Concerted loss of microRNAs uncovers multi-level restructuring of gene regulation in rodents

Felix Langschied[1]

Matthias S. Leisegang[2,3]

Stefan Günther[4]

Fabian Hahner[2,3]

Ralf P. Brandes[2,3]

Ingo Ebersberger[1,5,6]

[1]Goethe University, Applied Bioinformatics Group, Institute of Cell Biology and Neuroscience, Frankfurt, Germany.

[2]Goethe University, Institute for Cardiovascular Physiology, Frankfurt, Germany

[3]German Center of Cardiovascular Research (DZHK), Partner site RheinMain, Frankfurt, Germany

[4]Max-Planck-Institute for Heart and Lung Research, Bad Nauheim, Germany

[5]Senckenberg Biodiversity and Climate Research Centre (S-BIK-F), Frankfurt am Main, Germany.

[6]LOEWE Centre for Translational Biodiversity Genomics (TBG), Frankfurt am Main, Germany.

Correspondence to: langschied@bio.uni-frankfurt.de; ebersberger@bio.uni-frankfurt.de

The authors have declared that no conflict of interest exists.

Running Head: miRNA loss in rodents

Keywords: miRNA, phylogenetic profiling, iPSCs, gene regulation, gene loss

Abstract

MicroRNAs (miRNAs) are post-transcriptional regulators involved in key biological processes. Lineage-specific losses of multiple miRNA families during evolution are rare. If they occur, they often coincide with significant changes in gene regulation with consequences extending to body plan modifications. Recently, 15 mammalian miRNA families were found missing in the *Eumuroidea*, including mouse and rat. It remains unknown to what extent this has affected the gene regulatory network in this lineage. Here, we shed light on the implications of these losses. We integrate *in silico* characterizations of these miRNAs with differential gene expression analyses in human and mouse inducible pluripotent stem cells (iPSCs) overexpressing two representatives, Mir-197 and Mir-769. This revealed that partly the same genes are downregulated in response to the miRNA overexpression in mouse and human. We dated the emergence of the miRNA target sites in the corresponding transcripts to before the mammalian divergence. Therefore, the response to miRNA overexpression in mouse iPSCs likely represents remnants of an ancient gene regulatory network that existed already in the last common ancestor of human and mouse. This provides strong evidence that the miRNA loss disrupted established regulatory connections in *Eumuroidea*. Notably, we detected an accompanying loss of at least 37 transcription factors in in this lineage. Thus, *Eumuroidea* have modified their gene regulatory networks on two levels, transcriptionally and post-transcriptionally. It will now be interesting to precisely chart the differences in gene regulation and how this affects the use of mouse and rat as model systems for human disease.

Author summary

The phenotypic specifics of a species are jointly determined by its sets of structural and regulatory genes, where the latter control gene expression in a spatial and temporal manner. Micro-RNAs (miRNAs) are short regulatory genes and alter gene expression post-transcriptionally. Over evolutionary timescales, they are rarely lost in larger numbers on individual lineages, highlighting their functional relevance. The model species mouse and rat, together with their relatives in the Eumuroidea lack 15 miRNA families that are otherwise largely conserved throughout the mammals. If, and to what extent, the corresponding gene loss has affected gene regulation in the last common ancestor of Eumuroidea is unknown. Here, we provide evidence that the corresponding loss of miRNA genes is not compensated by other genes. We trace remnants of the ancestral regulatory network under control of two miRNAs in mouse, providing first time evidence that the miRNA loss in the Eumuroidea must have had consequences for gene expression regulation. We further show that protein-coding genes that have been co-lost with the 15 miRNAs are enriched for transcription factors. Jointly, this reveals that the diversification of Eumuroidea during mammalian evolution was accompanied by a change in gene expression regulation on the transcriptional and post-transcriptional level.

Introduction

Micro-RNAs (miRNAs) are post-transcriptional regulators, that each downregulate multiple mRNAs (1). The targeting process is mainly guided by the pairing of a 6-8 nt long seed sequence in the miRNA with a matching target site in the mRNA (2). Due to the shortness of the seed sequences, corresponding target sites are likely to occur in many mRNAs just by chance. It is therefore proposed that young miRNAs initially target a random set of genes (3). If the resulting knockdown of any of these initial targets is deleterious, two potential scenarios may occur. Either the new miRNA is removed from the population (4), or the deleterious target sites degrade, so that the novel miRNA gene can persist (5). Once a miRNA has become fixed in a population, its target specificity increases over time (6). Consequently, purifying selection likely influences the rates at which co-expressed mRNAs gain new target sites or lose existing ones, as either event can alter mRNA expression (7). Additionally, there is a trend that miRNAs acquire more targets over time (8), as they become expressed in an increasing number of tissues (4). This correlates well with the effect size of miRNA knockouts. Younger miRNAs that can be traced back only to the last common ancestor (LCA) of placental mammals show mostly modest knockout effects like growth defects (9) or anxiety-related behaviour (10). This is likely because they had less time to extend existing regulatory networks (8). In contrast, miRNAs which emerged prior to the diversification of the vertebrates assume key roles in cellular regulatory networks and their knock-out results in abnormal, and oftentimes lethal phenotypes (11). However, it remains largely unknown if these regulatory networks are shaped by cooperative effects of multiple younger miRNAs.

In the context of proteins, cooperative effects can be identified if the corresponding genes share a similar presence absence pattern across taxon collections (12). These patterns, also known as phylogenetic profiles, help in identifying genes that jointly establish, e.g., a metabolic pathway (13), or a protein complex (14). In such cases, the loss of one gene can abolish the function of the entire pathway/complex if it is not compensated for. This makes it likely that the remaining proteins become dispensable, and their subsequent loss leaves a characteristic footprint in their phylogenetic profiles. In turn, the analysis of even vague annotations of functionally integrated genes can provide a mosaic-like view of the overarching function they convey (15). However, it remains unknown whether concerted loss is also a signal of functional integration for miRNAs, or even how an overlap in miRNA function might manifest itself.

Over evolutionary time scales, miRNA families that are shared across multiple and taxonomically diverse species are generally resistant against gene loss (16,17). There are, however, a few exceptions, most notably in lineages that have significantly simplified their body plans, such as

flatworms and sea squirts (18,19). With respect to mammals, an analysis comprising 8 different species noted a loss of several miRNAs in mouse and rat that is not explained by body plan simplification (19). However, the limited taxonomic resolution of that study makes it difficult to determine when during the rodent diversification these losses occurred. Consequently, it is hard to connect the miRNA loss to likely phenotypic changes, obscuring functional consequences of the corresponding changes in the gene regulatory network.

To shed further light on the evolutionary dynamics of how miRNA loss impacts the regulatory network, the present study was conducted focusing on 15 mammalian miRNA families that we previously reported to be lost during the diversification of the rodents (17). We hypothesized that these losses flag a restructuring of the eumuroidean gene regulatory network. To investigate this, we first characterized the evolution and likely function of the absent miRNA families in silico. An overexpression of two miRNAs in human and murine inducible pluripotent stem cells (iPSCs) provided insights into primordial regulatory connections in the LCA of human and mouse that were likely affected by the miRNA loss. Scanning the entire protein-coding gene set of humans revealed an enrichment of transcription factors among the genes that have been co-lost with the miRNA families in the *Eumuroidea*.

Results

Characterization of miRNA loss and test for functional compensation

miRNAs are rarely lost in vertebrates. There are a few lineage-specific exceptions to this, with the absence of 15 miRNA families in rodents being among the most prominent one (17). To shed light on the evolutionary events that caused the shared absence of these families, we performed an in-depth characterization of their phylogenetic profiles. This indicated that the respective miRNA genes were lost at different points during the diversification of the *Glires* (*Rodentia* and *Lagomorpha*) (Figure 1A). Interestingly, some of these miRNA families were also independently lost in the insectivores (*Eulipotyphla*) and inside the bats (*Chiroptera*; Figure 1A). To determine whether a few or even a single large-scale event could explain the recurrent losses of these miRNA families, we analysed their position in the human genome. This revealed that the corresponding genes are not locally organised but instead are spread across multiple loci (Supplementary Table S 1). This strongly suggests that independent loss events account for the absence of these 15 miRNA families.

To ensure that the observed losses were not inferred due to sensitivity limits of the ortholog search or assembly artefacts, we compared the corresponding loci in the 100-vertebrate whole genome alignment in the UCSC Genome Browser (20). This revealed mutational changes ranging from

single nucleotide substitutions to small scale insertion/deletion events in the miRNA loci (Figure 1B, Supplementary Figure S2). To investigate whether the absence of the miRNA families is compensated by either redundant or newly emerged miRNAs, we analysed the lost seed sequences in more detail, as seed-pairing is the primary determinant of miRNA targeting specificity (2). We first confirmed that the seed sequences of the co-absent miRNA families are not shared by any of the rodent-specific miRNAs annotated in MirGeneDB (21). To increase the sensitivity of the analysis, we subsequently used each of the 15 mature miRNA sequences as a query for a BLASTn-short search in the collection of mature mouse and rat miRNAs in MirGeneDB. This obtained no significant hit (E-value < 0.01) indicating that there are no rodent-specific miRNAs that compensate for the observed loss using the same target sites.

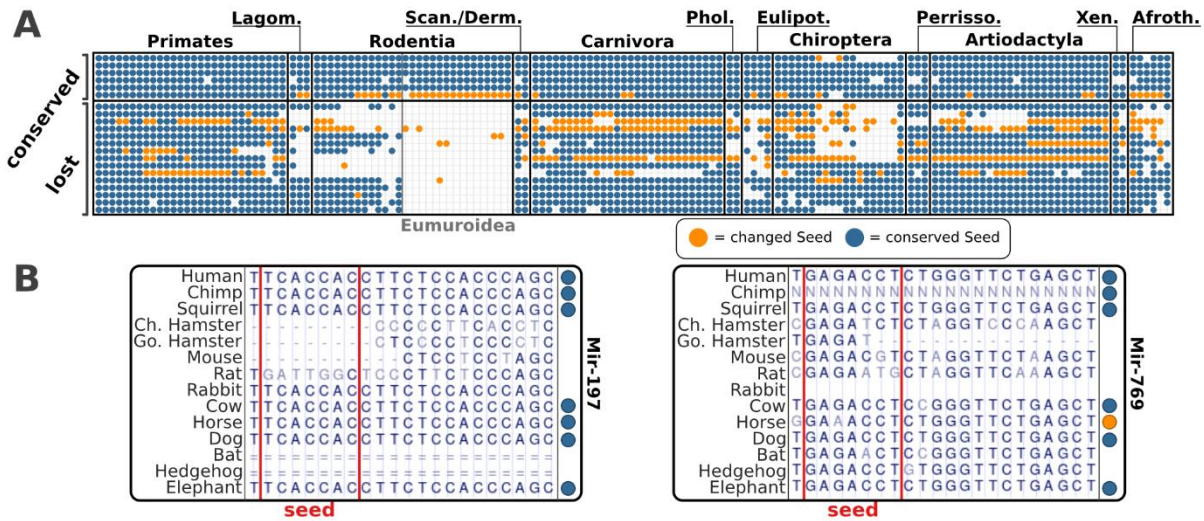

Figure 1: Characterization of miRNA families co-absent in the *Eumuroidea*. A) Phylogenetic profiles of 6 miRNA families that are conserved on the vertebrate level (top) and of 15 miRNA families lost in the Rodentia (bottom) (separated by a black line). Species are ordered according to increasing taxonomic distance to the reference species of the ortholog search (human) from left to right. A dot indicates that at least one ortholog of any pre-miRNA of the miRNA family was identified in the respective species. Dot color shows whether the human seed sequence was found in the ortholog (blue) or not (orange). Lagom. = Lagomorpha, Scan. = Scandentia, Derm. = Dermoptera, Phol. = Pholidota, Eulipot. = Eulipothyphla, Perrisso. = Perrissodactyla, Xen. = Xenarthra, Afroth. = Afrotheria. B) 14-species alignment for the genomic loci harboring miRNA families 197 and 769 in humans extracted from the 100-vertebrate whole genome alignments. The alignments confirm the loss of the miRNA in *Eumuroidea*.

Target genes of co-lost miRNAs are involved in the regulation of developmental processes

Given the collective absence of the 15 miRNA families in *Eumuroidea*, it is tempting to speculate about the functional integration of the respective miRNA genes. As a starting point, we tested functional integration via co-expression of the miRNA genes or co-regulation of mRNA targets. Comparing the expression levels of the corresponding miRNA genes across 45 human tissues (21) revealed that all 15 miRNA families are most highly expressed in testis and kidney, although expression was not exclusive

to these tissues (Supplementary Figure S3). Thus, correlation of gene expression across tissues provides no complementary evidence for the functional integration of these genes (Supplementary Figure S4). Likewise, we found no significant overlap in the set of target genes recorded in the miRTarBase database (22) (Supplementary Table S 2; Supplementary Figure S5). However, the combined set of target genes across all 15 miRNA families was significantly enriched with Gene Ontology (GO) terms associated with the regulation of cellular and developmental processes (Supplementary Figure S 6). This may provide an initial indication that there is a functional overlap of genes targeted by the 15 co-lost miRNAs, although the GO terms remain certainly very broad and are therefore of limited discriminative power.

miRNA overexpression affects similar numbers of genes irrespective of whether the host organism contains a native copy

All 15 miRNA families are at least as old as the placental mammals (see Fig. 1). Thus, it is conceivable that their regulatory connections were already established when the mouse and human lineages split, about 70 MY ago (23). Expressing human miRNA orthologs in mouse should allow investigating if and to what extent this regulatory network has evolved after the loss, and whether its remnants can still be traced in rodents. We selected Mir-197 and Mir-769 as candidates for the experiments as these were single-copy miRNA families with only moderate expression levels under native conditions in human (Supplementary Figure S7). An overexpression of the corresponding miRNAs should therefore impose a noticeable effect on the target genes' expression even in humans that contain a native copy. To test this, we performed differential gene expression analyses after overexpression of the two human miRNAs in both human and mouse iPSCs. iPSCs were used as their naïve state should have greater similarity between species than more differentiated cell types, even though truly comparable cell lines of organ origin do not exist between human and mice (24). In response to overexpression, the abundance of mature miRNAs Mir-197-3p and Mir-769-5p strongly increased in both human and murine iPSCs (Figure 2A, B). To reveal the effect of this overexpression on a transcriptome-wide level, RNA-Seq was performed in both human and murine iPSCs. The replicates from the four treatment conditions and the two controls form clusters in a principal component analysis of the normalized expression values, indicating a condition-specific impact of miRNA overexpression in both organisms (Figure 2C).

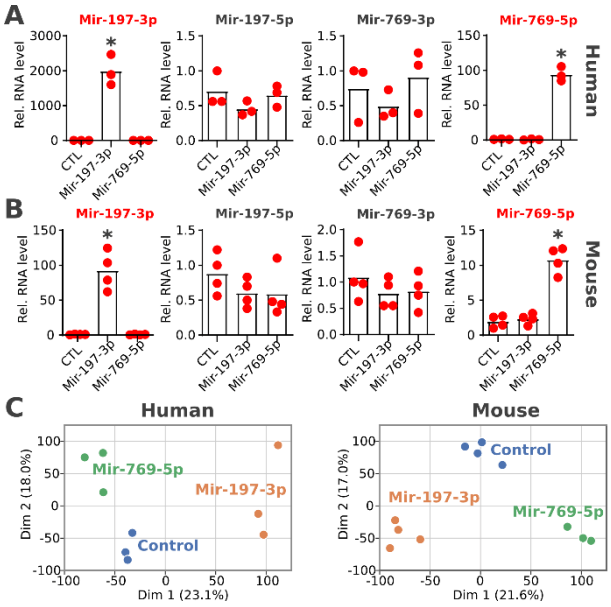

Figure 2: Effect of overexpression of human pre-miRNAs in human and murine iPSCs on gene expression. RNA levels relative to U6 snRNA (CTL) are shown for both miRNAs in human (A, n=3) and murine iPSCs (B, n=4). The name of the mature miRNA is highlighted in red; the name of the star miRNA is shown in black. Significantly altered expression levels are indicated with an asterisk (unpaired t-test, p-value < 0.01). C) Principal component analysis of normalized expression values after RNA-seq.

In human iPSCs, 194 genes (Mir-197) and 103 genes (Mir-769) showed significant changes in their expression levels in response to miRNA overexpression (Table 1, Supplementary Table S 3). In mice, the heterologous expression of the human miRNAs resulted in a significant change of expression levels for 216 (Mir-197) and 228 (Mir-769) genes. This confirms the expectation that the overexpression of the two miRNAs has a substantial effect on gene expression in human iPSCs. More importantly, this shows that the mouse transcriptome responds to the presence of the human miRNAs in the same order of magnitude.

Table 1: Differentially expressed genes after miRNA overexpression. Protein-coding genes were considered differentially expressed if their mRNAs have an absolute log2fold-change > 0.5, at least 100 reads in the negative control and an adjusted p-value <= 0.05 (FDR 5%)

Organism	miRNA	Up	Down
Human	Mir-197-3p	85	109
	Mir-769-5p	25	78
Mouse	Mir-197-3p	101	115
	Mir-769-5p	76	152

As expected, most genes that responded to miRNA overexpression were downregulated in all treatment conditions. However, a considerable number of genes increased their expression levels, which cannot be explained as a direct effect of the miRNA overexpression. Instead, upregulated genes

indicate the presence of secondary effects on the regulatory network. Indeed, we found 6-8 down-regulated transcription factors (TFs) in each treatment condition, which could mediate this effect (

Supplementary Table S 4; Lambert et al. 2018). It is conceivable that the miRNA-driven downregulation of these TFs propagates regulatory effects to further genes that are not directly targeted by the miRNATo reduce noise introduced by such secondary effects, we next identified the most likely primary targets of the two miRNAs in human and murine iPSCs.

Target prediction identifies physiologically relevant targets of lost miRNAs in both human and mouse

To identify primary targets of Mir-197 and Mir-769 in human and murine iPSCs, *in silico* target prediction was performed using the context++-score method implemented in TargetScan 7 (2). This predicted 1079 genes as potential targets of Mir-197 in human, and 792 targets in mouse. For Mir-769, TargetScan 7 predicted 935 and 839 targets for human and mouse, respectively. Notably, the genes we found up-regulated in the presence of the miRNAs contain no more targets than expected chance, which is in line with the secondary effect hypothesis (Figure 3B). In contrast, down-regulated genes were significantly enriched with predicted targets, which aligns with the expectation given the mode of action of miRNAs. The four sets of genes that are down-regulated as well as predicted as targets are thus considered primary targets of Mir-197 and Mir-769 in human or murine iPSCs, respectively.

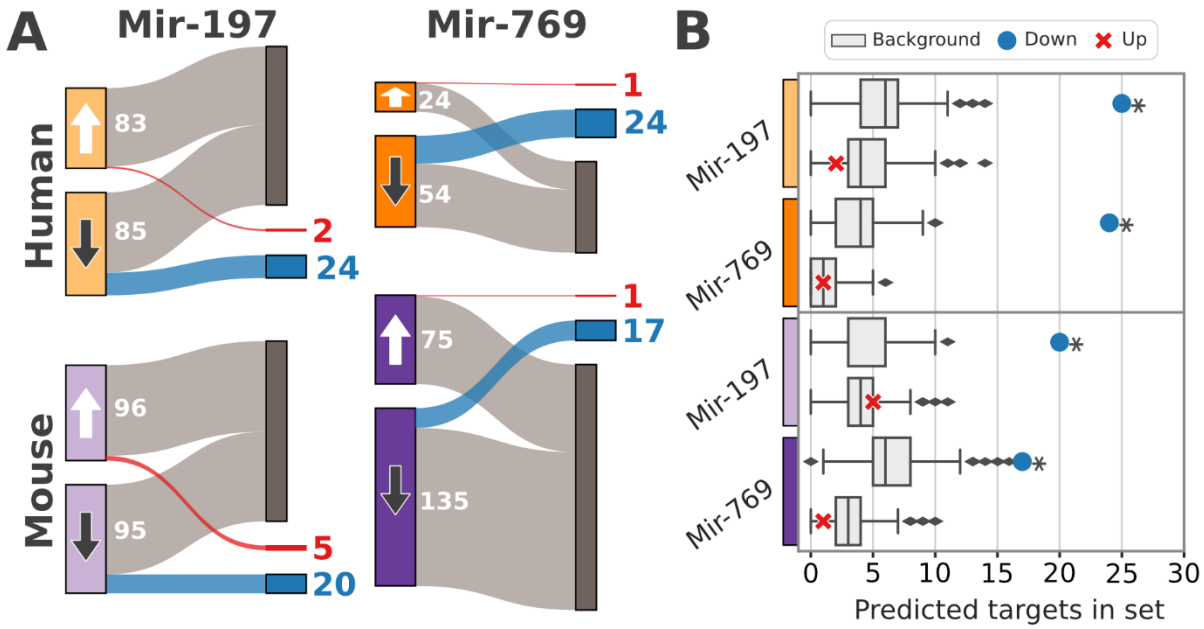

Figure 3: Overlap between predicted miRNA targets and differentially expressed genes. A) Sets of significantly up- and down-regulated genes are marked with white and dark arrows, respectively. Overlaps with predicted targets are marked in red for up-regulated genes and blue for down-regulated genes. B) Background distribution for the number of predicted targets was created by sampling 1,000 gene subsets matching the number of up- or down-regulated transcripts for each miRNA and species. Then, genes whose mRNAs are predicted as targets were counted and compared to the observed number. Observations that deviate significantly from the background distribution are marked with an asterisk (two-sided Z-score test; p-value <= 0.01).

Overlap of primary miRNA targets in human and murine cells indicate remains of ancient regulatory connections

Our analysis has identified four sets of 17 to 24 primary targets in human or murine iPSCs for each miRNA. Among these, none of the primary targets listed in miRTarBase for the two miRNAs were found, since they are not expressed in human iPSCs (Supplementary Table S 3, Supplementary Table S 5). No gene was shared between the sets for Mir-197 and Mir-769, which is in line with our previous analysis based on targets provided by miRTarBase. Consulting GO-annotations as well as available information about protein interactions (26) provided no statistically significant functional overlap between targets of the two miRNAs. We next pursued the orthogonal approach and compared the set of primary targets for the same miRNA between human and mouse. There are four genes, namely ARMC1, ATP6V1A, CCDC85C and TTPAL that are primary targets of Mir-197 in both human and murine iPSCs (Figure 4A). For Mir-769, no shared target genes were detected with the selected filter criteria, however several genes just fell below the selection threshold (Supplementary Table S 5). For example, when we only slightly reduce the minimum log2fold-change to 0.25, the overlap increases from 4 to 11 genes (Supplementary Table S 6). Thus, four overlapping genes seems a very conservative measure.

An overlap of primary targets of Mir-197 in human and mouse could be either a remnant of the ancient regulatory network, or the result of younger target sites gained independently in the human or rodent lineage. To differentiate between these scenarios, we dated the emergence of target sites in all primary targets using Mir-197 as an example (Figure 4B, C). This revealed that 8mer target sites of genes that respond to the overexpression of Mir-197 in only one of the two species emerged after the divergence of the human and mouse lineages (Figure 4B). Therefore, the regulatory network of Mir-197 appears to be highly flexible. In contrast, 8mer target sites in genes that are primary targets in both human and mouse are at least as old as the LCA of the two species (Figure 4B). The emergence of three 8mers even predates the diversification of the birds, making these target sites substantially older than the miRNA itself which is restricted to mammals. We repeated the analysis with 6mer target sites and found that they are gained throughout the vertebrates (Figure 4C). This is consistent with 6mer target sites having a markedly less pronounced impact on transcript abundance than 8mer sites (1). They are consequently more likely to be gained (and lost) as a stochastic effect of sequence evolution than 8mer target sites.

Taken together, our findings suggest that at least Mir-197 was part of an ancient regulatory network in the LCA of human and mouse. Individual ancient regulatory connections have been conserved on both evolutionary lineages such that Mir-197 still conveys a physiologically measurable effect on transcript abundance in contemporary human and mouse. This strongly indicates that Mir-197 had regulatory connections when it was lost during the diversification of the rodents.

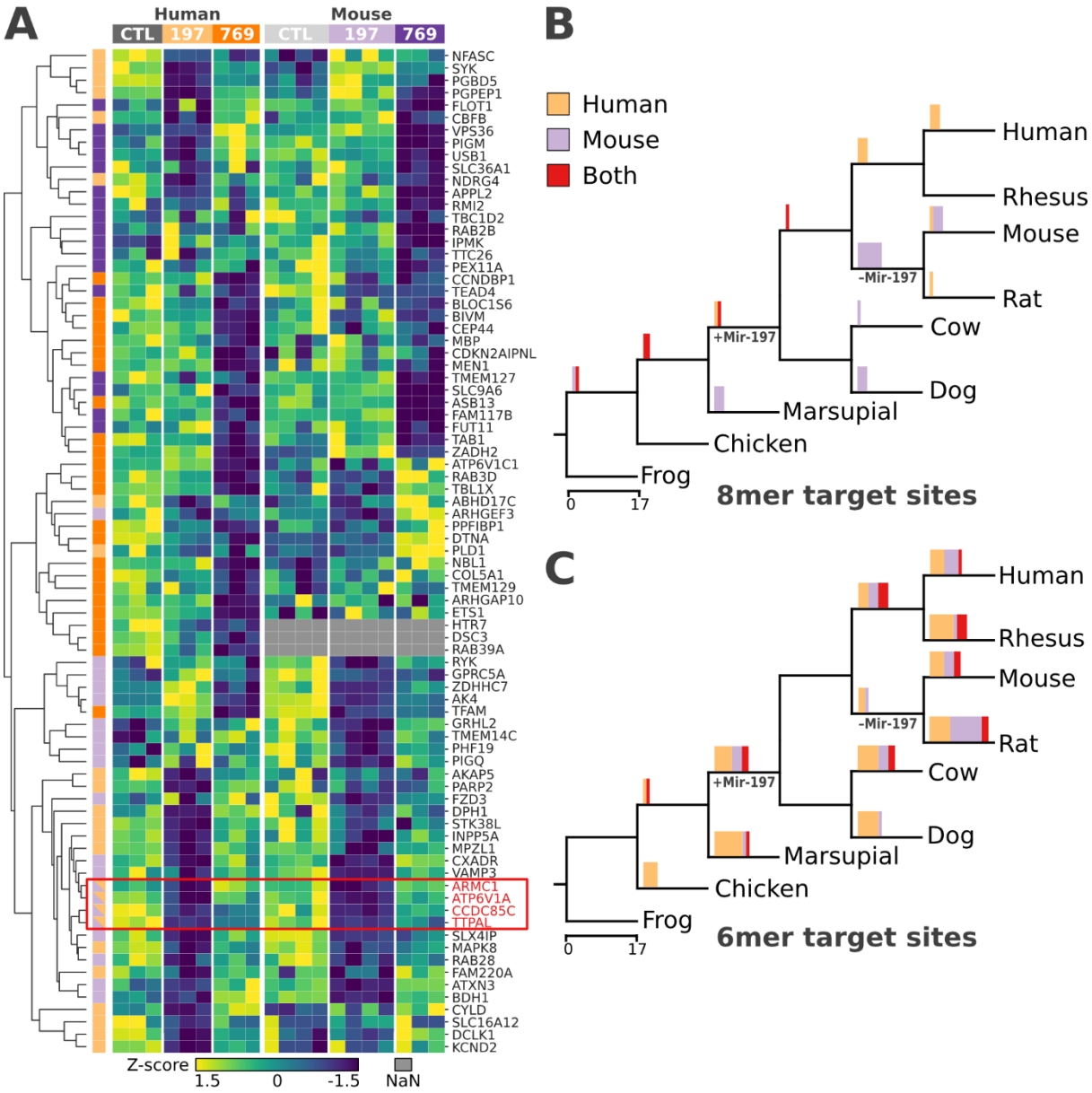

Figure 4: Primary targets in human and murine iPSCs partly overlap due to evolutionary old target sites. A) Z-score of normalized read counts between the negative control (CTL) and the overexpression of Mir-197-3p or Mir-769-5p per organism. The condition in which a gene is downregulated as well as predicted as a target for the respective miRNA are indicated with colored boxes on the left. The color code follows the top boxes where targets of Mir-197 and Mir-769, in human are colored in light orange and orange, respectively. Murine targets are colored in light purple for Mir-197 and purple for Mir-769. The red box highlights the four genes that are significantly down-regulated and predicted as targets for Mir-197 in both human and mouse. Rows are clustered by cosine similarity. Gene labels follow the human nomenclature, all names of the mouse orthologs can be derived by capitalization. B) Dating the emergence of target sites in species-specific and overlapping targets of Mir-197. Bar charts describe the number of target sites gained in the node to the right of the edge. The color of the bars indicates whether the target is downregulated in human (orange) or mouse (purple). Target sites in genes that are downregulated by Mir-197 in both human and murine cells (i.e. TTPAL, ATP6V1A, ARMC1 and CCDC85C) are marked in red. C) Origin of 6mer target sites. Color code follows B).

miRNAs are lost in tandem with TFs

Our results indicate that the miRNA loss must have had altered the gene regulatory network that existed in the LCA of human and mice on the eumuroidean lineage. We next investigated whether this change of gene regulation on the post-transcriptional regulation was accompanied by a corresponding (and hitherto overlooked) change at the transcriptional level. To this end, the phylogenetic profiles of human miRNAs were supplemented with the phylogenetic profiles of all human protein-coding genes. Phylogenetic profiles like those of the 15 co-lost miRNAs were identified (see Methods), resulting in a list of 127 protein-coding genes that were lost together with the miRNAs (Supplementary Table S 7, Figure 5A). We again ensured that the alleged loss does not represent sensitivity limits of the ortholog search (Supplementary Figure S8, Supplementary Figure S9). Additionally, there is no overlap of primary targets or miRTarBase targets with the lost protein-coding genes, yielding no evidence that the miRNA loss followed a loss of their respective target genes.

To assess a potential functional impact of the lost protein-coding genes, we performed a GO-term enrichment analysis for the corresponding human orthologs. This revealed a significant overrepresentation of genes involved in the regulation of transcription (Figure 5B). In line with this, the set contained 37 TFs listed in the Human Transcription Factor database (HTFD) (25), which is significantly more than expected by chance (Fisher's exact test, 4.2-fold enrichment, p = 3.8E-15). We next collected available TF-gene interactions for the 37 lost TFs to shed further light on the likely functional consequences of these gene losses. Five TFs were represented in the RegNetwork database of TF-gene interactions (27) and three were represented in the text-mining database of regulatory relationships TRRUST (28). For the 8 TFs recorded across both databases, a total of 28 interactions are recorded (Supplementary Table S 8). Known TF target genes are enriched for the regulation of DNA repair and the regulation of transcription (Supplementary Figure S 10), hinting at an even more deeply reticulated impact on the regulatory network.

To put the loss of the 37 TFs in the *Eumuroidea* into a larger context of TF evolution in mammals, we filtered the phylogenetic profiles of human protein-coding genes for entries in the HTFD. Out of the 1604 TFs, 1019 are almost ubiquitously present (> 95% of the investigated taxa). The remaining 585 have been lost on individual evolutionary lineages (Supplementary Table S 9), most prominently in the *Eumuroidea* (Supplementary Figure S 11). We therefore conclude that the *Eumuroidea* are unique in the extent with which their regulatory network was restructured on both TF- and miRNA-level compared to other mammalian lineages.

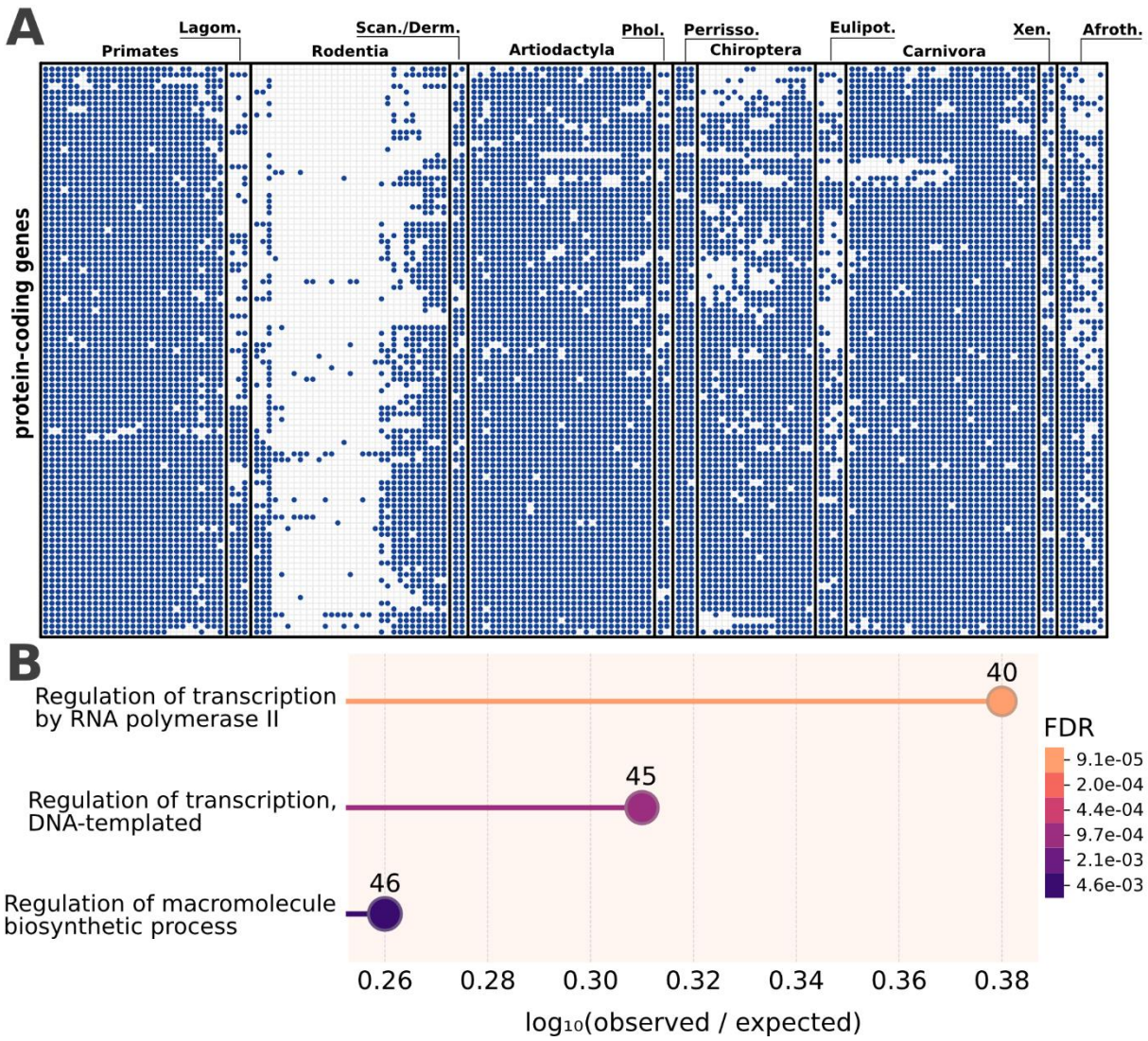

Figure 5: Protein-coding genes lost in tandem with the miRNAs are enriched for transcription-related biological processes. A) Phylogenetic profiles of orthologs to human protein-coding genes. The presence of an ortholog is indicated by a dot. B) Enrichment analysis for the "Biological Process" class, performed by the STRING database **(26)**. The number of genes annotated with the respective GO-term are shown above the node.

Discussion

After their stable integration into a regulatory network, miRNA genes are rarely lost. Here, we have investigated the striking absence of 15 mammalian miRNA families in the *Eumuroidea*. We provide substantial evidence for the coordinated loss of 15 miRNA families and could show that it coincides with the loss of 37 transcription factors along the same evolutionary lineage. This points to a lineage-specific shift in gene regulation at both transcriptional and post-transcriptional levels in species including the model organisms rat and mouse.

Functional integration of miRNAs

For protein-coding genes, a concerted loss indicates functional integration (12). Based on observations in the existing regulatory network of humans, the best indication of functional integration is the functional overlap of miRNA targets. Although the detectable signal for enrichment with development-related GO terms is faint, it aligns with the finding that expression patterns of half of human development-associated genes differ from those of their murine orthologs (29). Notably, many regulatory connections between miRNAs and transcripts evolve rapidly (7). This introduces noise to the inference of the ancient regulatory network in the eumuroidean LCA from available annotations in contemporary humans. Consequently, a potential functional overlap of ancient targets of the co-lost miRNAs may not be observable in the contemporary human regulatory network. Additionally, miRNAs are assumed to become expressed in more diverse tissues over time (4,5). Therefore, it cannot be excluded that the 15 miRNA families were tissue-specific when they were lost in the *Eumuroidea* LCA, potentially adding a layer of functional integration.

Identification of primary targets

A commonly used way to identify miRNA targets is to integrate the measured change in transcript number with predicted targets (30), because *in silico* target prediction alone infers many false positives (31). Notably, some methods for predicting miRNA targets are based on the evolutionary conservation of target sites (1). Alternative approaches like the context++-score implemented in TargetScan 7 take conservation-agnostic features of the miRNA and transcript sequence into account (2). While the former approach is most likely not suitable for detecting target sites of non-native miRNAs, we show that the latter is able to identify relevant target sites of human miRNAs in murine iPSCs.

Initial target repertoire of novel miRNAs

The existence of an initial target repertoire due to the small size of the seed region has been hypothesized previously (3–5). We here experimentally identify four genes that most likely contributed to Mir-197's initial target repertoire. Given that we derive our set of primary targets from strict filtering

steps, it is conceivable that we underestimate the extent to which targets of Mir-197 and Mir-769 overlap in human and murine iPSCs. Future studies may investigate this overlap more closely, e.g. by integrating RNA-seq with PRO-seq data (32).

It is tempting to speculate that the ancient regulatory connections that we report have remained observable because their target sites were conserved through additional factors like secondary structure constraints (33). Additional prerequisites for these four genes to contribute to the initial target repertoire include whether they were co-expressed with Mir-197 in the Eutherian LCA and whether their expression levels were high enough to down-regulate target transcripts. Still, these results are a strong indication that the lost miRNA families had regulatory connections when they were lost during the diversification of the rodents.

Impact of losing miRNAs on the *Eumuroidea* regulatory network

Next to the functional integration hypothesis, it is conceivable that the miRNAs were lost because they had only a limited impact on the regulatory network. miRNAs that are specific to mammals were previously found to only have 11 conserved targets on average (1). Additionally, their target sites appear to be less conserved across species compared to those of older miRNAs, suggesting that the corresponding regulatory network is still considerably plastic (7). These analyses have, however, not considered the lineage specific loss of miRNAs, which occurs more often for *Mammalia*-specific miRNAs than for more ancient ones (17). Consequently, the functional impact of mammalian miRNAs on the regulatory network has likely been underestimated in previous studies. Still, the observed loss could be a stochastic effect of a limited functional impact combined with the fast nucleotide substitution rate that is characteristic for the *Eumuroidea* (34,35). However, comparing the adjusted evolutionary distances of each respective species to human, eumuroidean species are not more distant than, for example, the Afrotherians (36). Given the overall rarity of miRNA loss across even larger evolutionary distances (17,19), the fast evolutionary rate in the *Eumuroidea* is unlikely to explain the concerted loss of 15 miRNA families that are largely conserved in mammals. Moreover, the rarity with which a gene is lost typically reflects its biological importance (37). A model where novel miRNAs exert little biological function (5) implies a frequent loss of miRNA genes that is not observed (17,19). Consequently, the rarity with which miRNA genes are lost indicates a functional importance of miRNAs once they become conserved across species level.

Restructuring of the eumuroidean regulatory network

The *Eumuroidea* lost 15 miRNA families together with 37 TFs, which are otherwise widely conserved in mammals. Of all investigated mammalian lineages, this is by far the most striking loss of regulators of gene expression, both on the transcriptional- and post-transcriptional level (Supplementary Figure

S 11, (17). On first sight, this multi-level modification suggests an additive if not multiplicative effect on gene expression. However, the opposite might be true. The loss of regulators on transcriptional as well as post-transcriptional level could be compensatory. In this scenario, the miRNA families are lost to balance the effect of a prior TF loss, or vice versa. According to this 'compensation hypothesis', the global transcriptome of eumuroidean species should be comparable to that of other mammals. Available data does not seem to support this assumption. A study of mammalian gene expression found the global transcriptome of rabbit, which branched of prior to the diversification of the rodents, to be more similar to human than that of rat and mouse (38). Similarly, the transcriptomes of human and canine natural killer cells are more alike than those of human and mouse (39). Both studies are therefore indicating that the losses of miRNAs and of TFs observed by us correlate with a considerable remodelling of the gene regulatory network in the *Eumuroidea*. Collectively, these findings support the general notion that mice represent a suboptimal host to model human diseases. In addition to the different size, life expectancy, metabolic activity, nutrition and microbiome of the two organisms (40), they also imply altered gene regulation principles. With this study, we have begun to investigate potential drivers of gene expression changes in the *Eumuroidea*. Given that only 8 out of the 37 TFs have recorded interactions in public databases, more studies are still needed for a comprehensive functional characterization of the lost TFs.

Conclusion

miRNAs are seldomly lost (16,17,19), directing attention to the absence of 15 miRNA families in the *Eumuroidea* lineage. Remnants of the ancient regulatory network shaped by the lost miRNAs indicate a regulatory shift that is specific to this lineage. This shift of the regulatory network extends to the regulation of gene transcription level where at least 37 regulators were lost. We show that the *Eumuroidea* are unique in the extent with which their regulatory network was restructured compared to other mammals. Additionally, we find no evidence that this shift was functionally compensated. These findings have important implications for studies that use mouse or rat as model organisms to analyze gene expression, for example in the context of investigating human diseases. Depending on the research question, it should be considered whether a non-eumuroidean model organism like rabbit, dog or cattle is more appropriate for the comparison with human than mouse or rat.

Material and Methods

Cell culture

Human induced pluripotent stem cells (iPSCs) (WSTIi081-A, EbiSC) were cultured in TeSR™-E8™ medium (#05990, STEMCELL™ Technologies) at 37°C and 5% CO_2 in a humidified atmosphere.

Mouse embryonic fibroblasts (MEF) were isolated from C57B6 mice and cultured as described previously (41). Isolated mouse embryonic fibroblasts were reprogrammed into induced pluripotent stem cells (iPSCs) using the STEMCCA system (42). Briefly, 20.000 mouse embryonic fibroblast (were seeded into culture dishes. After 24 h cells were infected with STEMCCA virus for 24 h. Media was changed for five consecutive days. Cells were then reseeded on mitomycin (Serva, 10 µg/mL, 3 h) - inactivated feeder cells until murine induced pluripotent colonies emerged. Individual colonies were subsequently picked and expanded. Cells were passaged (DMEM GlutaMAX, #10566016, Gibco; 20 % Knockout Serum Replacement, #10828028, 25 µg mLIF, #250–02, Peprotech; 0.1% β-mercaptoethanol, 200 µM Glutamine, #11539876, Gibco) by 70% before colonies touched and reseeded between 2.5 × 10^3 and 5 × 10^3 cells per cm^2.

FACS sorting of mouse iPSCs

Mouse induced pluripotent stem cells (iPSC) cultured on feeder cells (MEF) or only feeder cells (negative control) were dissociated with accutase (#A6964, Sigma-Aldrich) centrifuged and resuspended to single cell suspension in FACS Buffer (PBS, 5 % FCS). Cells were sorted using the Sony SH800 at 4°C (nozzle size 100 um, flow rate 6.0). Cells were selected using forward scatter and back scatter (4, 25 %). Gating was done by using autofluorescence of cells (FITC-A-Compensated vs. PE-A-Compensated). Feeder used as negative control to differentiate between cell types in mixed culture of feeder and miPSC. Gating was set to sort for miPSC (100.000 events).

Transfection of human and murine iPSCs with miRNA precursors

For miRNA overexpression treatments, 250.000 human or murine iPSCs were transfected with Lipofectamin RNAiMAX (Cat. No. 13778075) according to the manufacturers protocol (ThermoFisher Scientific). Ambion® Pre-miR™ miRNA Precursor for hsa-miR-197-3p (Assay ID PM10354, Cat. No. AM17100, ThermoFisher Scientific) and hsa-miR-769-5p (Assay ID PM11974, Cat. No. AM17100, ThermoFisher Scientific) were used. As negative control, Pre-miR™ miRNA Precursor Negative Control #1 (Cat. No. AM17110, ThermoFisher Scientific) was used. All miRNA precursor transfections were performed with a concentration of 8 nM for 48 h.

RNA isolation, Reverse transcription and RT-qPCR

Total RNA isolation was performed with the RNA Mini Kit (Bio&Sell) according to the manufacturers protocol and reverse transcription was performed with the SuperScript III Reverse Transcriptase (Thermo Fisher) using a combination of oligo(dT)23, random hexamer as well as U6 snRNA- and miRNA-specific RT primers (Sigma). The resulting cDNA was amplified in an AriaMX cycler (Agilent) with the ITaq Universal SYBR Green Supermix and ROX as reference dye (Bio-Rad, Cat. No. 1725125). Relative expression of human target genes was normalized to U6 snRNA. Expression levels were analyzed by

the delta-delta Ct method with the AriaMX qPCR software (Agilent, Version 1.7). The primers for the detection of miRNAs were designed with miRprimer2 (43). The following oligonucleotide sequences were used for the miRNAs: miR-197-5p RT 5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC CTC CC-3', forward 5'- GCG GCG GCG GGT AGA GAG GGC AG-3'; miR-197-3p RT 5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACG CTG GG-3', forward 5'-GCG GCG GTT CAC CAC CTT CTC C-3'; miR-769-5p RT 5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA GCT CA-3', forward 5'-GCG GCG GTG AGA CCT CTG GGT TC-3'; miR-769-3p RT 5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA ACC AA-3', forward 5'-GCG GCG GCT GGG ATC TCC GGG G-3'. For all miRNAs, a universal reverse primer was used: 5'-ATC CAG TGC AGG GTC CGA GG-3'. For U6 snRNA, the following oligonucleotides were used: RT 5'-CGC GCC TGC AGG TCG ACA ATT AAC CCT CAC TAA AGG GTT GCG TGT CAT CC-3', forward 5'-GTA ATA CGA CTC ACT ATA GGG AGA AGA GCC TGC GCA AGG-3' and reverse 5'-CAG GTC GAC AAT TAA CCC TC-3'.

RNA-Seq

RNA amounts were normalized and 500-100ng of total RNA was used as input for SMARTer Stranded Total RNA Sample Prep Kit HI Mammalian (Takara Bio). Sequencing was performed on the NextSeq2000 platform (Illumina) using P3 flowcell with 72bp single-end setup. Trimmomatic version 0.39 was employed to trim reads after a quality drop below a mean of Q15 in a window of 5 nucleotides and keeping only filtered reads longer than 15 nucleotides (44). Reads were aligned versus Ensembl human genome version hg38 (release 104) or Ensembl mouse genome version mm39 (Ensembl release 104) with STAR 2.7.9a (45). Alignments were filtered to remove: duplicates with Picard 2.25.2 (46)(https://github.com/broadinstitute/picard), multi-mapping, ribosomal, or mitochondrial reads. Gene counts were established with featureCounts 2.0.2 by aggregating reads overlapping exons on the correct strand excluding those overlapping multiple genes (47). The raw count matrix was normalized with DESeq2 version 1.30.0 (48).

miRNA targets

Experimentally validated targets of all 15 lost miRNAs were downloaded from MirTarBase 9 (22) and filtered for interactions with strong experimental evidence. Targets of Mir-197 and Mir-769 were predicted with TargetScan7 in the 3'-UTR alignments supplied by TargetScan that are constructed with either human or mouse as reference species (2). TargetScan provides the option to predict target sites using the context++-score method which is independent of the conservation status of the miRNA in 10 vertebrate species. We calculated the total unweighted context++-score (tc++) as described in Agarwal et al. (2015) and set a minimum tc++ threshold of -0.2 to define predicted targets (Supplementary Figure S1). Overlapping target sites in the 3'-UTRs of orthologous genes were considered homologous,

and we used this information to date the emergence of these sites. The most parsimonious branch of target site origin was determined using the Count software package (49).

Phylogenetic profiles of protein-coding genes

We used fDOG (50) to identify orthologs to all human protein-coding genes in all 169 mammals for which miRNA ortholog assignments are available (17). Phylogenetic profiles of all protein-coding genes and miRNA genes from the 16 families lost in Eumuroidea were represented as binary vectors. miRNA orthologs with non-conserved seed sequence were considered to be absent since they likely have changed in function. The top percentile of protein-coding genes with the highest Pearson correlation coefficient to each miRNA profile were extracted and genes whose profile is similar to at least half of the lost miRNAs were identified as sharing an evolutionary history with the lost miRNA families.

Data availability

The RNA-Seq datasets have been deposited and are available at NCBI GEO. The accession number for human is GSE281793 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE281793) and the mouse dataset is available with the accession number GSE281795 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE281795).

Data analysis scripts are available at https://github.com/felixlangschied/rodent_loss.

Author contributions

Felix Langschied (Conceptualization [equal], Data Curation [equal], Formal Analysis [lead], Investigation [equal], Software [lead], Validation [equal], Visualization [lead], Writing – Original Draft Preparation [lead], Writing – Review & Editing [equal]), Matthias Leisegang (Conceptualization [supporting], Data Curation [equal], Formal Analysis [supporting], Investigation [equal], Validation [equal], Visualization [supporting], Writing – Review & Editing [supporting]), Stefan Günther (Data Curation [equal], Formal Analysis [supporting], Investigation [supporting]), Fabian Hahne (Investigation [supporting]), Ralf P. Brandes (Conceptualization [equal], Funding Acquisition [equal], Resources [equal], Supervision [supporting], Validation [equal], Writing – Review & Editing [supporting]), Ingo Ebersberger (Conceptualization [equal], Funding Acquisition [equal], Resources [equal], Supervision [lead], Validation [equal], Writing – Original Draft Preparation [supporting], Writing – Review & Editing [equal])

Funding

This work was supported by the Alfons und Gertrud Kassel-Stiftung; Research Funding Program Landes-Offensive zur Entwicklung Wissenschaftlich-ökonomischer Exzellenz (LOEWE) of the State of Hessen, Research Center for Translational Biodiversity Genomics (TBG) (to IE); Goethe University Frankfurt am Main and the DFG excellence cluster Cardiopulmonary Institute (CPI) EXS2026 (to RPB). This project was funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation)—Project-ID 403584255–TRR 267 (to MSL and RPB).

Acknowledgments

We thank Judit Izquierdo Ponce and Manuela Späth for excellent technical assistance.

Competing interests statement

The authors have declared that no conflict of interest exists.

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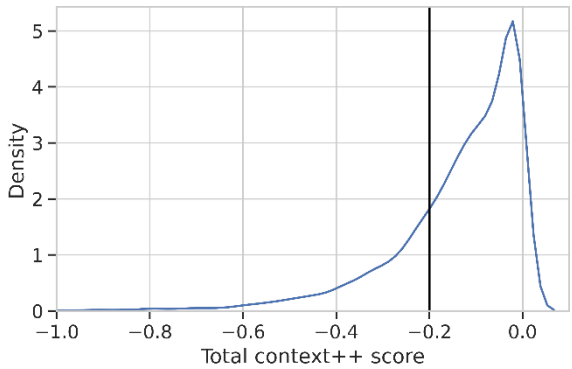
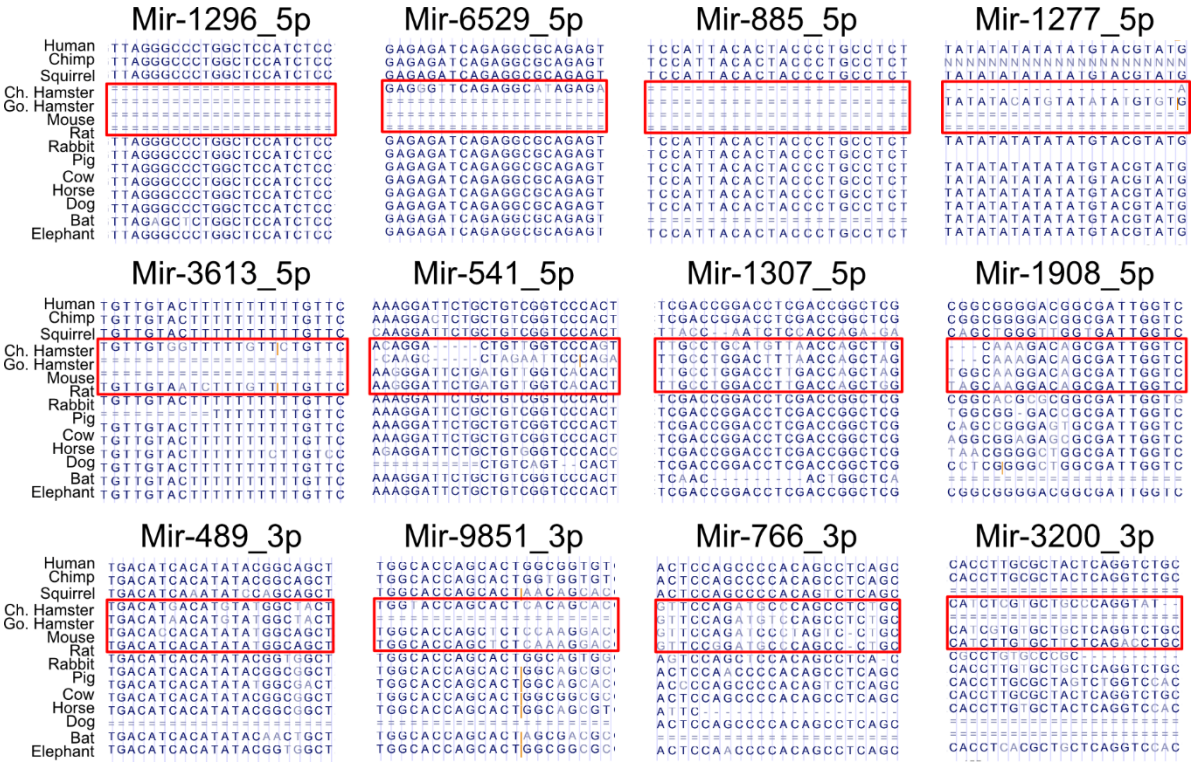
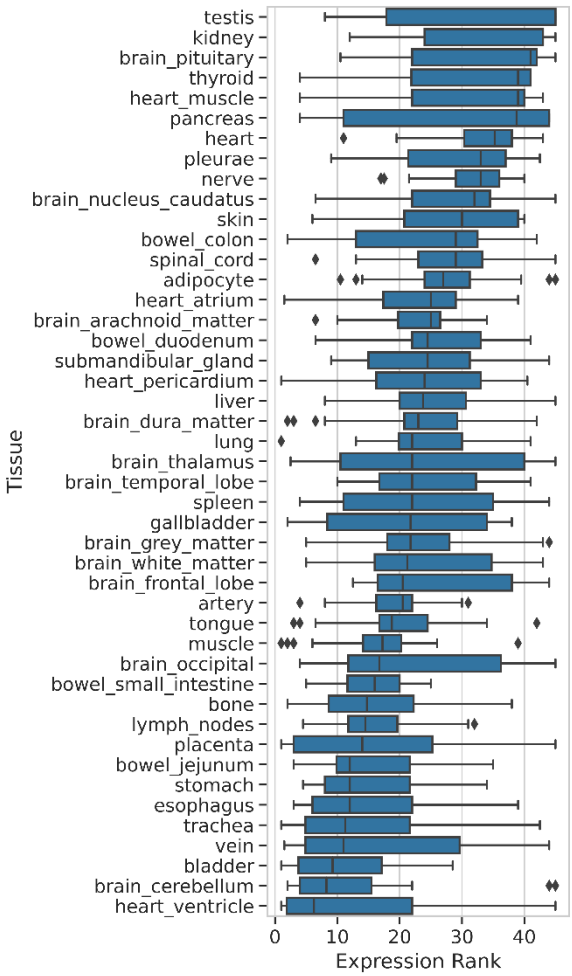
Supplementary Figures



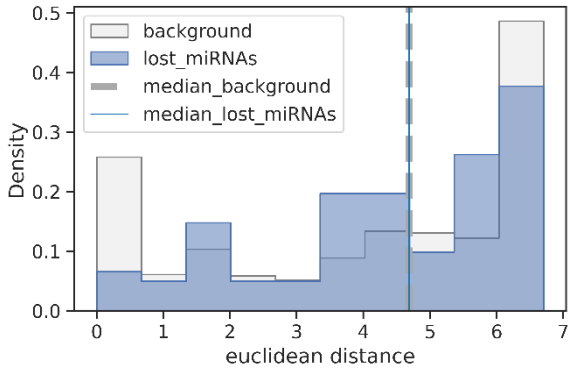
Supplementary Figure S1: Distribution of total context++ scores of miRNA targets predicted with TargetScan7. The minimum score threshold used for finding primary miRNA targets of -0.2 is marked with a black line.



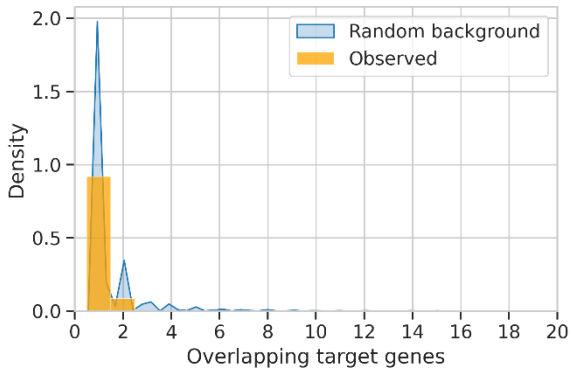
Supplementary Figure S2: 14-species whole-genome alignments of miRNA families lost the Eumuroidea. Eumuroidean species are marked with a red box.



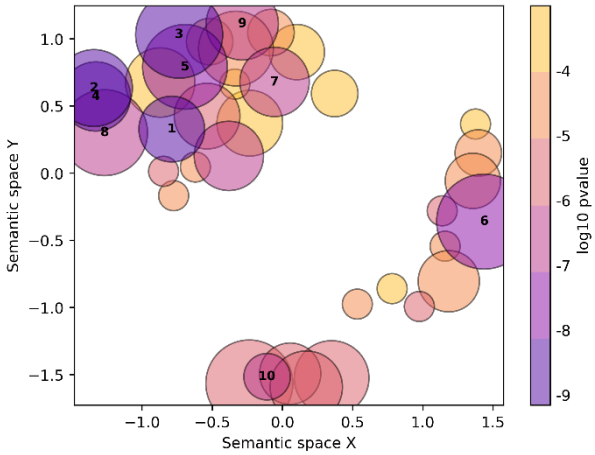
Supplementary Figure S3: Expression level of miRNA genes from families co-absent in the Eumuroidea.



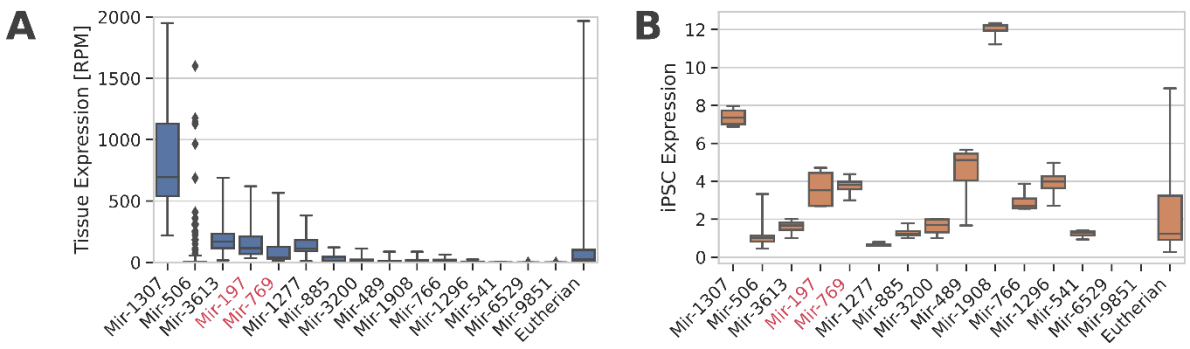
Supplementary Figure S4: Pairwise distances of miRNA expression vectors across 45 human tissues. Normalized reads of miRNA expression were downloaded from MirGeneDB 2.1 **(21)**. A minimum threshold of 15 was set to determine presence or absence of a miRNA in a tissue. If a miRNA family was represented by multiple genes, the gene with the highest expression in the family was chosen as representative.



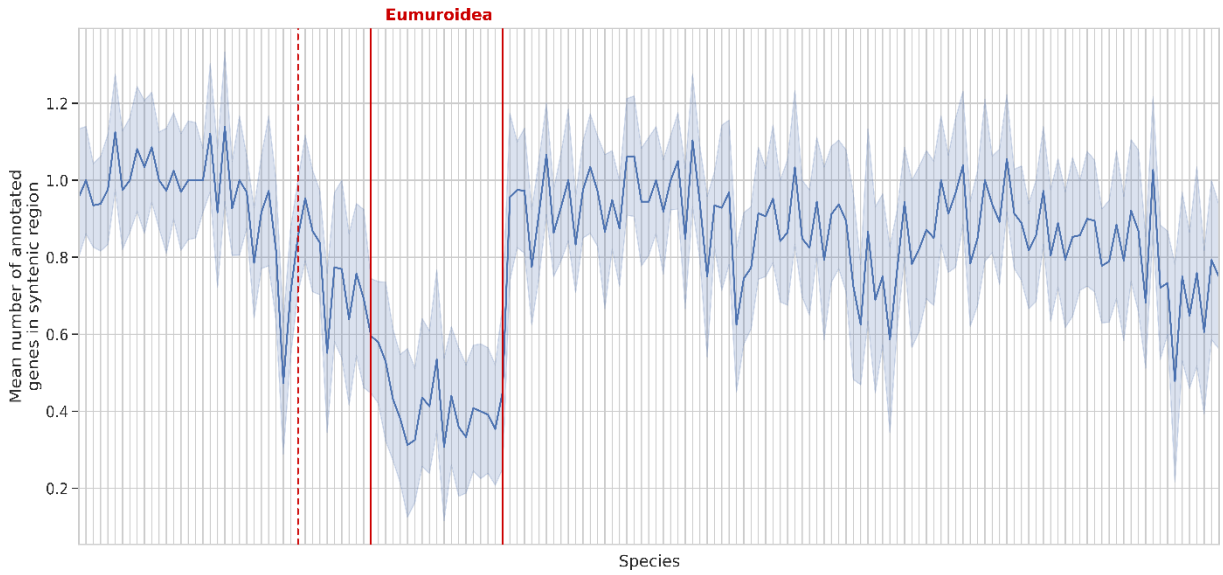
Supplementary Figure S5: Overlap of miRTarBase targets of lost miRNAs.



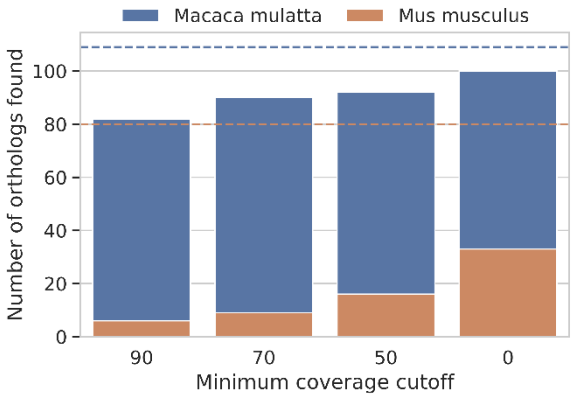
Supplementary Figure S 6: Significantly enriched GO-terms of miRTarBase targets of lost miRNAs. Visualized using semantic clustering performed by GO-Figure **(51)**.



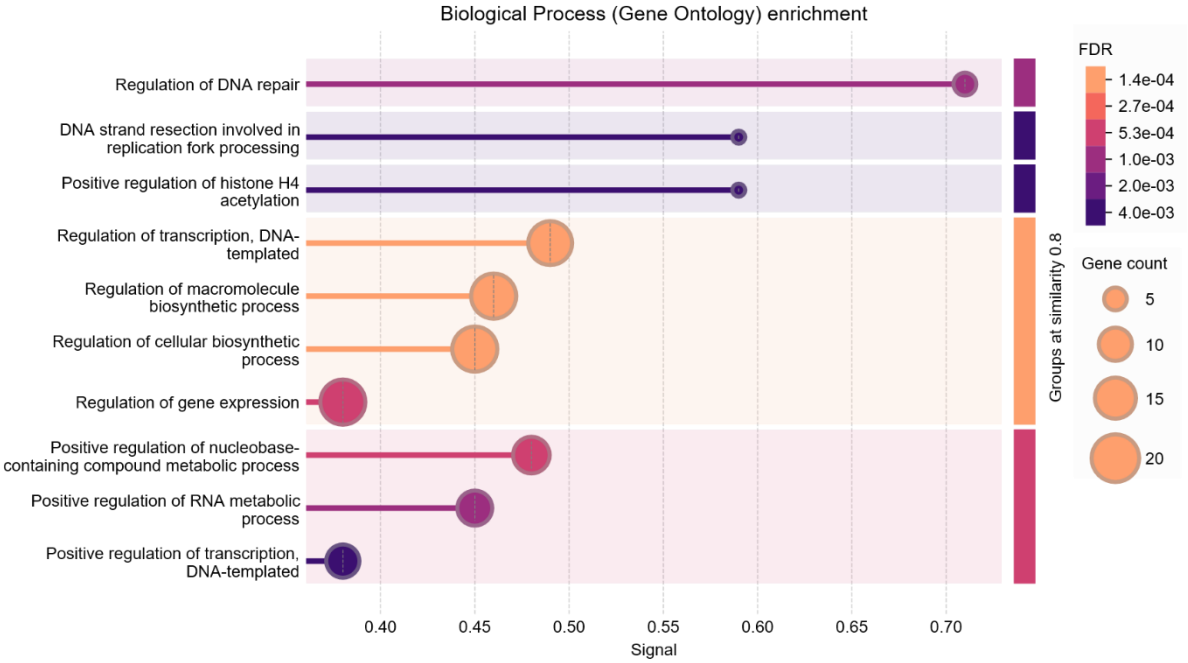
Supplementary Figure S7: Expression of miRNA genes from the 16 families of interest and across all Eutheria-specific miRNAs. A) RNAseq data of 45 tissues from MirGeneDB **(21)**. B) Microarray expression levels of the same miRNA genes in iPSC cells reported by **(52)**



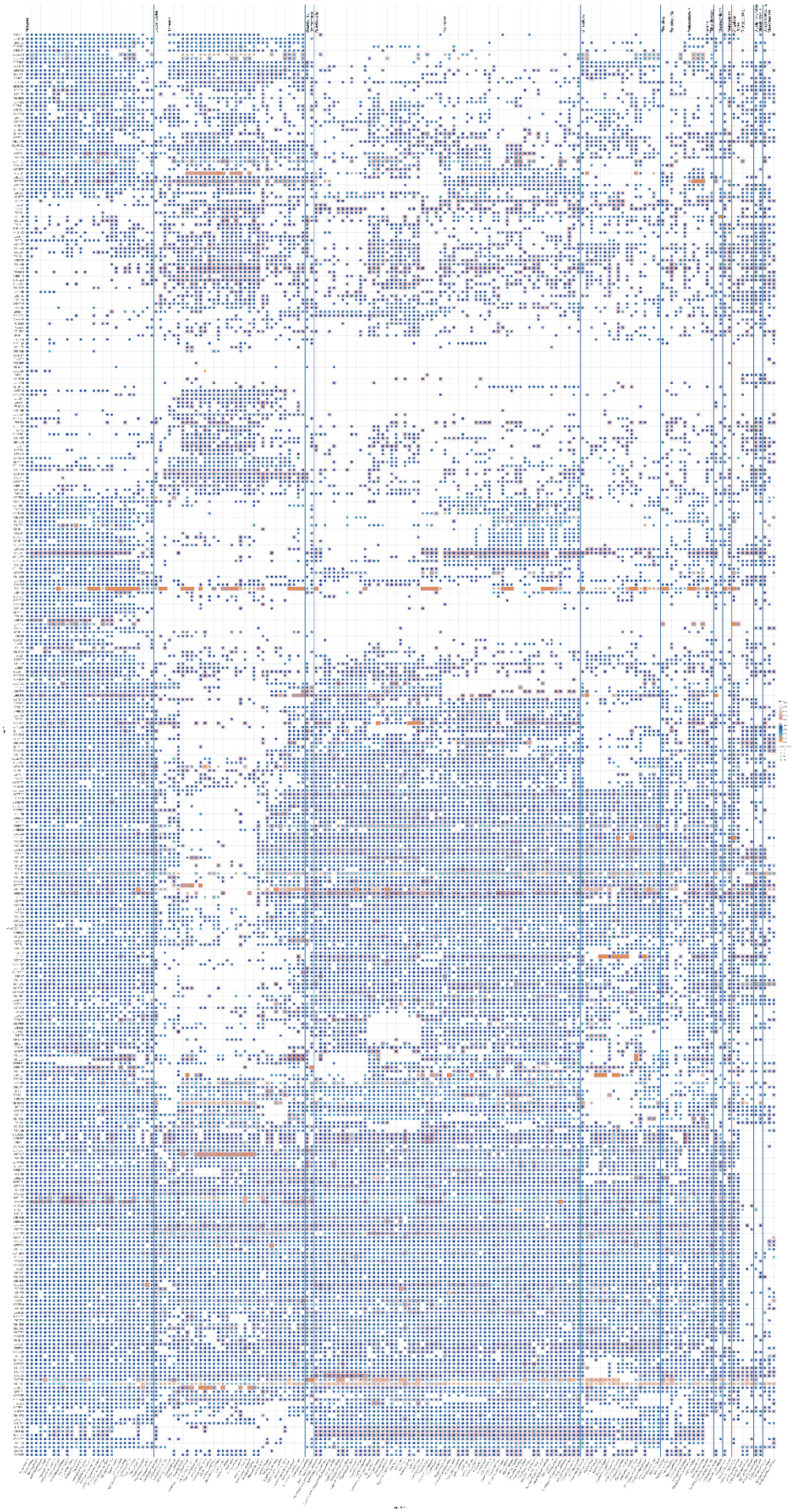
Supplementary Figure S8: Number of annotated genes in genomic region that is shared syntenic to human reference proteins. First, the number of annotated genes in all identified syntenic regions was counted. In taxa where no ortholog was found (i.e. the Eumuroidea), this value drops below 1, indicating that there is no annotated gene in the respective region.



Supplementary Figure S9: Sequence similarity search identifies ruins of lost genes. A tBLASTn search of the human protein-sequence was performed in the respective shared syntenic region of the mouse genome. Number of identified syntenic region per species are marked with a dashed line. This identified short fragments that share a significant sequence similarity to the lost genes. Thus, the syntenic regions in mouse still harbour remnants of the lost genes but they are sufficiently degraded so that no gene was inferred in the region. This strongly indicates that the genes were indeed lost **(53)**, and that the functions encoded by them are most likely absent in the Eumuroidea.



Supplementary Figure S 10: GO-Enrichment analysis of the genes targeted by TFs lost in the Eumuroidea. Performed by the STRING database **(26)**.



Supplementary Figure S 11: Phylogenetic Profile of non-conserved human transcription factors.

Supplementary Tables

https://github.com/felixlangschied/rodent_loss/blob/master/publication/supplement_tables/supplementary_tables.xlsx

Supplementary Table S 1: miRNA overview

Supplementary Table S 2: miRTarbase_targets

Supplementary Table S 3: Differentially expressed genes

Supplementary Table S 4: downregulated TFs

Supplementary Table S 5: Downregulated + predicted as targets (no threshold)

Supplementary Table S 6: Overlap with relaxed threshold

Supplementary Table S 7: Lost proteins

Supplementary Table S 8: TF interactions

Supplementary Table S 9: Gain and conservation of human TFs