 spermatogenesis (GO)	10	9.2E-4
3. Secreted protein	3.1 extracellular region (GO)	64	1.8E-15
	3.2 secreted (Uniprot)	59	2.8E-14
	3.3 signal peptide (Uniprot)	60	7.0E-10

Table 2. Main functions of mammalian-specific genes. The results shown are for human genes classified as 'mam-basal' (class 2).

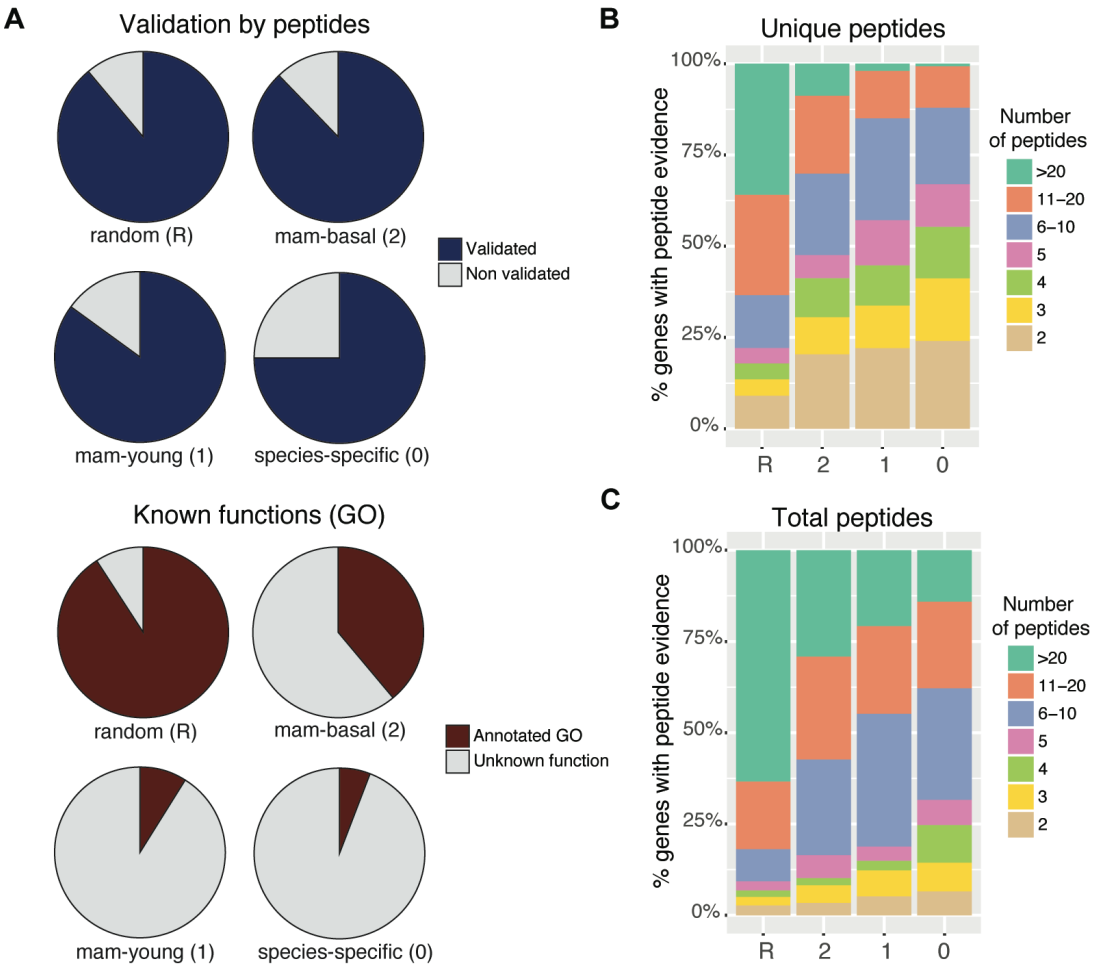

Figure 4. Proteomics and Gene Ontology information. A. Proportion of mouse genes with proteomics or Gene Ontology (GO) data for different gene groups. Validated by proteomics were proteins that had at least two different peptides with a perfect match and these peptides did not map to any other protein allowing for up to two mismatches. **B.** Number of unique peptides for proteomics validated proteins from different groups. **C.** Number of total peptides for proteomics validated proteins from different groups.

Origin of mammalian-specific proteins

Mammalian-specific protein-coding genes may have originated from the recycling of already existing coding sequences or completely *de novo* from previously non-coding genomic regions [8,17]. In the context of new genes for which ancestors cannot be traced back, as described in this paper, the first process often involves complex sequence rearrangements and the cooption of genomic sequences into a coding function. One example is the

caseins, which transport calcium in the milk. Comparative genomics studies have shown that these proteins probably originated from genes encoding teeth proteins during the early evolution of mammals, although they would have undergone a very large number of sequence modifications [28]. Another example is the mammalian-specific Late Cornified Envelope (LCE) group of proteins. The LCEs are part of a gene cluster shared by mammals, birds and reptiles, known as the epidermal differentiation complex (EDC). In this cluster, multiple episodes of sequence duplication and divergence have resulted in a highly diversified set of epidermal proteins in mammals [49].

In other cases the genes may have originated *de novo* from a previously non-coding sequence of the genome [17,21,24,27,29,30,50,51]. The definition of these genes usually includes the requirement that the syntenic genomic regions from closely related species lack a coding sequence of a similar length. This means that *de novo* genes that have originated a long time ago will be very difficult to identify, as genome synteny will be no longer detectable. Our dataset contained 15 human and 13 mouse *de novo* protein-coding genes identified in previous studies (Tables S15 and S16, respectively; Table 1 for selected examples). As expected, they corresponded to recent nodes, containing one or a few closely related species.

DISCUSSION

The sequencing of complete genomes has resulted in a more accurate view of the number of genes in each species and how these genes relate to genes in other species. A puzzling discovery has been that a sizable fraction of genes does not have homologues in other species [9,11,17,39,52,53]. In general, the existence of these "orphan" genes cannot be explained by gene duplication, as gene duplicates retain similarity to their parental copies and thus homologues exist in other species. Rather, their origin is often linked to sequences that were not previously coding but that have been coopted for a coding function [20,21,54,55]. These new genes are likely to drive important species- and lineage-specific adaptations [56,57] but we still know very little about their functions.

In order to advance our knowledge in this field we have performed the first integrated analysis of mammalian-specific gene families. We have performed extensive sequence similarity comparisons using proteomes and RNA-Seq-derived data, constructed thousands of families, and assigned each of them to one of the 29 internal nodes or 30 terminal branches of the tree. This is different from the classical "phylostratigraphy" approach, which is based on the homologues found for a single species [6,58]. The integration of data from multiple species is expected to result in a more robust classification of the node of origin of each gene. Besides, the gene families that are specific to each group of organisms can be directly retrieved and studied.

In order to be as conservative as possible we considered both protein-based and transcript-based homologies. We distinguished between three groups of mammalian-specific genes: those present in many different groups of mammals ('mam-basal'), those present in specific groups ('mam-young') and those found in a single species. The group 'mam-basal' was composed of 439 gene families, in general represented by a large number of species (>15 species in 78% of cases). Although functional annotations were quite incomplete even for these genes, they provided a very clear picture of the key role of new genes in mediating mammalian adaptations.

In mammals, the skin needs to be flexible and thin enough to allow muscles to produce an elastic deformation. The outermost layer of the skin, known as *stratum corneum*, is particularly flexible in this group when compared to the stiff reptile skin [59]. Several mammalian-specific genes identified here were involved in the formation of skin structures, such as proteins from the Late Cornified Envelope (LCE) family, Dermokine, Keratinocyte Differentiation-Associated Protein, and Corneodesmosin (CDSN). For example CDSN participates in the specialized junctions known as corneodesmosomes, which bridge together corneocytes in the lower part of the *stratum corneum* [60]. Additionally, we found a gene associated with psoriasis (PSOR-1), localized in the same genomic region than CDSN. Another important adaptation in mammals is the production of milk. In most mammals, the most abundant milk proteins are caseins, which form micelles in which they transport calcium. Our method identified several caseins (alpha S1, beta, kappa), these are part

of a larger family of secretory calcium-binding phosphoproteins (SCPP), which has diverged extensively in a common ancestor of current day mammals [28].

Mammalian-specific genes are often expressed in testis and are significantly enriched in testis-related functional terms such as 'spermatogenesis' and 'sperm motility'. The over-representation of new genes in this organ has been noted before, both in the case of duplicated genes [61] and *de novo* evolved genes [20,27]. For genes conserved across many mammalian groups ('mam-basal'), this enrichment probably reflects the importance of sexual selection in driving changes in the reproductive organs, both at the anatomical and molecular levels [62,63]. As a result of sexual selection, new genes that increase sperm competitiveness will rapidly reach fixation in the population, and subsequently be preserved by purifying selection.

It is well-documented that germ cells express a large variety of transcripts, including conserved coding genes, *de novo* genes and transcripts derived from intergenic regions [27,36]. This permissive chromatin environment is likely to favor the birth of new genes [64,65]. This is generally consistent with our observations that many young mammalian genes show maximum expression in this organ. However, as many of the youngest genes may disappear over time [14], one may expect that the testis enrichment will tend to decrease for older gene sets. In contrast, we found a similar enrichment for genes present in many mammalian species ('mam-basal') and for younger genes ('mam-young' and 'species-specific'). Intriguingly, the 'mam-basal' genes were expressed at much higher levels than younger genes. This probably reflects the presence of many 'mam-basal' genes with important sperm cell functions. It also supports the idea that the expression level of recently evolved genes may tend to increase over time.

Another large group of mammalian-specific genes encoded proteins involved in the immune response. One example was the IgA-inducing protein (IGIP), a short secreted protein of only 52 residues. This protein is produced in the dendritic cells and stimulates the production of immunoglobulin A in B cells [66]. Although its sequence is highly conserved across mammals, no homologues have been found in other vertebrates. Our set of mammalian-

specific genes also included several previously described antimicrobial peptides (AMPs). The antimicrobial active regions in AMPs, even if evolved independently, are often enriched in certain residues, including arginines and cysteines [67]. This can be used to predict the propensity of a protein to be an AMP [45]. We observed higher AMP propensities in mammalian-specific genes than in non mammalian-specific genes, suggesting that there may be additional mammalian-specific AMPs that have not yet been characterized.

The mechanism of origin of the majority of 'mam-basal' gene families is unknown. There are however a number of cases in which the genes are part of multi-gene families and have probably originated, at least partially, from the recycling of duplicated coding sequences [28,49,68]. In general, we expect the divergence of a new gene to be very fast at first, due to joint effects of relaxation of selection and positive selection, and become slower at latter stages, when the gene has fully acquired the new function. At this point any similarity to the ancient parental gene may have been erased [17,69]. Aside from the 'mam-basal' genes, the classification of genes in different nodes was based on the lack of homologues in diverse mammalian groups, using not only gene annotations but also RNA-Seq data. Due to the high sensitivity of BLASTP in closely related species such as mammals [70], and the large number of genomes employed, many of these genes may have originated completely *de novo* during mammalian evolution. Indeed, our set of 'mam-young' genes contained several *de novo* genes previously reported in the literature. The vast majority of these genes had no known functions, but they were in general supported by proteomics data. There were nearly a hundred of human- or primate-specific genes with clear proteomics support. The lack of a mosue model may explain why these genes are understudied, despite their importance for understanding lineage-specific adaptations. Young mammalian genes were preferentially expressed in testis. This may simply reflect permissive transcription in the germ cells, the relevant site of expression may have remained unsampled. However, it also seems possible that some of the youngest genes are involved in sperm-related processes, as this is something we can observe for older, and better characterized, mammalian-specific genes.

Species-specific genes were surprisingly abundant in comparison to the other classes. The number was variable depending on the species, which is expected given that the genomes have been annotated by different Consortia. For example, whereas for most primate genomes the annotations are primarily based on human, in other species, such as the lynx, extensive RNA-Seq data has also been employed. Additionally, the genetics distances between species differ considerably. For example the genetic distance between mouse and rat, is of about 0.2 substitutions/site, which is comparable to the genetic distance separating the most evolutionary distant primate species. It is thus not surprising that mouse had a very large number of species-specific genes, of which we validated 291 by proteomics. An excess of species-specific genes in phylostratigraphy-based studies has been previously observed [8]. These observations are consistent with a high rate of *de novo* gene emergence accompanied by frequent gene loss in the first stages of the evolutionary history of a gene [14,71].

An important question is whether genes from different conservation levels (or gene ages) form a continuum, meaning that the observations are not independent but reflect different phases of the same process [6,39]. Our results show that this is likely to be the case, as there are many similarities between the genes of the different mammalian-specific classes. In the first place, independently of the specific age of the gene, the encoded proteins tend to be much shorter than average. It is easy to understand why new coding sequences, specially those originated *de novo*, will tend to be shorter than proteins that have evolved for a long time. In the first case the open reading frame (ORF) is derived from a previously non-coding sequence, and it will be difficult to obtain long ORFs by chance. In contrast, proteins which have evolved for a long time usually have several domains, which may have been gained at different evolutionary periods [72–74].

Mammalian-specific proteins are depleted of aromatic and negatively charged residues. The trend is more extreme in the youngest proteins, supporting the idea of a continuum. One possibility is that these residues are more often associated with deleterious effects, at least in the context of short proteins with a large solvent contact surface. Interestingly, young proteins in plants

also tend to have higher isoelectric point values than older proteins [12]. To our knowledge there has been no studies addressing the relationship between aromaticity and gene age *per se*.

Contrary to early predictions [75], the sequencing of new genomes has not solved the mystery of orphan genes –genes with no homologues in other species- but now we have a larger number of them. Besides, the cell expresses many transcripts with low coding potential, or long non-coding RNAs, which are species- or lineage-specific, and which can be potentially translated [55]. Whereas there is ample evidence for continuous new gene emergence, it is unclear which functions these genes contribute to. On the basis of over 400 mammalian-specific genes that are relatively well-conserved and have functional annotations we have found that many new genes are secreted and may have been advantageous in the response against pathogens or mating. The collection of gene families obtained here should accelerate future studies on the evolutionary dynamics and functions of novel genes.

METHODS

Sequence sources

We initially identified 68 mammalian species that had fully sequenced genomes and which showed a relatively sparse distribution in the mammalian tree. The proteomes and cDNA sequences were downloaded from Ensembl v.75 [76], the National Center for Biotechnology Institute (NCBI Genbank March 2014)[77] and the *Lynx pardinus* Genome Sequencing Consortium web server (http://denovo.cnag.cat/genomes/iberian_lynx). The tree topology, and the approximate number of substitutions per site in each branch, were obtained from previous studies [53,78,79]. We obtained RNA sequencing (RNA-Seq) data for 30 different species using the public resource Gene Expression Omnibus (GEO) [80]. The RNA-Seq samples were from different body tissues depending on the species. The total number of RNA-Seq samples was 434, with a median of 10 samples per species.

Identification of mammalian-specific genes

We run BLASTP sequence similarity searches for each of the mammalian proteomes against a set of 34 non-mammalian proteomes (Table S1). All BLASTP searches were run with version 2.2.28+ using an e-value threshold of 10^{-4} and the filter for low complexity regions (LCRs) activated (seg = 'yes') [81]. Consequently, we did not consider proteins that were extensively covered by LCRs (with less than 20 contiguous amino acids devoid of LCRs as measured by SEG with default parameters [82]). We also discarded genes encoding a protein that had significant sequence similarity to non-mammalian species, as well as genes that had paralogues with homologues in non-mammalian species, as previously described [17].

We generated two control sets of non mammalian-specific genes. The first set, which we named 'ancestral' (A) contained proteins that had homologues in all the 34 non-mammalian species mentioned above. This set of genes is well-conserved across eukaryotes, having originated in a common ancestor. The second set, named 'random' (R), contained 1,000 proteins (for each species) that were not in the mammalian-specific group.

Building gene families based on gene annotations

We built a mammalian tree for the species with complete genomes using previous published data [78,79]. The next step was to develop a pipeline to construct gene families and to assign them to a node in the tree. We wanted the gene families to be as large as possible and include both orthologues and paralogues. The node represented the point from which no further ancestors could be traced back.

We first ran BLASTP searches for every set of mammalian-specific genes in each species against the mammalian-specific genes in the other species (e-value$<10^{-4}$). This resulted in a node of origin for each individual gene and a list of homologous proteins in the same and other species. Next we collated the information for all the homologous proteins, progressively expanding the gene families until no more members could be added. The group then was

assigned to the oldest possible node considering the species present in the gene family.

Transcript reconstructions from RNAseq

The RNA-Seq sequencing reads of each sample underwent quality filtering using ConDeTri (v.2.2) [83] with the following settings (-hq=30 -lq=10). Adaptors were trimmed from filtered reads if at least 5 nucleotides of the adaptor sequence matched the end of each read. In all paired-end experiments, reads below 50 nucleotides or with a single pair were not considered. We aligned the reads to the corresponding reference species genome using Tophat (v. 2.0.8) [84] with parameters –N 3, -a 5 and –m 1, and including the corresponding parameters for paired-end and stranded reads if necessary. We performed gene and transcript assembly with Cufflinks (v 2.2.0) [85] for each individual tissue. We only considered assembled transcripts that met the following requirements: a) the transcript was covered by at least 4 reads. b) transcript abundance was >1% of the abundance of the most highly expressed gene isoform. c) <20% of reads were mapped to multiple locations in the genome. d) The reconstructed transcripts were at least 300 nucleotides long. Subsequently, we used Cuffmerge to build a single set of assembled transcripts for each species. We use Cuffcompare to compare the coordinates of our set of assembled transcripts to gene annotation files from Ensembl (gtf format, v.75) or NCBI (gff format, December 2014), to identify annotated transcripts and, to generate a set of novel, non-annotated, transcripts.

Refinement of the age of the families using transcriptomics data

We performed TBLASTN (e-value < 10^{-4}) searches of the proteins in each gene family against the set of novel transcripts assembled from RNA-Seq samples. If we found any homologous sequence in a species that was more distant to the members of the family than the originally defined node we reassigned the family accordingly, to an earlier node. This was based on conservative criteria, the presence of an homologous expressed sequence, even if not annotated as a gene, was considered as evidence that the gene

had originated at an earlier node connecting all homologous transcripts.

In order to minimize the biases due to the different availability of RNA-Seq data in different parts of the tree we decided to focus only on the 30 species with RNA-Seq data for all analyses. Besides, we only considered families with sequence representatives in at least half of the species derived from the node to which the family had been assigned. The use of transcriptomics data filled many gaps in the tree and resulted in a deepening of the node of origin in some cases. The use of RNA-Seq data allowed us to expand the range of species for 1,126 of the gene families initially defined as species-specific (~22%) and to increase the number of species per family in 617 multi-species families (68%). The procedure resulted in 2,013 multi-species gene families and 3,972 species-specific gene families (mostly singletons).

Gene expression data

We retrieved gene expression data from GTEx [33] and mouse ENCODE [86] tissue expression panels. We only analyzed genes which were expressed in at least one tissue sample. We computed the FPKM (Fragments per Kilobase Per Million mapped reads) median expression for each gene and tissue available in GTEX (release 2014, Ensembl v.74). For Mouse ENCODE, which was based on Ensembl v.65 (mm9), we computed the FPKM mean for each gene and for tissues with 2 replicas we only used the data if reproducibility indexes were lower than 0.1. We identified the tissue with the highest expression value and calculated the tissue preferential expression index as previously described [27,35]. Genes for which this index was 0.85 or larger were classified as tissue-specific.

Sequence features

The analysis of the sequences from different sets was performed using Python scripts. We employed Biopython embedded functions to calculate the isoelectric point (IP) and aromaticity. The proteomics analysis was based on peptides extracted from the PRIDE database for mouse and human [87]. Proteins with positive proteomics evidence were defined as those matching

at least two different peptides. Besides, the peptides had to match the sequence perfectly and could not match any other protein in the same species, allowing this time up to two mismatches.

Functional analysis

We downloaded Gene Ontology (GO) terms from Ensembl v.75 for all human and mouse genes (Tables S11 and S12, respectively). In order to estimate the number of gene with known functions in each conservation class we counted how many genes were associated with at least one GO term. We did not consider the terms 'biological process', 'nucleus', 'cellular_component', 'molecular_function', 'cytoplasm', 'plasma membrane', 'extracellular space', 'protein binding', 'extracellular region', or 'integral to membrane' as they were very general and did not imply knowledge of the function of the protein.

We used DAVID [42] to assess enrichment of particular functions or subcellular locations in mammalian-specific genes from human and mouse (Tables S13 and S14, respectively).

Prediction of antimicrobial activity

We wanted to test if mammalian-specific genes were enriched in AMP-like features. This was motivated by the enrichment of immune response proteins among mammalian-specific genes (Table 2), including three known AMPs (dermcidin, mucin 7, C10orf99 protein) and the skew towards high isoelectric point values observed in these proteins (Figure 3). We measured the antimicrobial activity potential of all human proteins using the program AMPA [88]. In the majority of proteins from a set of 59 genes encoding known AMPs, which we gathered from the literature, AMPA was able to predict stretches with AMP activity (Table S15, AMP status 'known'). We then used this program to calculate scores and number of putative AMP stretches in mammalian-specific proteins (Table S15, type 'mammalian-specific') as well as in a large set of size-matched non mammalian-specific proteins (Table S15, type 'conserved').

The peptides for testing the antimicrobial activity were prepared by Fmoc solid phase synthesis methods, purified by HPLC and characterized by mass spectrometry, as previously described [89]. We assayed the activity of mucin-7 peptideson reference strains of *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), *E. faecalis* (ATCC 29212), and *S. aureus* (ATCC 29213) at the Microbiology Service of Hospital Clínic (Universitat de Barcelona). Minimal inhibitory concentration (MIC) assays were performed by the microdilution method in Mueller–Hinton broth according to Clinical and Laboratory Standards Institute (CLSI) guidelines. As a positive control the Cecropin A-Melittin peptide CA(1-8)M(1-18) was employed [90]. This peptide exhibited CMI values ranging from 0.5 to 64 in the four bacteria strains tested.

Statistical Data Analyses

We used Python 2.7 to parse the data from different programs and files, cluster the genes into gene families, and calculate sequence-based statistics. The ete2 package was used to perform analyses using the phylogenetic tree structure [91]. We generated plots and performed statistical tests with R [92].

DATA AVAILABILITY

Supplementary tables and figures can be found in Supplementary file 1 and 2, respectively. The list of mammalian-specific gene families with annotations on the node of origin, protein identifier and number of species is available at http://dx.doi.org/10.6084/m9.figshare.4239404. Protein sequences for the gene families classified at different nodes are available from http://dx.doi.org/10.6084/m9.figshare.4239440. Information on the RNA-Seq datasets employed for transcript assembly, including species and tissue data, is available from http://dx.doi.org/110.6084/m9.figshare.4239431. Transcripts assemblies generated from the RNA-Seq data are available from http://dx.doi.org/10.6084/m9.figshare.4239449.

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