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Post-fertilization transcription initiation in an ancestral LTR retrotransposon drives lineage-specific genomic imprinting of *ZDBF2*

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eLife Assessment

The authors analyses describe a novel mechanism by which a retrotransposon-derived LTR may be involved in genomic imprinting and demonstrate imprinting of the ZDBF2 locus in rabbits and Rhesus macaques using allele-specific expression analysis. This imprinting of the ZDBF2 locus correlates with transcription of GPR1-AS orthologs. The accompanying genomic analysis is very well executed allowing for the conclusions reached in the manuscript. The revisions made at the request of the reviewers in this **important** manuscript strengthen the evidence from the genomic analyses, and as a result, the evidence is now **convincing** and will be informative to the genomics and developmental biology communities.

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Abstract

The imprinted *ZDBF2* gene is controlled by oocyte-derived DNA methylation, but its epigenetic regulation is quite different from that of other canonically imprinted genes that are dependent on DNA methylation deposited in the gametes. At the *ZDBF2* locus, maternal DNA methylation in the imprinted differentially methylated region (DMR) does not persist after implantation. Instead, a transient transcript expressed in the early embryo exclusively from the unmethylated paternal allele of the DMR, known as *GPR1-AS* in humans and *Liz* in mice, contributes to establishing secondary DMRs that maintain paternal expression of *ZDBF2* in the somatic lineage. While the imprinting of *ZDBF2* is evident in humans and mice, whether this process is conserved in other mammals has not been addressed. Here, we show that the first exon of human *GPR1-AS* overlaps with that of a long terminal repeat (LTR) belonging to the MER21C subfamily of retrotransposons. Although this LTR family appears and is amplified in Boroeutherians, the magnorder of placental mammals that includes the Euarchontoglires and Laurasiatheria superorders, the MER21C insertion into the *GPR1-AS* orthologous region occurred specifically in the common ancestor of Euarchontoglires, a clade that includes extant primates, rodents, and rabbits. The first exon of mouse *Liz* does not overlap with an annotated LTR in standard repeat annotation; however, promoter activity assay and multiple sequence alignment suggests that it retains a functionally conserved relationship with the MER21C-overlapping first exon of *GPR1-AS*. Furthermore, directional RNA sequencing of placental tissues from rabbits and nonhuman primates also revealed *GPR1-AS* orthologs, with their first exon embedded within the same ancestral LTR. In contrast, allele-specific expression profiling of cow and tammar wallaby, mammals outside the Euarchontoglires group, revealed expression from both alleles in all tissues analyzed. Taken together, these observations suggest that imprinting of *ZDBF2* in Euarchontoglires had its genesis in the insertion of a MER21C element in their common ancestor. Our previous studies showed that LTRs reactivated in oocytes contribute to lineage-specific imprinting during mammalian evolution. The data presented here suggest that post-fertilization activation of an ancestral LTR-derived sequence can also contribute to the lineage-specific establishment of imprinted genes.

Introduction

Genomic imprinting is a well-established biological phenomenon that refers to the differential expression of imprinted genes depending on their parental origin (Tucci et al. 2019; Kobayashi 2021). Epigenetic mechanisms, including DNA methylation, histone modification, and noncoding RNA molecules, regulate this process, establishing and maintaining parent-of-origin-specific gene expression patterns. Imprinted genes play vital roles in various biological processes such as fetal growth and development, placental function, metabolism, and behavior. These imprinted genes are often clustered within imprinted domains and regulated by epigenetic marks in specific imprinting control regions (ICRs). The inheritance of parent-of-origin-specific DNA methylation marks from the oocyte or sperm occurs in germline differentially methylated regions (germline DMRs), which are propagated in all somatic tissues of the next generation and function as ICRs (therefore, referred to as canonical imprinting). Imprinting patterns can vary significantly among species. Although some genes are imprinted in only one or a few species, others are conserved in many mammals. For example, nearly 200 imprinted genes have been identified in mice and humans. However, one comparative analysis suggested that fewer than half of these genes are imprinted in both species, suggesting the existence of many species- or lineage-specific imprinted genes (Tucci et al. 2019). The establishment of species-specific imprinting at specific loci can be driven by various evolutionary events, including retrotransposition of genes or

transposable elements, which may lead to the acquisition of new genes as imprinted genes or the formation germline DMRs at CpG- rich regions (CpG islands)(Kobayashi 2021; Kaneko-Ishino and Ishino 2022). These CpG islands, often conserved as orthologous sequences across species, may become germline DMRs in the female germline as a consequence of transcription across the CpG island (Chotalia et al. 2009; Kobayashi et al. 2012a). Such transcription may be initiated within transposable elements which have integrated nearby. In such cases, the retrotransposition of genes or transposable elements is not directly responsible for the creation of CpG islands themselves but instead facilitates their recruitment into the imprinting system, contributing to the establishment of species-specific imprinting. Indeed, our previous studies have shown that oocyte-specific transcription from upstream promoters, such as integrated LTR retrotransposons, likely played a critical role in the establishment of lineage-specific maternal germline DMRs (Brind'Amour et al. 2018; Bogutz et al. 2019). An alternative form of imprinting involves the Polycomb marks H3K27me3 and H2AK119ub1, initially deposited in oocytes (Mei et al. 2021). Originally described in rodents, such "non-canonical" imprints are maternally inherited and in turn silence associated genes during preimplantation. Subsequently, these Polycomb marks are converted into DNA methylation over LTR elements, thus maintaining silencing of maternal alleles in the extraembryonic lineage (Hanna et al. 2019 ; Richard Albert et al. 2023). In addition, interspecies differences in the regulators of ICRs, such as *ZFP57* and *ZNF445*, as well as species-specific orthologs of DNA methyltransferases, including *DNMT3C,* are potential species-specific features: *ZFP57* and *ZNF445* cooperatively maintain ICR methylation, with *ZNF445* being more dominant in humans and *ZFP57* in mice; and *DNMT3C* is crucial for a paternally methylated DMR and appears to be specific to Muroidea. These features might also have contributed to the establishment of species- specific imprinted genes (Barau et al. 2016; Takahashi et al. 2019). However, these reports alone have not been sufficient to propose a comprehensive model for the emergence of all species-specific imprinted genes.

Zinc finger DBF-type containing 2 (*ZDBF2*), a paternally expressed gene located on human chromosome 2q37.1, has been reported to regulate neonatal feeding behavior and growth in mice, although its molecular function remains unknown (Kobayashi et al. 2009; Glaser et al. 2022). There are at least three imprinted genes, *ZDBF2*, *GPR1* (alternatively named *CMKLR1*), and *ADAM23* around this locus (Hiura et al. 2010; Morcos et al. 2011). Recently, the *Zdbf2* gene was also shown to be paternally expressed in the rat, another member of the Muridae family (Richard Albert et al. 2023)(**Supplemental file 1**). Studies have shown that *ZDBF2* imprinting is regulated by two types of imprinted DMRs: a germline DMR located in the intron of the *GPR1* gene and a somatic (secondary) imprinted DMR upstream of *ZDBF2* (Kobayashi et al. 2012b; Kobayashi et al. 2013; Duffie et al. 2014). The germline DMR is methylated on the maternal allele, leading to silencing of *GPR1* antisense RNA (*GPR1-AS*, alternatively named *CMKLR1-AS*) expression on that allele. In contrast, the paternal allele is hypomethylated, allowing for the expression of *GPR1-AS*, which is transcribed in the reverse direction of *GPR1* (in the same direction as *ZDBF2*) from the germline DMR. Although it is not known whether *GPR1-AS* encodes a protein, a fusion transcript of *Gpr1-as* (*Platr12*) and *Zdbf2* (alternatively named *Zdbf2linc* or *Liz*; a long isoform of *Zdbf2*) has been identified in mice (Kobayashi et al. 2012b; Duffie et al. 2014). Notably, this fusion transcript exhibited an imprinted expression pattern similar to that of human *GPR1-AS* (**Supplemental file 1**). *Liz* transcription counteracts the H3K27me3-mediated repression of *Zdbf2* by promoting the deposition of antagonistic DNA methylation at the secondary DMR (Greenberg et al. 2017). Germline DMRs that function as ICRs generally maintain uniparental methylation throughout development; however, differential methylation of the germline DMR is no longer maintained at this locus after implantation because the paternally derived allele also becomes methylated after implantation. Thus, the secondary DMR, rather than germline DMR, is thought to maintain the imprinted status of this locus in somatic cell lineages. The unique regulatory mechanism responsible for imprinting at the *ZDBF2* locus appears to be conserved between humans and mice; however, whether this conservation extends to other mammals remains unknown.

Here, we focus on *GPR1-AS/Liz*, which is critical for the imprinting of *Zdbf2* in mice, and identified orthologous transcripts of *GPR1-AS* in primates and rabbits using RNA-seq- based transcript analysis. Strikingly, the first exon of these orthologs overlaps with a common LTR retrotransposon, suggesting that LTR insertion in a common ancestor led to the establishment of *ZDBF2* imprinting. In support of this hypothesis, *ZDBF2* expression is not imprinted in a mammalian outgroup to the Euarchontoglires that lack this LTR, including cattle and tammar wallabies. Taken together, our findings indicate that in the branch of the mammalian lineage that shows imprinting of *ZDBF2*, the key evolutionary event was the insertion at this locus of an LTR-derived sequence that becomes active only after fertilization.

Results

Detection of *GPR1-AS* orthologs using RNA-seq data sets

GPR1-AS and *Liz* expression have been observed in placental tissues, blastocyst-to- gastrulation embryos, and/or ES cells in humans and mice (Kobayashi et al. 2009; Kobayashi et al. 2012b; Kobayashi et al. 2013; Duffie et al. 2014). Public human tissue RNA-seq data sets show that *GPR1-AS* transcription is detectable in the placenta, albeit at a relatively low level (RPKM value of around 0.45–1.8), but is significantly suppressed in other tissues (**Figure 1 – supplement figure 1A**) (Fagerberg et al. 2014; Duff et al. 2015). To determine whether *GPR1-AS* orthologs are present across different mammalian species, we performed transcript prediction analysis using short-read RNA- seq data from the placenta or specific extra-embryonic tissues of Eutheria (placental mammals) and Metatheria (marsupials).

First, we downloaded the placental RNA-seq data from 15 mammals: humans, bonobos, baboons, mice, golden hamsters, rabbits, pigs, cattle, sheep, horses, dogs, bats, elephants, armadillos, and opossums (Armstrong et al. 2017; Mika et al. 2022). These datasets were generated using conventional non-directional RNA-seq (also known as non-stranded RNA-seq), which does not retain information regarding the genomic element orientation of the sequenced transcripts. In addition, library preparation methods and the amount of data varied significantly among the datasets, ranging from millions to tens of millions (**Supplemental file 2**). Although transcriptional analysis of these datasets identified transcripts such as *GPR1-AS* in human and baboon placentas, the structures of these predicted transcripts were so fragmented that their full-length forms could not be robustly determined (**Figure 1 – supplement figure 1B-C**). Furthermore, we did not observe such a transcript at the *Gpr1-Zdbf2* locus in the mouse placental data, where *Liz* is known to be expressed. This suggests that conventional RNA-seq datasets may not be sufficient to accurately detect *GPR1-AS* in different species.

Next, we downloaded directional RNA-seq data (also known as strand-specific or stranded RNA-seq, which can identify the direction of transcription) from the human placenta, rhesus macaque trophoblast stem cells, and mouse embryonic placenta (Nesculea et al., 2014; Rosenkrantz et al. 2021). In each of these deeply sequenced datasets, which include a minimum of 50 million reads, transcripts originating from the *GPR1* intron were detected in the opposite direction to *GPR1*, extending towards the *ZDBF2* gene (i.e., *GPR1-AS* in humans and rhesus macaques, and *Liz* in mice) (**Figure 1**). Thus, *GPR1-AS* transcripts can be identified in placental tissues using deep directional RNA-seq data.

To identify *GPR1-AS*-like transcripts in other mammalian species, we generated deep directional RNA-seq datasets from the whole placenta of chimpanzees and the trophectoderm (TE) of rabbit, bovine, pig, and opossum post-gastrulation embryos, which yielded a total of 50–100 million reads (**Supplemental file 2**). Among these datasets, *GPR1-AS* orthologs were identified in chimpanzees and rabbits but were not observed in cows, pigs, or opossums (**Figure 2**). Additionally, we performed directional RNA-seq of tissues from the embryonic proper (embryonic disc: ED) of

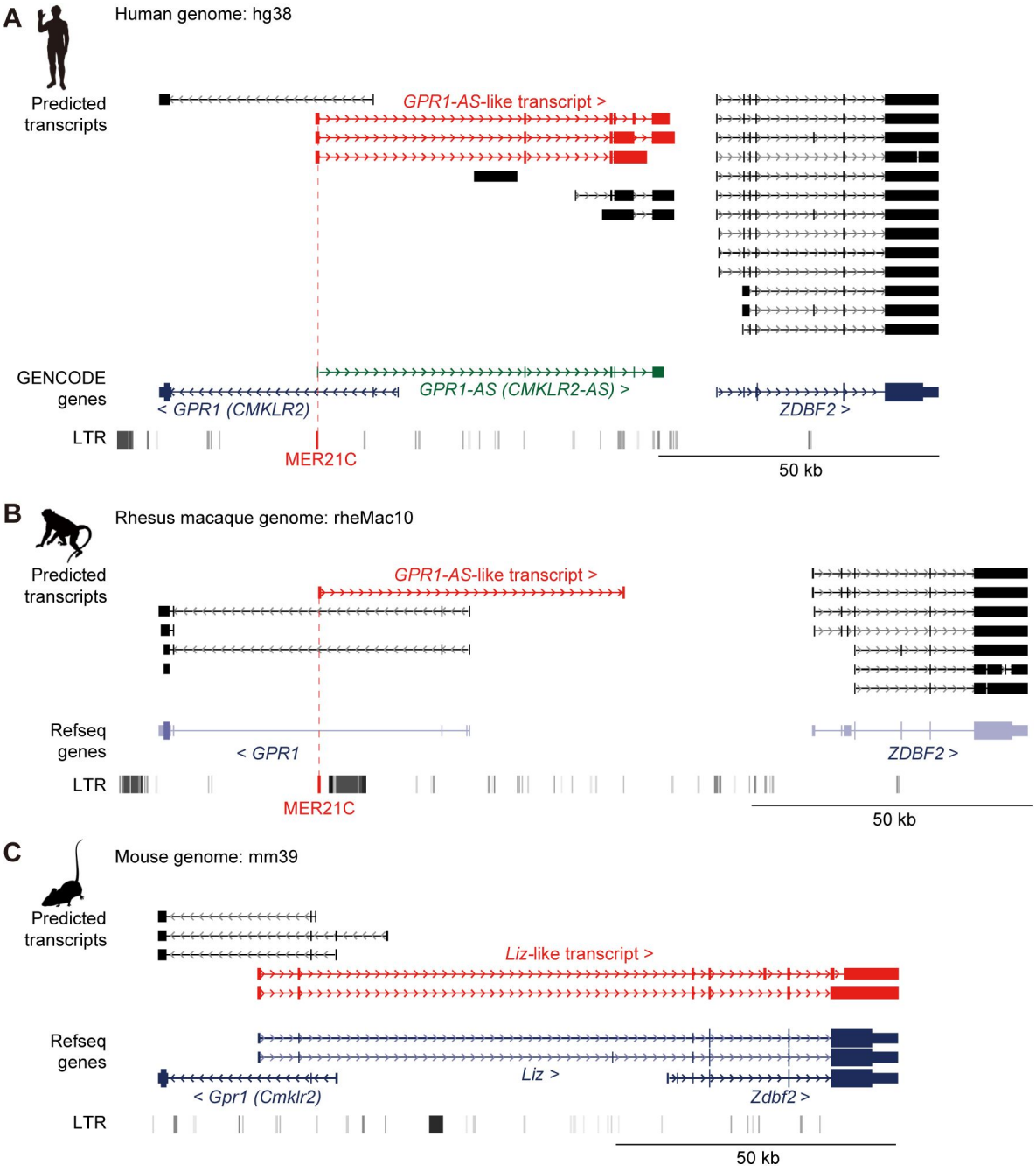


Figure 1.

Identification of *GPR1-AS* orthologs from public placental transcriptomes

UCSC Genome Browser screenshots of the *GPR1-ZDBF2* locus in humans (**A**), rhesus macaques (**B**) and mice (**C**). Predicted transcripts were generated using public directional placental RNA-seq datasets (accession numbers: SRR12363247 for humans, SRR1236168 for rhesus macaques, and SRR943345 for mice) using the Hisat2-StringTie2 programs. Genes annotated from GENCODE or RefSeq databases and LTR retrotransposon positions from UCSC Genome Browser RepeatMasker tracks are also displayed. Among the gene lists, only the human reference genome includes an annotation for *GPR1-AS* (highlighted in green). *GPR1-AS*-like transcripts and MER21C retrotransposons are highlighted in red. Animal silhouettes were obtained from *PhyloPic* (mouse silhouette by *Katy Lawler*, available under a *CC BY 4.0* license).

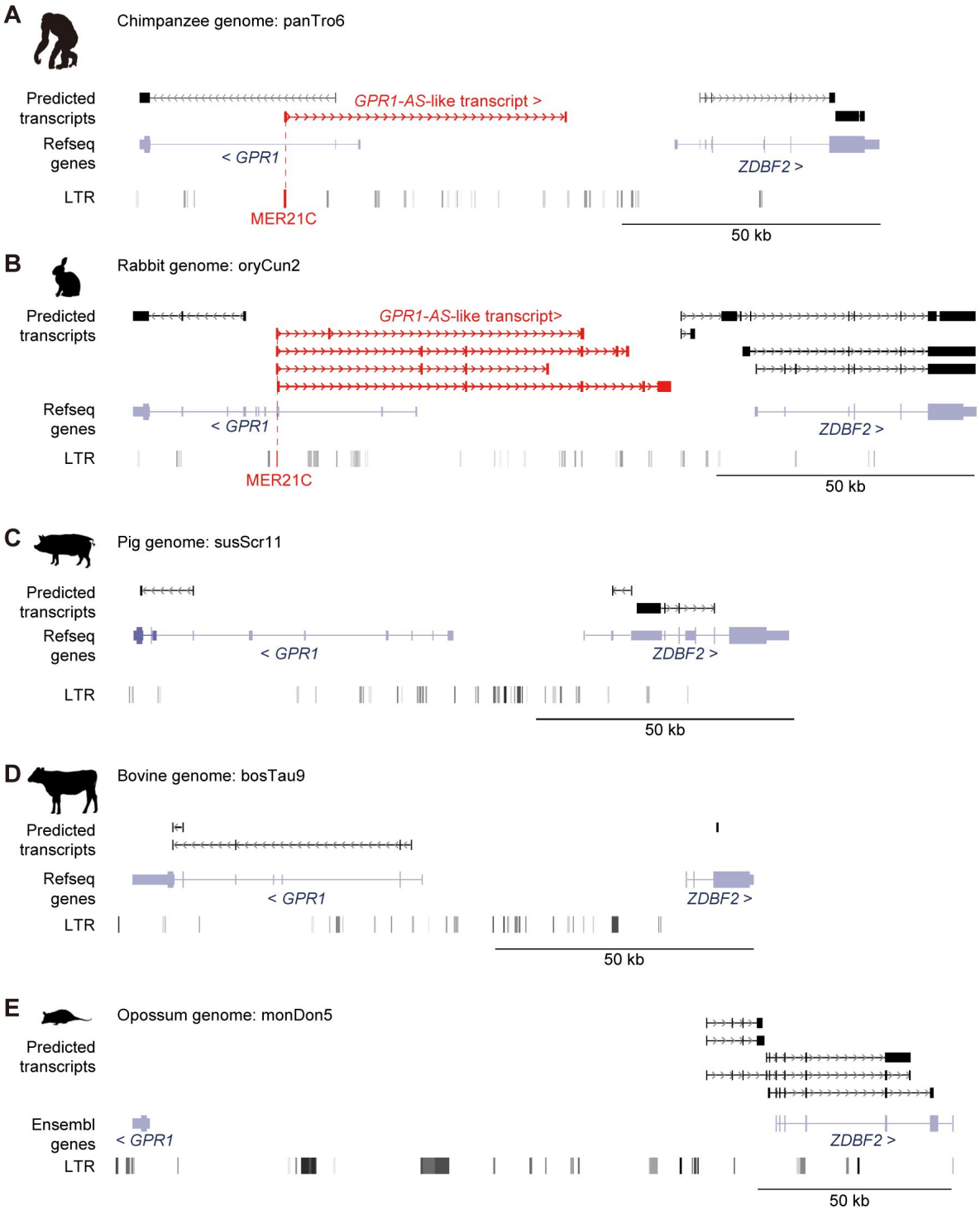


Figure 2.

Identification of *GPR1-AS* orthologs from original placental and extra-embryonic transcriptomes

Predicted transcripts were generated from placental and extra-embryonic directional RNA-seq datasets of chimpanzee (**A**), rabbit (**B**), pig (**C**), cow (**D**), and opossum (**E**) with the Hisat2-StringTie2 programs. Genes annotated from RefSeq or Ensembl databases and their LTR positions are also shown. *GPR1-AS*-like transcripts and MER21C retrotransposons are highlighted in red. Animal silhouettes were obtained from *PhyloPic* (opossum silhouette by *Sarah Werning*, available under a *CC BY 3.0* license).

multiple individual animal embryos but did not identify any *GPR1-AS*-like transcripts in any of these samples (**Figure 2– figure supplement 1**). Thus, rabbit *GPR1-AS* is expressed only in placental lineages. Identification of *GPR1-AS* in several primate species as well as mice and rabbits but not in cows, pigs, or opossums indicates that the regulatory region driving *GPR1-AS* transcripts likely originated in the common ancestor of the Euarchontoglires group.

Origin of *ZDBF2* imprinting and *GPR1-AS* transcript

Since *GPR1-AS*/*Liz* is essential for *ZDBF2* imprinting in mice, it is assumed that *ZDBF2* imprinting system via *GPR1-AS* transcription is not established in other eutherians and marsupials that lack *GPR1-AS* (Greenberg et al. 2017). To test this hypothesis, we performed allele-specific expression analysis of the *ZDBF2* gene in embryonic, fetal and adult tissues of tammar wallabies and cattle, which are outside the Euarchontoglires group. We identified available SNPs at the 3'-UTR of *ZDBF2* in each of these mammals, and Sanger-sequencing-based polymorphism analysis showed the expression of both *ZDBF2* alleles in all bovine and wallaby tissues analyzed (**Figure 3A-B**) (**Supplemental file 1**). Similar analyses were performed using blood samples from rhesus macaques and rabbits, in which *GPR1-AS* was identified. Consistent with our findings in humans and mice, we observed paternal allele-specific expression of *ZDBF2* in these species (**Figure 3C-D**), in clear contrast to the bi-allelic expression observed in the embryonic, fetal, and adult tissues of tammar wallabies and cattle.

To investigate a potential initiating mechanism in the germline for *ZDBF2* imprinting, which is established prior to the secondary DMR, we examined the presence of a germline DMR in both species with and without *ZDBF2* imprinting and *GPR1-AS* expression. Analysis of published whole-genome bisulfite sequencing data from rhesus macaque oocytes and sperm revealed DMRs between both germ cells, including an oocyte-methylated germline DMR at the first exon of *GPR1-AS* (Figure 3 – figure supplement 1A) (Gao et al. 2017). This finding aligns with previous observations in humans and mice (Kobayashi et al. 2012b; Kobayashi et al. 2013; Duffie et al. 2014). In contrast, analysis of published whole-genome bisulfite sequencing data for porcine and bovine oocytes and sperm (Ivanova et al. 2020) showed no oocyte-methylated germline DMRs in the *GPR1* intragenic region, where *GPR1-AS* is transcribed from an intron of *GPR1* in humans and rhesus macaques (Figure 3 – figure supplement 3B). Taken together, these results support the hypothesis that *ZDBF2* imprinting is restricted to mammals within the Euarchontoglires group.

Notably, the first exon of all *GPR1-AS* orthologs, except for mouse *Liz*, overlapped with an annotated MER21C element, a member of the LTR retrotransposon subfamily (**Figure 1**, **2**, and **Figure 1 – figure supplement 1C**). While MER21C retrotransposons are broadly present in Boroeutherians, among the mammals tested, only primate and rabbit genomes have MER21C in the *GPR1* intron, where the first exon of *GPR1-AS* is located. Therefore, we compared the LTR sequence location information of the syntenic region of the *GPR1* and *GPR1-AS* loci to profile MER21C insertion sites in several mammalian genomes, excluding marsupials. Among eutherians, we found the insertion of MER21C (or MER21B retrotransposons that showed high similarity to MER21C) in the introns of *GPR1* in five groups of Euarchontoglires, including primates, colugos, treeshrews, rabbits, and rodents, with the exception of mouse, rat, and hamster (**Figure 4**). As the default parameters of RepeatMasker may not detect degenerate LTR sequences, we reanalyzed these three rodent species using less stringent RepeatMasker parameters but again failed to reveal the presence of an MER21C insertion in the relevant regions (**Figure 4 – figure supplement 1**). However, multiple genome alignments from UCSC/Cactus track indicated that the MER21C-derived sequence on the human *GPR1- AS* is likely conserved in mammals belong to Euarchontoglires, including mouse, rat, and hamster (**Figure 4 – figure supplement 2**) (Armstrong et al. 2020; Zoonomia 2020), albeit with a high level of degeneracy in the latter.

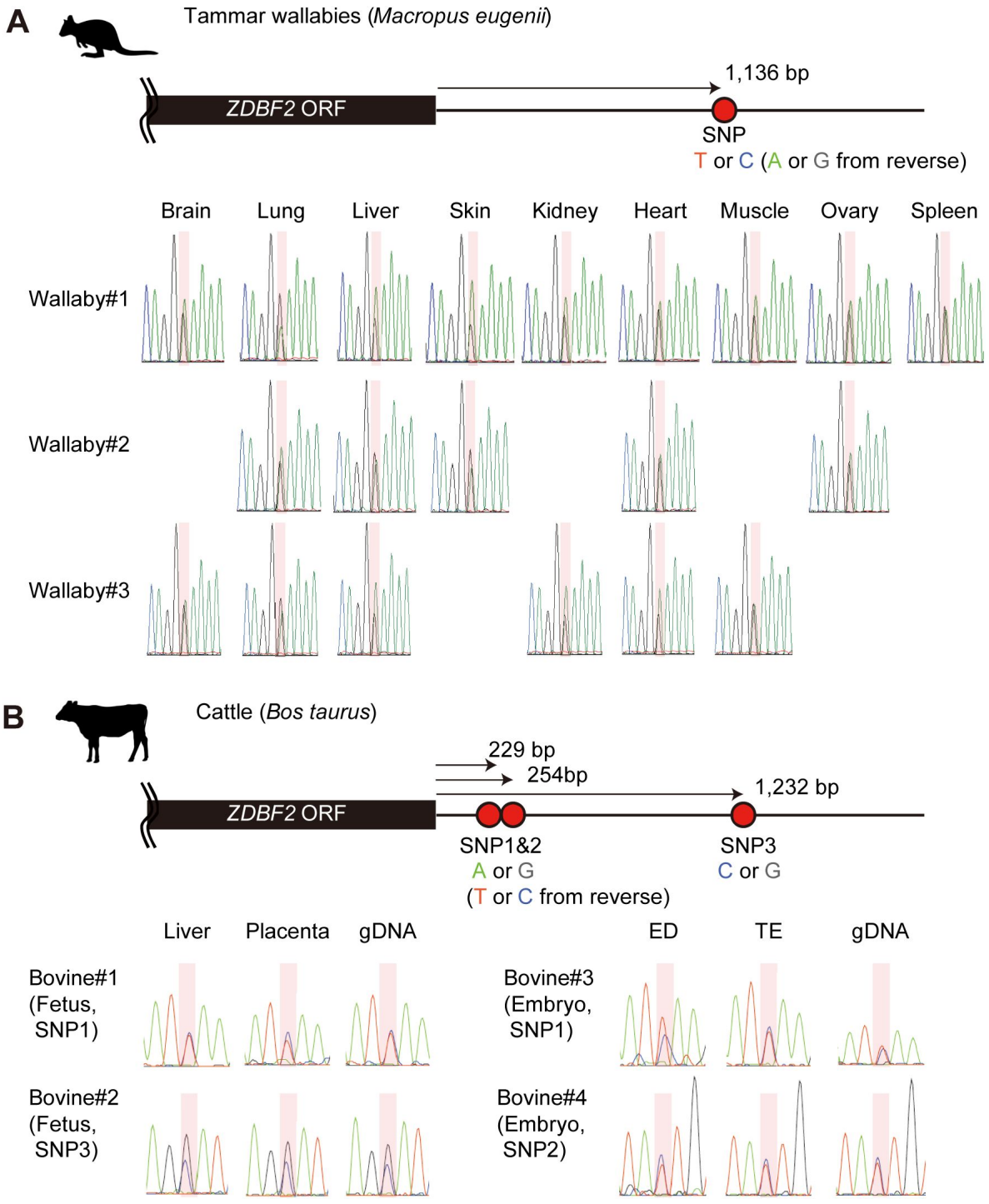


Figure 3.

Allele-specific RT-PCR sequencing of *ZDBF2* in various mammals

Heterozygous genotypes were used to distinguish between parental alleles in adult tissues from tammar wallabies (**A**), fetal/embryonic tissues from cattle (**B**), blood samples from rhesus macaques (**C**) and rabbits (**D**), respectively. Primers were designed to amplify the 3'-UTR regions of *ZDBF2* orthologs and detect SNPs. Each SNP position is highlighted in red. Reverse primers were also used for Sanger sequencing. Animal silhouettes were obtained from *PhyloPic*.

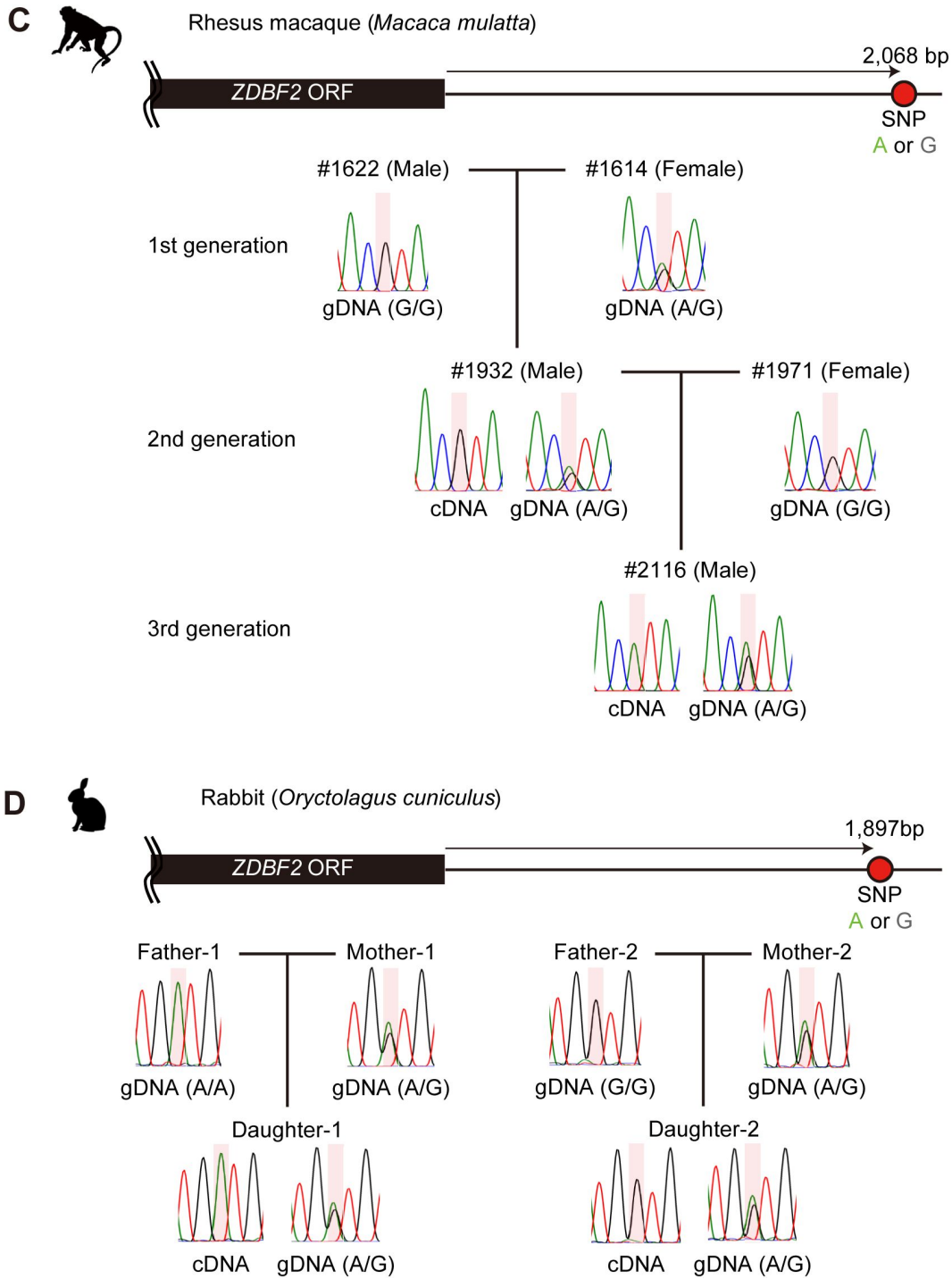


Figure 3. (continued)

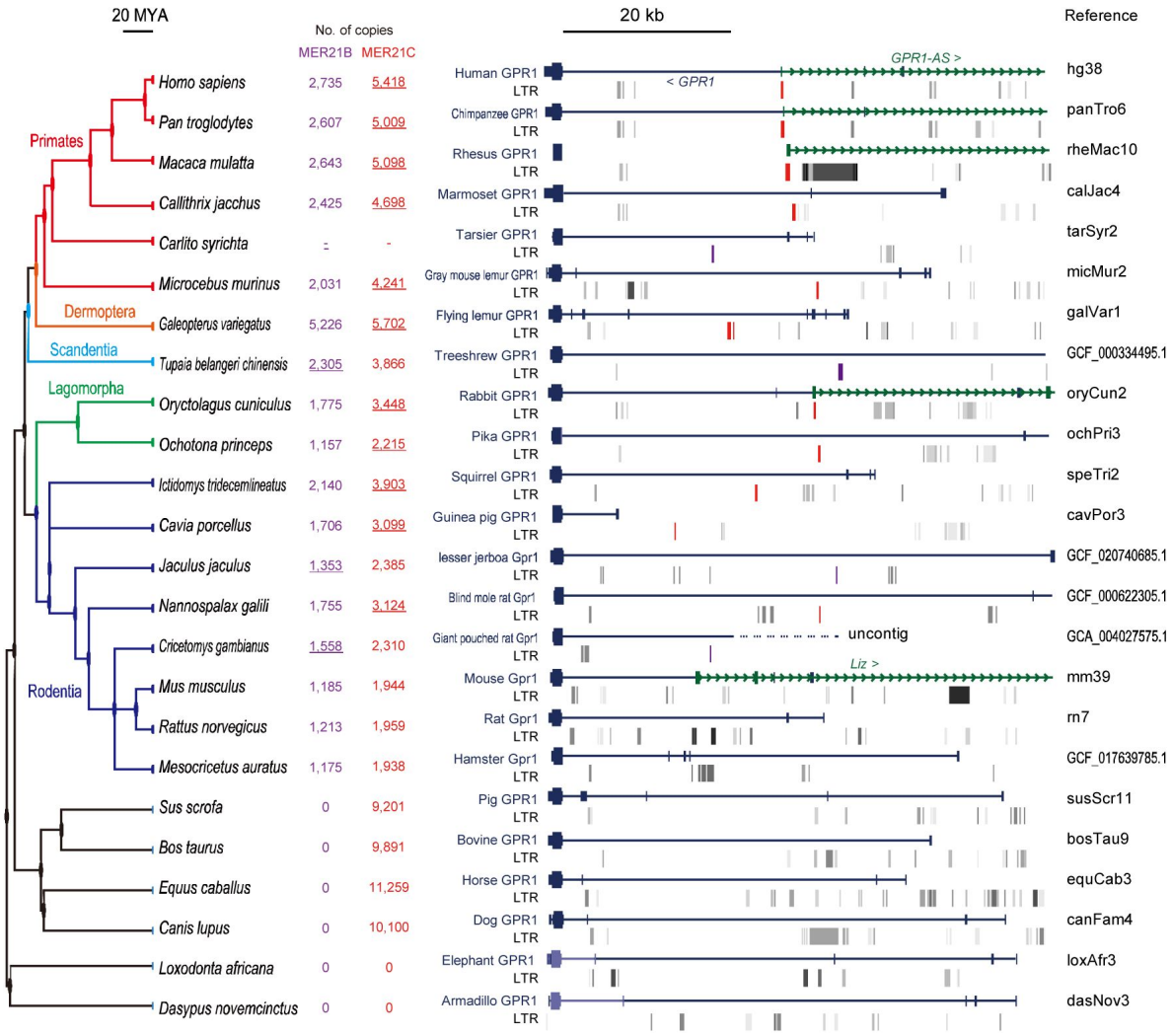


Figure 4

Multi-species comparison of LTR retrotransposon locations at *GPR1* locus

A total of 24 mammalian genomes were compared, including six primates (human, chimpanzee, rhesus macaque, marmoset, tarsier, and gray mouse lemur), one colugo (flying lemur), one treeshrew (Chinese treeshrew), two lagomorphs (rabbit and pika), eight rodents (squirrel, guinea pig, lesser jerboa, blind mole rat, giant pouched rat, mouse, rat, and golden hamster), and six other eutherians (pig, cow, horse, dog, elephant, and armadillo). Among the selected genomes, LTRs that can be considered homologous to MER21C, which corresponds to the first exon of *GPR1-AS*, are marked in red. In tarsier, treeshrew, lesser jerboa, and giant pouched rat, the orthologous LTRs were annotated as MER21B, which exhibits 88% similarity with MER21C in their consensus sequences through pairwise alignment. MER21B are marked in purple. According to Dfam, the MER21C and MER21B subfamilies are specific to the genomes of Boroeutherians and Euarchontoglires, respectively. The copy number of MER21C/B in selected species is shown in red and purple (LTRs likely matching the *GPR1-AS* exon are underlined). There are 5,418 and 2,529 copies of MER21C and 2,894 and 1,535 copies of MER21B in human and mouse genomes, respectively.

To analyze this syntenic region in greater detail, we compared MER21C sequences that overlapped with the first exon of *GPR1-AS* in each primate (345 bp, 339 bp, and 506 bp from humans, chimpanzees, and rhesus macaques, respectively) as well as rabbits (218 bp), with the first exon of *Liz* in mice (317 bp), and the common sequence of MER21C (938 bp). Pairwise alignment revealed high identities between primate sequences (identity: 71–72%) and the consensus MER21C sequence compared to rabbit (60%) and mouse sequences (46%) (**Figure 5 – figure supplement 1A**). The sequence identity in mice is so low that it cannot be distinguished from other LTRs or retrotransposons, which explains why it was not classified as MER21C by RepeatMasker analysis (**Figure 5 – figure supplement 1B-C**). Multiple sequence alignments and phylogenetic tree reconstruction reveal that the rabbit and mouse sequences deviate to a greater extent from the consensus MER21C sequence than the cluster formed by the primate sequences, indicating that a greater number of mutations have accumulated in the non- primate lineage of Euarchontoglires since their divergence from primates (**Figure 5A**). This observation aligns with the shorter generation time of lagomorphs and rodents compared to primates (Wu and Li 1985; Huttley et al. 2007).

To evaluate whether the putative degenerate MER21C-derived regulatory region driving mouse *Liz* expression confers transcriptional activity in the mouse comparable to that of the MER21C element that drives human *GPR1-AS expression*, we performed a dual reporter assay in the human cell line HEK293T. Strikingly, the first exon of mouse *Liz* exhibits promoter activity greater than that of the human *GPR1-AS* promoter, despite the relatively low sequence similarity between the *Liz* first exon and the consensus MER21C sequence (**Figure 5 – figure supplement 2**). Taken together, these findings suggest that despite substantial sequence divergence, the functional elements responsible for initiating transcription of Liz in the mouse are derived from the same MER21C relic annotated in the human locus and can drive robust expression in human cells.

If this is indeed the case, then specific transcription factor binding motifs are likely shared between species in this regulatory region. To identify common cis-motifs, we compared these sequences with known transcription factor-binding motifs using the TOMTOM program in the MEME suite. Through this analysis, we found a common region that contained an ETS family transcription factor (ELF1 and ELF2) binding site, which had significant matches with E-values < 0.01 and q-values < 0.01 (**Figure 5B,C**). Additionally, we identified the TFAP2 and ZSCAN4 binding motifs (with p-values = 1.58×10^{-5} and 7.24×10^{-7}) from the JASPAR database at the first exon of human *GPR1-AS* and mouse *Liz*, respectively (**Figure 5B,D**). Since *TFAP2C* and *ZSCAN4C* are activated in the placental lineage and preimplantation embryos, respectively (Zhang et al. 2019; Papuchova and Latos 2022) (**Figure 5 – figure supplement 3**), binding of these transcription factors may play a role in the transcriptional activation of *GPR1-AS* or *Liz* during embryogenesis, and in turn imprinting of *ZDBF2* .

GPR1-AS transcription and LTR reactivation in human

GPR1-AS is reported to be expressed only briefly during embryogenesis, both before and after implantation, with the exception of the placental lineage. For instance, human *GPR1-AS* has been identified in ES cells, and mouse *Liz* expression has been observed in blastocyst-to-gastrulation embryos but not in gametes (Kobayashi et al. 2012b; Kobayashi et al. 2013; Duffie et al. 2014). Therefore, it is likely that the expression of *GPR1-AS*/*Liz* is activated during preimplantation. To investigate the timing of *GPR1-AS* activation during embryonic development, we obtained public human RNA-seq data from the oocyte to blastocyst stages and performed expression analysis and transcript prediction (Kai et al. 2022; Zou et al. 2022b). Among the datasets from oocytes; zygotes; 2-, 4-, and 8-cell embryos; inner cell mass (ICM); and TE from blastocysts, *GPR1-AS* was only detected at the 8-cell stage, ICM, and TE; *GPR-AS-like* transcripts were detected as predicted transcripts, with TPM values above 1 (**Figure 6**). This indicates that *GPR1-AS* expression begins at the 8-cell stage in humans. However, at the *GPR1- ZDBF2* locus, both *GPR1* and *ZDBF2* were expressed in oocytes, but only *GPR1* was downregulated following fertilization and became undetectable beyond the 8-cell stage (**Figure 6 and Figure 5 – figure**

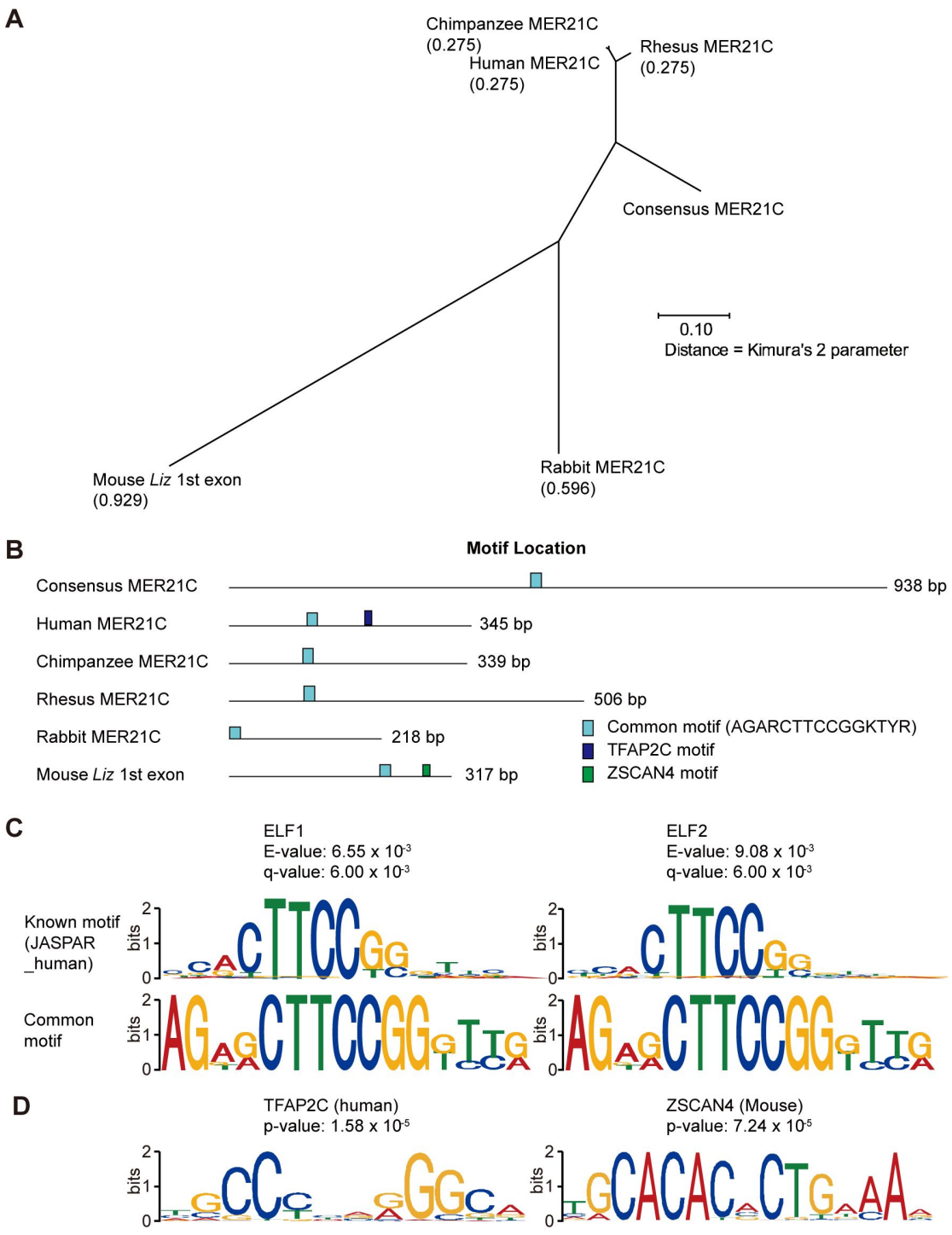


Figure 5.

Comparison of MER21C-derived sequences overlapping the first exon of *GPR1- AS* orthologs

(**A**) Phylogenetic tree of MER21C-derived sequences estimated by multiple sequence alignment (MSA) using multiple sequence comparison by log-expectation (MUSCLE) program. (**B**) Positions of common and unique cis-acting elements at each sequence. (**C**) Motif structures of the common region that contains E74-like factor 1 and 2 (ELF1 and ELF2) binding motifs. (**D**) Motif structures of transcription factor AP-2 gamma (TFAP2C) and Zinc finger and SCAN domain containing 4 (ZSCAN4).

supplement 3). We also examined MER21C expression at each stage and found that it increased from the from 4-cell to 8-cell stage and peaked at the TE, coinciding with the appearance of *GPR1-AS* (**Figure 6 – figure supplement 1**). Moreover, focusing on Kruppel-associated box zinc-finger proteins (KRAB-ZFPs) involved in the suppression of transposable elements, we observed that *ZNF789*—a key KRAB-ZFP that binds MER21C—was specifically downregulated at the 4-cell stage (**Figure 5 – figure supplement 3**)(Imbeault et al. 2017). Taken together, these results suggest that *GPR1-AS* expression begins in the preimplantation embryo, concurrently with a transcription factor/repressor milieu permissive for expression from its MER21C-derived promoter.

To determine whether specific epigenetic marks are associated with the LTR-derived promoter located in the first exon of human *GPR1-AS* and mouse *Liz*, we surveyed the ChIP-Atlas database (Zou et al. 2022a) (**Figure 7 – figure supplement 1**). Both sequences that constitute the oocyte-derived germline DMR exhibit an enrichment of H3K4me3 in sperm or male germ cells, consistent with DNA hypomethylation in sperm. These sequences also show an enrichment of H3K27ac in pluripotent stem cells (such as ES and iPS cells). Although as discussed above, the first exon of mouse *Liz* has not been computationally determined as an LTR (according to the RepBase database), the mouse sequence is also enriched for H3K9me3, a repressive mark which is deposited at retrotransposons, in multiple cell types and tissues. Additionally, human and mouse germline DMRs coincided with the TFAP2C peak (trophoblast stem cells) and ZSCAN4C peak (ES cells), respectively, and aligned with the binding motif predictions found in the JASPAR database (**Figure 5B,D**). These data indicate that the first exons of human *GPR1-AS* and mouse *Liz* exhibit similar epigenomic modification variations from gamete to embryonic development, and their promoter activities are triggered after fertilization. However, the specific regulatory pathways governing this activation may differ between species, likely reflecting differences in transcription factor involvement and regulatory networks (**Figure 7**).

Discussion

The *ZDBF2* gene, the last canonical imprinting gene identified in mice and humans, was recently shown to regulate neonatal feeding behavior (Glaser et al. 2022). This discovery has garnered attention owing to its implications for behavioral phenotypes, aligning with the conflicting hypothesis of genomic imprinting mechanisms. The conflict hypothesis proposes that the evolutionary significance of genomic imprinting mechanisms lies in the functional conflicts between paternally and maternally expressed genes, resulting from the conflicting genomic survival strategies of the father, child, and mother (Moore and Haig 1991). *ZDBF2* does not directly influence cell differentiation or ontogeny, such as embryogenesis, but rather affects individual growth through an indirect pathway involving feeding behavior. In humans, “imprinting diseases”, such as Prader-Willi syndrome and Angelman syndrome, cause psychiatric disorders and growth retardation (Nicholls 2000). Considering that many imprinted genes show specialized expression patterns in the brain, *ZDBF2* is particularly interesting as a factor that can induce behavioral effects. However, the evolutionary origin of *ZDBF2* imprinting had remained unclear. In this study, we report that *ZDBF2* does not exhibit imprinted expression in cattle and tammar wallabies, mammalian species that do not belong to the Euarchontoglires clade. In contrast, we identified *GPR1-AS* orthologs in all the Euarchontoglires species examined, including humans, chimpanzees, baboons, rhesus macaques and rabbits. We propose that the first exon of *GPR1-AS* is derived from the MER21C retrotransposon and that the insertion of this LTR at the *GPR1* intragenic region in the common ancestor of Euarchontoglires was the crucial event in the genesis of imprinting of the *ZDBF2* gene (**Figure 7**).

Two paternally expressed imprinted genes, *PEG10*/*SIRH1*, and *PEG11*/*RTL1*/*SIRH2*, that encode GAG-POL proteins of *sushi-ichi* LTR retrotransposons have been identified in mammals and are essential for placenta formation and maintenance (Kaneko-Ishino and Ishino 2012). The presence of these genes provides evidence that LTR insertions have played a significant role in

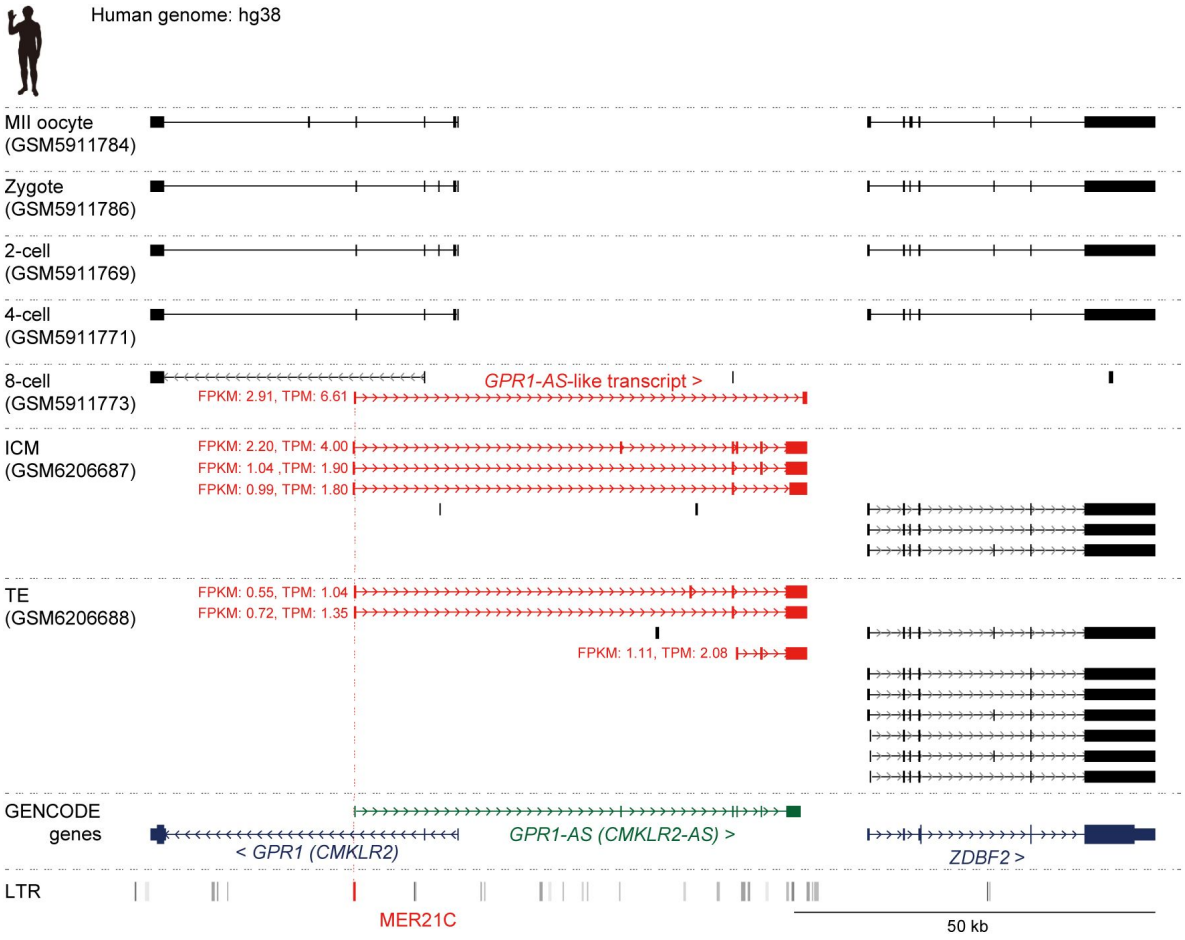


Figure 6.

Initiation of *GPR1-AS* transcription before implantation

Genome browser screenshots of the *GPR1-ZDBF2* locus in humans at preimplantation stages, including the MII oocyte, zygote, 2-cell, 4-cell, 8-cell, inner cell mass (ICM), and trophectoderm (TE) from the blastocyst. Predicted transcripts were generated from publicly available full-length RNA-seq datasets, with detected *GPR1-AS*-like transcripts and their FPKM and TPM values highlighted in red. Silhouette was obtained from *PhyloPic*.

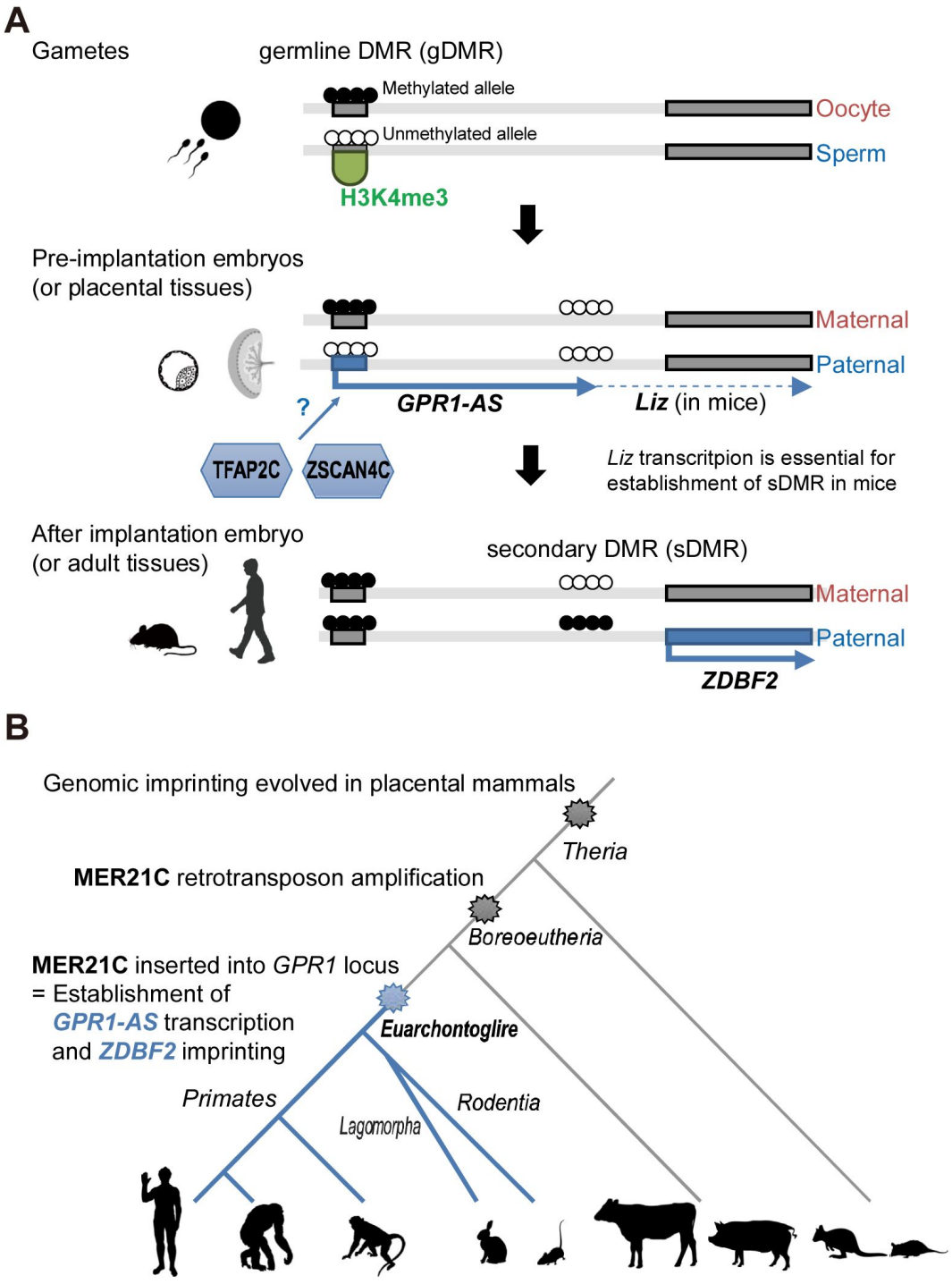


Figure 7.

Establishment of *ZDBF2* imprinted domain in evolution and genome biology

(A) Scheme of epigenetic and transcriptional changes at the first exon of mouse *Liz* and human *GPR1-AS*. (B) Timescale of the evolution of *ZDBF2* imprinting and LTR (MER21C) insertion. Animal silhouettes were obtained from *PhyloPic* (mouse silhouette by *Katy Lawler*, available under a *CC BY 4.0* license; opposum silhouette by *Sarah Werning*, available under a *CC BY 3.0* license).

genomic imprinting as well as placentation throughout evolution of placental mammals. In contrast, while the *ZDBF2* gene is present in all vertebrate genomes, it is only imprinted in mammals belonging to the Euarchontoglires superorder of placental mammals. This suggests that a nonimprinted gene acquired imprinting status through the insertion of an LTR retrotransposon. Indeed, we previously demonstrated that transcriptional activity of specific LTRs in oocytes results in species- specific imprinting of numerous genes in rodents and primates (Bogutz et al. 2019). These LTRs act as alternative promoters and produce unique transcripts in the oocyte, which we referred to as LITs (LTR-initiated transcripts). Such transcription leads to a high level of methylation in the transcribed region, similar to gene body methylation, and is responsible for extensive interspecies differences in DNA methylation (Brind'Amour et al. 2018). The key difference between these LTRs and the LTR retrotransposon focused on here is the stage in development when LTR (re)activation occurs, i.e. before or after fertilization, respectively. Some LITs, which are driven by LTR families that are particularly active in oocytes, become active during oogenesis and establish oocyte- derived gDMRs of species-specific imprinted genes, such as *Impact* and *Slc38a4* genes in mice. Strikingly, deletion of these LTRs causes loss of imprinting in mouse (rodent)- specific imprinted genes (Bogutz et al. 2019). *GPR1-AS* is a (likely noncoding) RNA whose transcription is initiated from an LTR-derived sequence, similar to the LITs found in oocytes; however, transcription in this case starts after fertilization. In mice, *Liz*, a long isoform of *Zdbf2* and putative *Gpr1-as* ortholog, is essential for establishing a somatic secondary DMR upstream of *Zdbf2* (Greenberg et al. 2017). Although the first exon of *Liz* does not overlap with an annotated LTR, MER21C (or MER21B, a closely related LTR family) insertions are found in the homologous region in most rodents, with the exception of mice, rats, and hamsters. Our analyses suggest that a MER21C relic is also present in these rodents, but has accumulated a number of mutations and in turn is no longer recognized as a MER21C element using optimal pairwise alignment algorithms such as RepeatMasker. Indeed, multiple genome alignment using Cactus reveals that the first exon of human *GPR1-AS* is homologous across all selected rodents, including mouse, rat, and hamster. Based on these observations, we propose that *Liz* and *GPR1- AS* are bona fide LITs with a MER21C-derived sequence serving as an alternative promoter. A combined approach involving the annotation of repetitive elements with tools like RepeatMasker and the reconstruction of ancestral genomes using multiple-genome alignments can uncover highly degenerated LTR relics. For instance, Didier Trono's group recently identified numerous previously unannotated transposable elements in the human genome using a similar methodology (Matsushima et al. 2024). Advances in such computational analyses could enable the identification of additional functional LTR relics and provide insights into the origin of lineage-specific regulatory sequences that are embedded within newly discovered lineage-specific LTR relics.

Transposable elements are recognized by DNA-binding factors and their co-repressors and are suppressed through epigenetic and post-transcriptional regulation in both germline and somatic tissues to protect host genome stability. For example, LTR- retrotransposons are bound by KRAB-ZFPs, which recruit the KAP1/SETDB1 co- repressor complex, promoting deposition of the repressive histone modification H3K9me3 (Matsui et al. 2010; Karimi et al. 2011). However, numerous retrotransposons are activated and robustly expressed during maternal to zygotic transition, where dynamic epigenetic reprogramming occurs. Indeed, a subset of transposable elements have been co-opted by the host during this window of development, including in support of embryonic development (Sakashita et al. 2023). In humans, retrotransposons are activated at the 8-cell stage and gradually downregulated in later developmental stages, coinciding with embryonic genome activation. In addition to the full-length endogenous retrovirus (ERV) sequences with partial coding potential, approximately 85% of human ERVs (HERVs) exist as solitary LTRs, known as "solo LTRs," which provide a rich source of cis-regulatory elements for gene expression during human embryonic development. Retrotransposons located near host genes can act as alternative promoters or regulatory modules, such as enhancers, to activate

embryonic genes (Grow et al. 2015; Hashimoto et al. 2021). The discovery of the *GPR1-AS* as a transcript that initiates in an ancestral LTR reveals a new aspect of the functional role of these ectopic regulatory elements, with activation in this case occurring after fertilization.

In summary, this study reveals that the origin of imprinting of *ZDBF2* can be traced back to a common ancestor of Euarchontoglires, in which an LTR element inserted in the locus. The solo LTR derived from this element (following recombination between 5' and 3' LTRs) drives expression of *GPR1-AS*, which in turn plays a critical role in establishing imprinting of the *ZDBF2* locus, including in both mice and humans. Although the house mouse is used widely in the laboratory, and the *Mus musculus* genome has been extensively characterized, the retroviral origin of this promoter would not have been identified if this analysis had been performed solely on mice, as the annotated exon 1 of *Liz* (alternatively named *Gpr1-as, Platr12*, or *Zdbf2linc*) is highly degenerate and in turn not recognized as an LTR in this species. Comparative analysis of multi-omics data, including genomes, transcriptomes, and epigenomes, across species was critical for the identification of the central role of a MER21C LTR in imprinting of *ZDBF2*. This study adds to a growing list of LTR elements that have been domesticated in mammals, with their transcriptional activity serving as a mechanism for the genesis of imprinting, including for a number of genes during gametogenesis and in the case of *ZDBF2,* following fertilization.

Methods

Ethical approval for animal work

Animal experiments (chimpanzee, rhesus macaque, rabbit, pig, cow, and opossum) were conducted in accordance with the guidelines of the Science Council of Japan and approved by the Institutional Animal Care and Use Committee of the University of Tokyo, the National Institute for Physiological Sciences, RIKEN Kobe Branch, Meiji University, Shinshu University, Hokkaido University, the Center for the Evolutionary Origins of Human Behavior of Kyoto University, and Nara Medical University. Experimental procedures of tammar wallaby conformed to Australian National Health and Medical Research Council (2013) guidelines and were approved by the Animal Experimentation Ethics Committees of the University of Melbourne.

Animals for transcriptome analysis

Placental samples from chimpanzees (*Pan troglodytes verus*) were provided by Kumamoto Zoo and Kumamoto Sanctuary via the Great Ape Information Network. The samples were stored at −80 °C before use. Total RNA was isolated from the placenta using an AllPrep DNA/RNA Mini Kit (Qiagen, Netherland). Bovine (*Bos taurus*, Holstein cow) embryos were prepared by in vitro oocyte maturation, fertilization (IVF), and subsequent in vitro embryo culture (Akizawa et al. 2018). Briefly, presumptive IVF zygotes were denuded by pipetting after 12 h of incubation and cultured up to the blastocyst stage in mSOFai medium at 38.5 °C in a humidified atmosphere of 5% CO_2 and 5% O_2 in air for 8 days (D8). D8 blastocysts were further cultured on agarose gel for 4 dayss as described previously (Akizawa et al. 2018; Saito et al. 2022). The embryo proper (embryonic disc: ED) and TE portions of IVF D12 embryos were mechanically divided using a microfeather blade (Feather Safety Razor, Japan) under a stereomicroscope. Total RNA from ED and TE samples was isolated using the ReliaPrepTM RNA Cell Miniprep System (Promega, MA, USA) according to the manufacturer's instructions. Rabbit embryos were prepared on embryonic day 6.75 (E6.75) by mating female Dutch and male JW rabbits (Kitayama Labes, Japan). Pig embryos (*Sus scrofa domesticus*: Landrace pig) at E15 and opossum embryos at E11 were prepared (Kiyonari et al. 2021). Individual embryo proper and TE were mechanically divided using a 27-30G needle and tweezer under a stereomicroscope with 10% FCS/M199 -medium in rabbits, HBSS medium in pigs, and 3% FCS/PBS in opossums. Total RNA was isolated from individual tissues using the RNeasy

Mini and Micro Kit (Qiagen). The quality of the total RNA samples was assessed using an Agilent 2100 Bioanalyzer system (Thermo Fisher Scientific, MA, USA) and high-quality RNA samples (RIN≧7) were selected for RNA-seq library construction.

Strand-specific RNA library preparation and sequencing

Embryonic total RNA (10 ng) from opossums and pigs, 5 ng of placental total RNA from chimpanzees, and 1 ng of embryonic total RNA from rabbits and cattle were reverse transcribed using the SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian (Takara Bio, Japan) according to the manufacturer's protocols, where Read 2 corresponds to the sense strand due to template-switching reactions. RNA-seq libraries were quantified by qPCR using the KAPA Library Quantification Kit (Nippon Genetics, Japan). All libraries were pooled and subjected to paired-end 75 bp sequencing (paired- end 76 nt reads with the first 1 nt of Read 1 and the last 1 nt of Read 2 trimmed) using the NextSeq500 system (Illumina, CA, USA). For each library, the reads were trimmed using Trimmomatic to remove two nucleotides from the 5' end of the Read 2.

RNA-seq data set download

Strand-specific RNA-seq datasets from the human bulk placenta, rhesus macaque trophoblast stem cells, and mouse bulk placenta at E16.5, were downloaded from the accession numbers SRR12363247 and SRR12363248 for humans, SRR1236168 and SRR1236169 for rhesus macaques, and SRR943345 for mice (Necsulea et al. 2014; Rosenkrantz et al. 2021). Non-directional RNA-seq data from placentas or extra- embryonic tissues of 15 mammalian species, including humans, bonobos, baboons, mice, golden hamsters, rabbits, pigs, cattle, sheep, horses, dogs, bast, elephants, armadillos, and opossums, were downloaded (Armstrong et al. 2017; Mika et al. 2022).

Full-length RNA-seq (based on the smart-seq method) data from human oocytes, zygotes, 2-, 4-, and 8-cell, ICM, and TE were downloaded from previous studies (Kai et al. 2022; Zou et al. 2022b). The corresponding accession numbers are shown in **Supplemental file 2**.

RNA-seq data processing

All FASTQ files were quality-filtered using Trimmomatic and mapped to individual genome references (**Supplemental file 2**) using Hisat2 with default parameters and Stringtie2 with default parameters and "-g 500" option. Fragments per kilobase million (FPKM) and transcripts per kilobase million (TPM) were calculated using StringTie2 with "-e" option and the GENCODE GTF file, selecting lines containing the "Ensembl_canonical" tag. Read counts of human transposable element were computed at the subfamily level using the TEcount. The read counts were normalized by the total number of mapped reads to retrotransposons in each sample as Reads per kilobase million (RPKM).

Data visualization

All GTF files (StringTie2 assembled transcripts) were uploaded to the UCSC Genome Browser, and the predicted transcripts, annotated genes (GENCODE, Ensembl, or RefSeq), and LTR locations were visualized. The expression profiles of human *GPR1*, *GPR1-AS*, and *ZDBF2* in the somatic tissues (Fagerberg et al. 2014; Duff et al. 2015), and these imprinted genes, the associated transcription factors (*TFAP2C*, *ZSCAN4*, *ELF1*, and *ELF2*), and LTR subfamilies in early embryo (Kai et al. 2022; Zou et al. 2022b) were downloaded or calculated, and visualized as heatmaps using the Morpheus software. The estimated evolutionary distance between the selected mammals was visualized as a physiological tree with a branch length of millions of years (MYA) using Timetree. The pairwise alignment, multiple sequence alignment, and physiological tree of MER21C sequences overlapping *GPR1-AS* were conducted with Genetyx software (Genetyx, Japan) using the MSA and MUSCLE programs. The common cis-motif was identified and compared with known transcription factor binding motifs from JASPAR CORE database using the XSTREAM and TOMTOM programs in the MEME suite. JASPAR motifs, logos, and *p*-values were obtained from the JASPAR

hub tracks of the UCSC Genome Browser and JASPAR database. LTR positions of human (hg19) and mouse (mm10) were downloaded from UCSC Genome Browser and re-uploaded to Integrative Genomics Viewer with epigenetic patterns from ChIP-Atlas (Zou et al. 2022a) and oocyte/sperm DNA methylomes (Brind'Amour et al. 2018).

Allele-specific expression analysis

Tammar wallabies (*Macropus eugenii*) of Kangaroo Island origin were maintained in our breeding colony in grassy, outdoor enclosures. Lucerne cubes, grass and water were provided ad libitum and supplemented with fresh vegetables. Pouch young were dissected to obtain a range of tissues. DNA/RNA was extracted from multiple tissues of the Tammar wallaby using TRIzol Reagent (Thermo Fisher Scientific), and cDNA synthesis was performed using Transcriptor First Strand cDNA Synthesis Kit (Roche, Switzerland). PCR was performed using TaKaRa Ex Taq Hot Start Version (TaKaRa Bio) with primers designed at 3'UTR of wallaby *ZDBF2* according to the manufacturer's instructions. Fetal tissues of pregnant healthy Holstein cattle were obtained from a local slaughterhouse. DNA/RNA was extracted from bovine fetus (liver), placentas, and *in vitro* cultured embryos using ISOGEN (Wako, Japan), and cDNA synthesis and PCR were performed using SuperScript IV One-Step RT-PCR System (Thermo Fisher Scientific) with primers designed at 3'UTR of bovine *ZDBF2* according to the manufacturer's instructions. Target regions were also amplified using genomic DNA and Takara EX Taq Hot Start Version (Takara Bio). Whole blood samples of rhesus macaque (*Macaca mulatta*) and rabbit (Japanese White, *Oryctolagus cuniculus*) were obtained from PRI of Kyoto University and purchased from Oriental Bio Service (Japan), respectively. DNA/RNA was extracted from blood samples using ISOSPIN Blood & Plasma DNA (Nippon Gene, Japan) and Nucleospin RNA Blood (Takara Bio) and cDNA synthesis and PCR were performed using SuperScript IV One-Step RT-PCR System (Thermo Fisher Scientific) with primers designed at 3'UTR of rhesus macaque and rabbit *ZDBF2* according to the manufacturer's instructions. Sanger sequencing was performed using a conventional Sanger sequencing service (Fasmac, Japan) and ABI 3130xl Genetic Analyzer (Thermo Fisher Scientific). Sanger sequencing results were visualized using 4Peaks. Primers used in this analysis are listed in **Supplemental file 3**.

DNA Methylome Analysis

The DSS R package (v2.54.0) was used to identify DMRs from oocyte and sperm CpG report files (accession numbers GSM1466810, GSM1466811, and GSE143849), with smoothing enabled and customized parameters. These parameters included a minimum CpG methylation difference of 50%, a p-value threshold of 0.05, a minimum DMR length of 200 bp, and other default settings. All BED files were uploaded to the UCSC Genome Browser and CpG sites with DNAme levels and called DMR positions were visualized.

Dual reporter assay

Luciferase reporter plasmids were constructed using the pGL4.13 vector (Promega). Sequences containing the first exon and upstream regions of both mouse *Liz* and human *GPR1-AS* were amplified by PCR using TaKaRa HS Perfect Mix (Takara Bio) and specific primers with NheI and HindIII recognition sites at their 5' ends (**Supplemental file 3**). The PCR products were inserted between the NheI and HindIII sites of the pGL4.13 vector using DNA Ligation Mix (Takara Bio). To evaluate the promoter activity of these sequences, the SpectraMax DuoLuc Reporter Assay Kit (Molecular Devices, CA, USA) was employed. Human embryonic kidney 293T (HEK293T) cells were cultured in Dulbecco's Modified Eagle's Medium (Thermo Fisher Scientific, Cat. No. 10313021) supplemented with 10% fetal bovine serum (NICHIREI Biosciences, Tokyo, Japan), 0.1 mg/mL penicillin-streptomycin (Thermo Fisher Scientific), and GlutaMax (Thermo Fisher Scientific, Cat. No. 35050061), on gelatin-coated dishes. For luciferase reporter assays, HEK293T cells were seeded in gelatin-coated 96-well plates at a density of 4×10^5 cells/well prior to transfection. Transient transfections were carried out using Lipofectamine 2000 Transfection Reagent (Thermo Fisher

Scientific) according to the manufacturer's instructions. Each well was co-transfected with 10 ng of the pGL4.75 plasmid (Promega) as an internal transfection control reporter and 100 ng of the pGL4.13 plasmid with or without the candidate regulatory elements. After 48 hours of transfection, dual-luciferase assays were performed as per the manufacturer's protocol (Molecular Devices). Luminescence signals were measured using a SpectraMax iD3 Multi-Mode Microplate Reader (Molecular Devices). Firefly luciferase activities (pGL4.13) were normalized to Renilla luciferase activities (PGL4.75).

Softwares used

Trimmomatic (*http://www.usadellab.org/cms/?page=trimmomatic*)

Hisat2 (*http://daehwankimlab.github.io/hisat2/*)

StringTie2 (*https://ccb.jhu.edu/software/stringtie/*)

TEcount (*https://github.com/bodegalab/tecount/*)

Integrative Genomics Viewer (IGV: *https://software.broadinstitute.org/software/igv/*)

4Peaks (*https://nucleobytes.com/4peaks/*)

DSS (*https://bioconductor.org/packages/release/bioc/html/DSS.html*)

Web-tools used

UCSC Genome Browser (*https://genome.ucsc.edu/*)

Morpheus (*https://software.broadinstitute.org/morpheus/*)

Timetree (*http://timetree.org/*)

MEME Suite (*https://meme-suite.org/meme/*)

JASPAR (*https://jaspar.genereg.net/*)

ChIP-Atlas (*https://chip-atlas.org/*)

PhyloPic (*https://www.phylopic.org/*)

RepeatMasker (*https://www.repeatmasker.org/*)

PipMaker and MultiPipMaker (*http://pipmaker.bx.psu.edu/pipmaker/*)

Data access

All raw sequencing data generated in this study were submitted as FASTQ files to the NCBI SRA (*https://www.ncbi.nlm.nih.gov/sra*). The accession numbers are shown in **Supplemental file 2**.

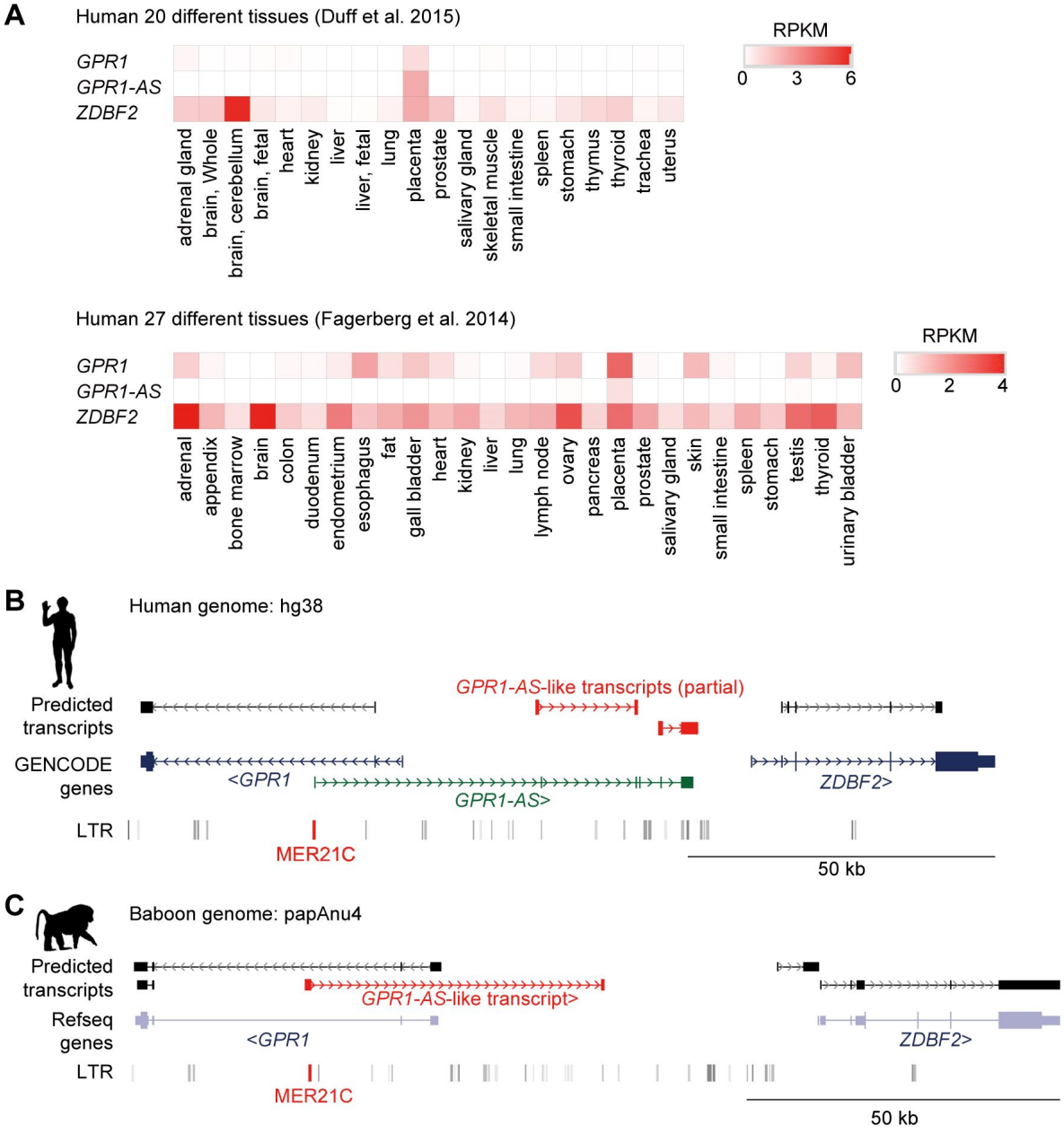


Figure 1 - figure supplement 1.

Identification of *GPR1-AS* orthologs using public and non-directional RNA-seq data

(A) Heat map showing the expression levels of *GPR1*, *GPR1-AS*, and *ZDBF2* in different human tissues, including the placenta. Genome browser screenshots of the GPR1- *ZDBF2* locus in humans (B) and baboons (C). Predicted transcripts were generated using public non-directional placental RNA-seq datasets (accession numbers: SRR1850957 for humans, GSM4696517 for baboons). Transcript/gene information and LTR retrotransposon positions are shown. *GPR1-AS*-like transcripts and MER21C retrotransposons are shown in red. Animal silhouettes were obtained from *PhyloPic*.

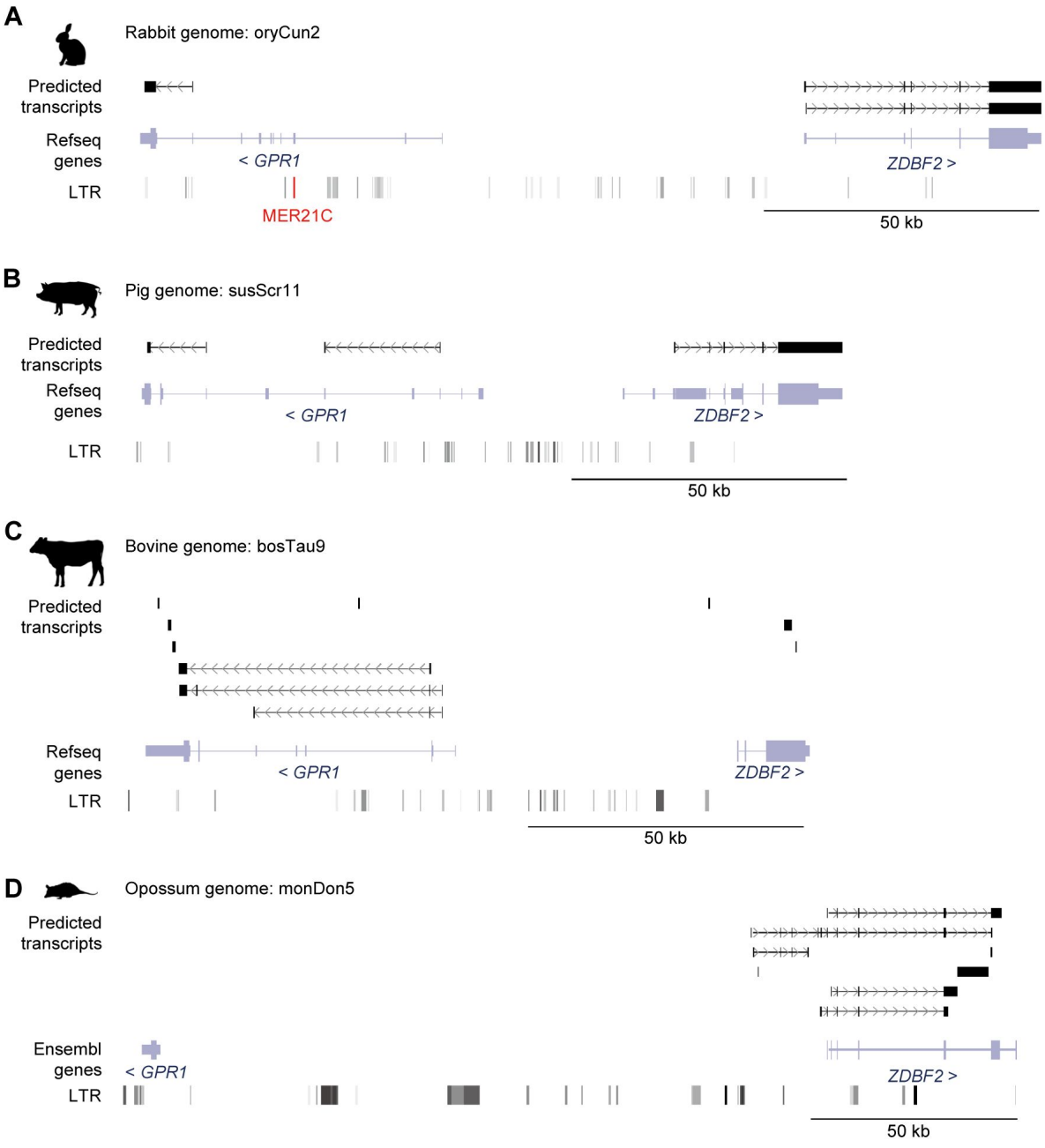


Figure 2 – figure supplement 1.

Search for *GPR1-AS* orthologs from embryonic transcriptomes

Predicted transcripts were generated using directional RNA-seq datasets of embryonic proper tissues from rabbit (A), pig (B), bovine (C), and opossum (D) embryos. Transcript/gene information and LTR retrotransposon positions are displayed and the annotated MER21C retrotransposon (only in rabbit) is highlighted in red. Animal silhouettes were obtained from *PhyloPic* (opossum silhouette by *Sarah Werning*, available under a *CC BY 3.0* license).

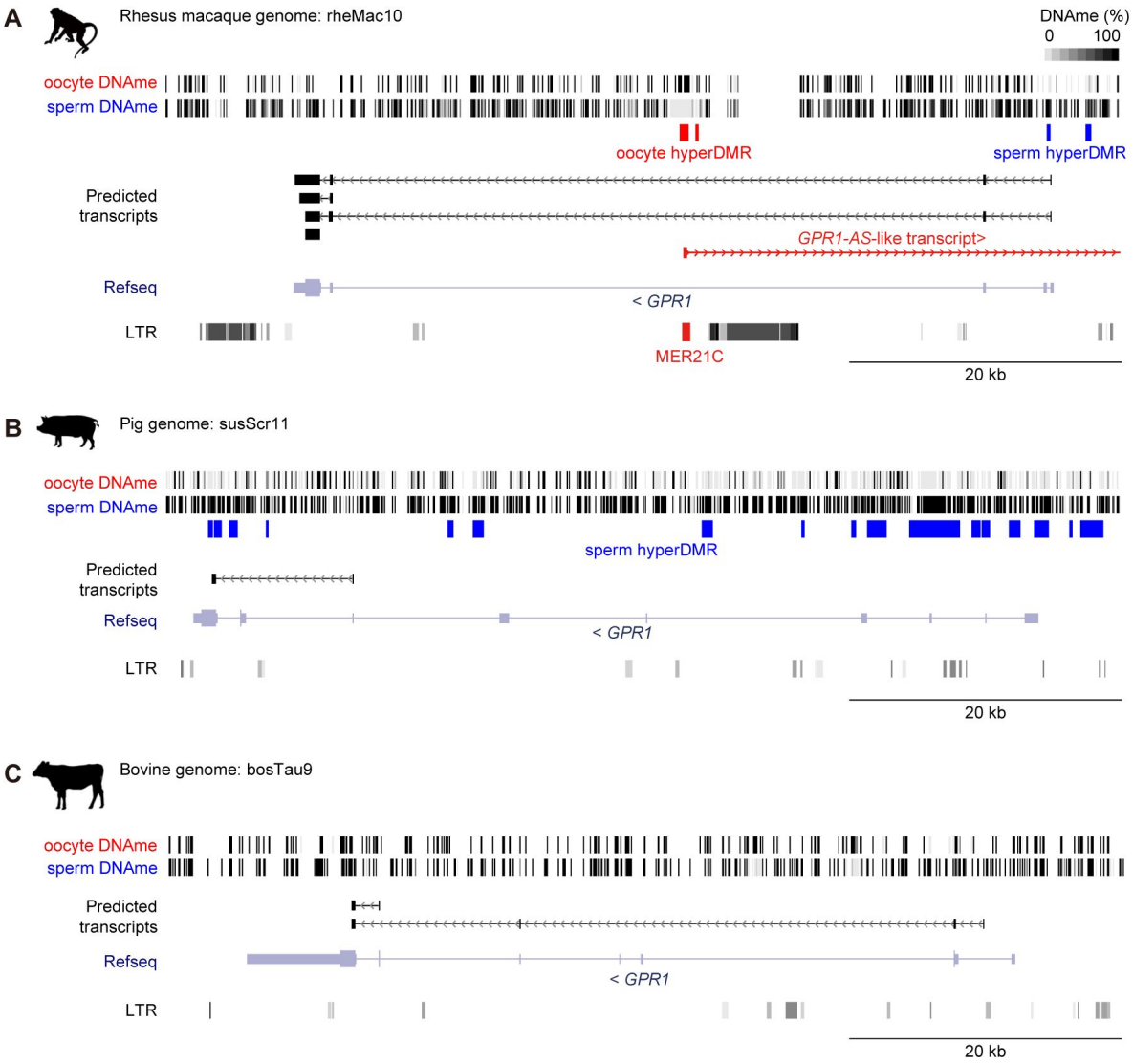


Figure 3 – figure supplement 1.

Search for germline DMRs from oocyte and sperm DNA methylomes

The DNA methylation (DNAme) levels of individual CpG sites in oocyte and sperm from rhesus macaque (**A**), pig (**B**), and bovine (**C**) whole genome bisulfite sequencing datasets are shown. Oocyte-methylated and sperm-methylated DMRs are highlighted in red and blue, respectively. Predicted transcripts from placental and extra-embryonic directional RNA-seq datasets (shown in **Figure 1**, **2**), genes annotated from RefSeq databases, and LTR positions from UCSC/RepeatMasker are included, with a MER21C retrotransposon overlapping rhesus macaque *GPR1-AS* highlighted in red. Animal silhouettes were obtained from *PhyloPic*.

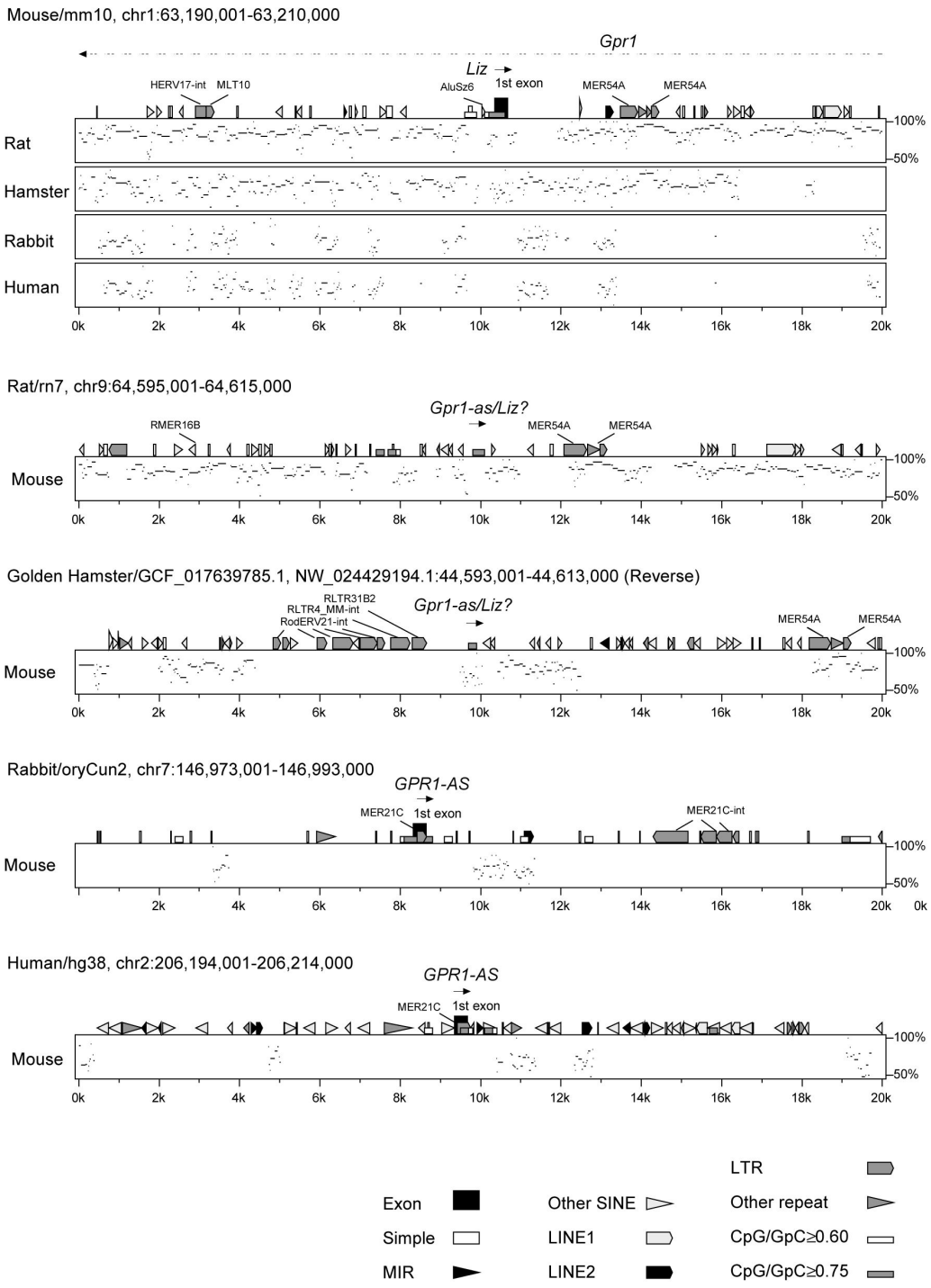


Figure 4 – figure supplement 1.

Reanalysis of repeat positions using RepeatMasker

Repetitive elements were re-identified in five mammalian species: mouse, rat, and hamster—where MER21C, which overlaps the first exon of human *GPR1-AS*, was not found in the homologous region—and rabbit and human, where it was detected. The Percent Identity Plot (PIP, showing a conservation scale between sequences from 50% to 100% on the y-axis) illustrates the order and alignment of the 20 kb region surrounding the *GPR1-AS* (*Liz*) transcription start site in each mammalian chromosome. Detected repeat elements are displayed above each plot. RepeatMasking was performed under less stringent settings, including switching search engines from RMblast to HMMER and adjusting speed/sensitivity settings from default to slow. Despite these adjustments, MER21C insertion was not detected in the three rodent species.

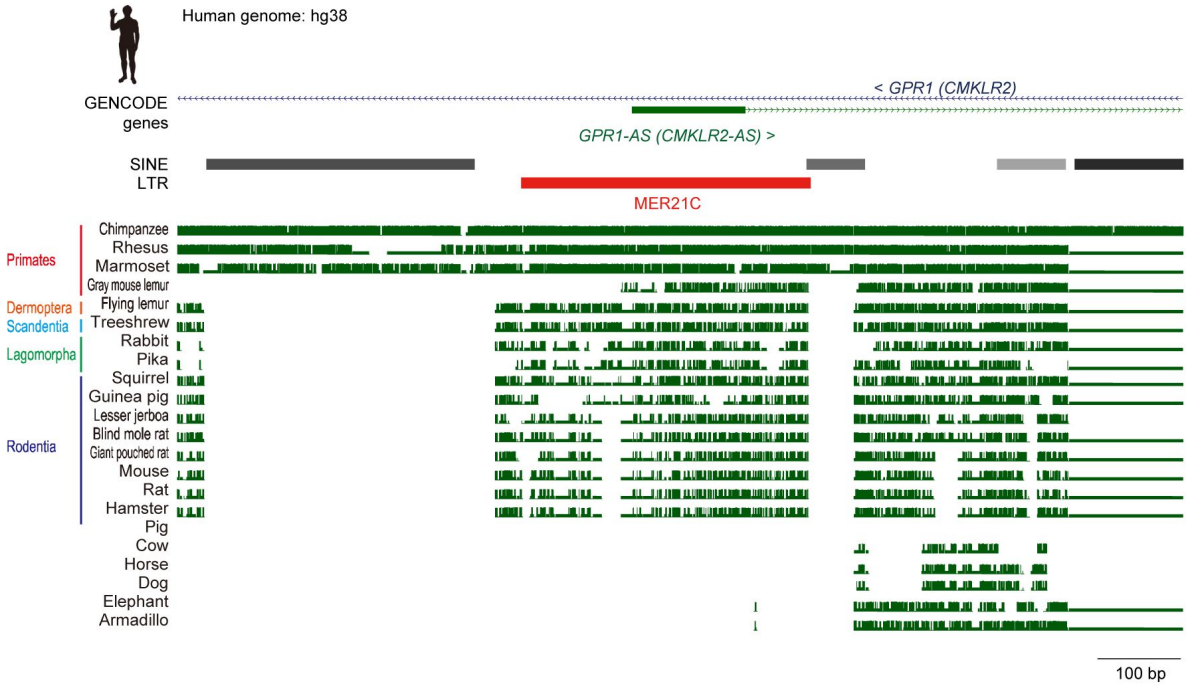


Figure 4 – figure supplement 2.

Multiple genome alignments at the first exon of *GPR1-AS* locus

Cactus generates reference-free, whole-genome multiple alignments (Armstrong et al. 2020). The Cactus track from UCSC Genome Browser displays multiple alignments across vertebrate species and evolutionary conservation metrics from the Zoonomia Project (Zoonomia 2020). Green square brackets indicate shorter alignments where DNA from one genomic context in the aligned species is nested within a larger alignment chain from a different genomic context. The alignment within these brackets may represent a short misalignment, a lineage-specific insertion of a retrotransposon in the human genome that aligns to a paralogous copy in another species. SINE and LTR retrotransposon positions from UCSC Genome Browser are also displayed. Silhouette obtained from *PhyloPic*.

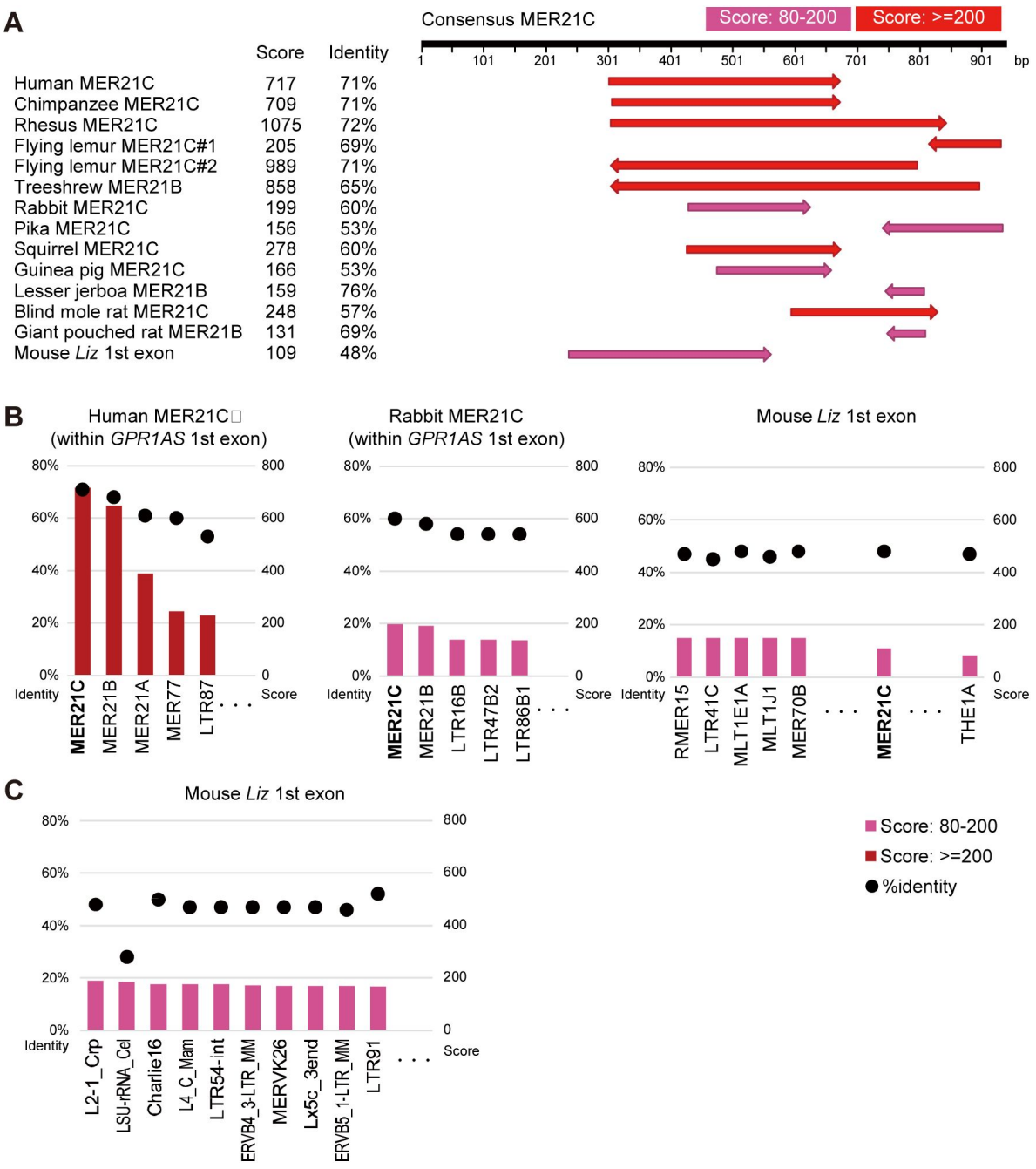


Figure 5 – figure supplement 1.

Pairwise alignment between consensus sequences of retrotransposons and *GPR1-AS*-exonic MER21 sequences

(A) MER21C (or MER21B) sequences located in the *GPR1* intron of eutherian genomes and the first exon of mouse *Liz* were compared with the consensus MER21C sequence.
(B) Human and rabbit MER21C sequences overlapping the first exon of *GPR1-AS* and the first exon of mouse *Liz* were compared with the consensus sequences of ERV3/ERVL solo-LTRs present in human and mouse (n = 182). Each graph displays the identity percentages and alignment scores for the top five LTRs with the highest scores. In humans and rabbits, MER21C showed the highest identity with the exonic sequences.
(C) The first exon of mouse *Liz* was compared with the consensus sequences of all retrotransposons present in mice (n = 1,361). The graph represents the top 10 retrotransposons with the highest scores. In mice, MER21C does not show sufficient sequence identity to the first exon of *Liz* to distinguish it from other retrotransposons. Pairwise alignment scores and percent identity values for each sequence pair were calculated using Genetyx software.

Figure 5 – figure supplement 2.

Promoter activities of first exons of mouse *Liz* and human *GPR1-AS*

(**A**) Constructs (inserted sequences) used for dual luciferase reporter assays in HEK293T cells. A promoter-less vector served as the negative control. (**B**) Results of dual luciferase reporter assays. Relative fold changes in Firefly luciferase activity (Firefly/Renilla) were normalized to the Firefly/Renilla ratio of the negative control. Error bars indicate mean ± s.e.m. Statistical significance was determined using unpaired t-tests: *P < 0.05, **P < 0.01. Data represent four biological replicates.

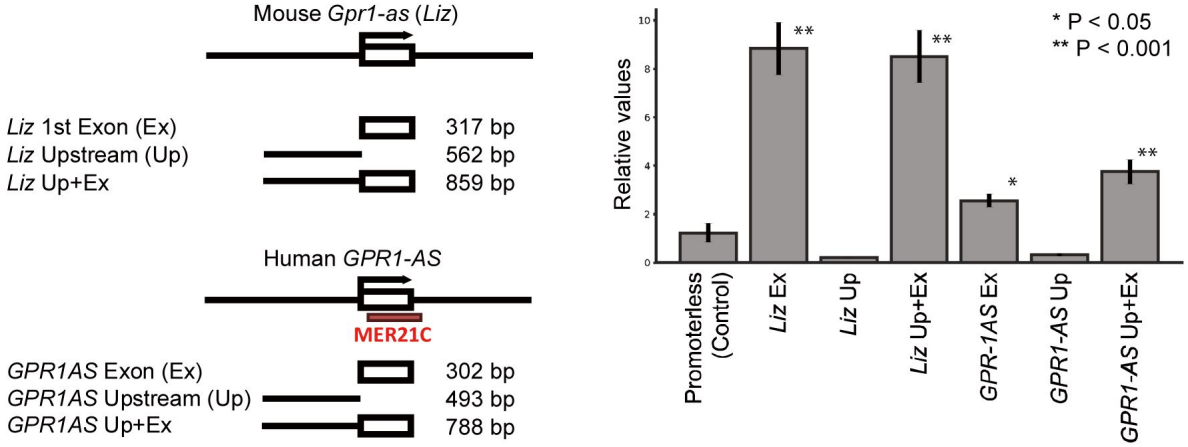
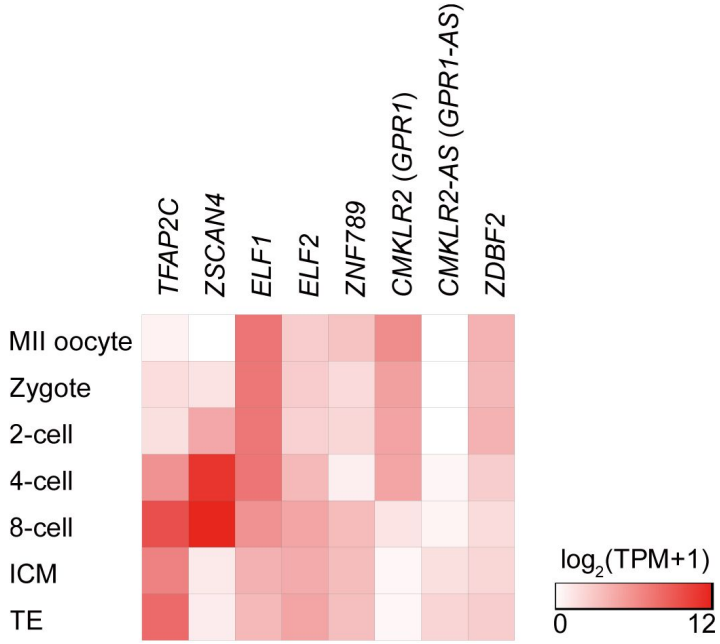


Figure 5 – figure supplement 3.

Expression patterns of transcription factors and imprinted genes during human preimplantation development

A heat map displaying the average expression of four transcription factors associated to human *GPR1-AS* or mouse *Liz* transcription, a primary KRAB-ZFP that binds to MER21C, and three imprinted genes surrounding the *ZDBF2* locus.



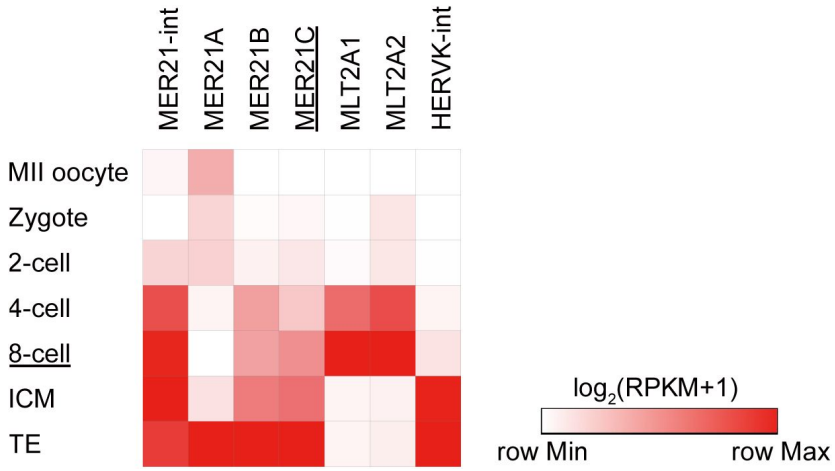


Figure 6 – figure supplement 1.

Human LTR reactivation during preimplantation development

Heat map displaying the average expression of select LTR retrotransposon families in human oocytes and early embryos. MLT2A1/MLT2A2 and HERVK are reactivated between the 4- to 8-cell stage and after the 8-cell stage, respectively (Grow et al. 2015; Hashimoto et al. 2021).

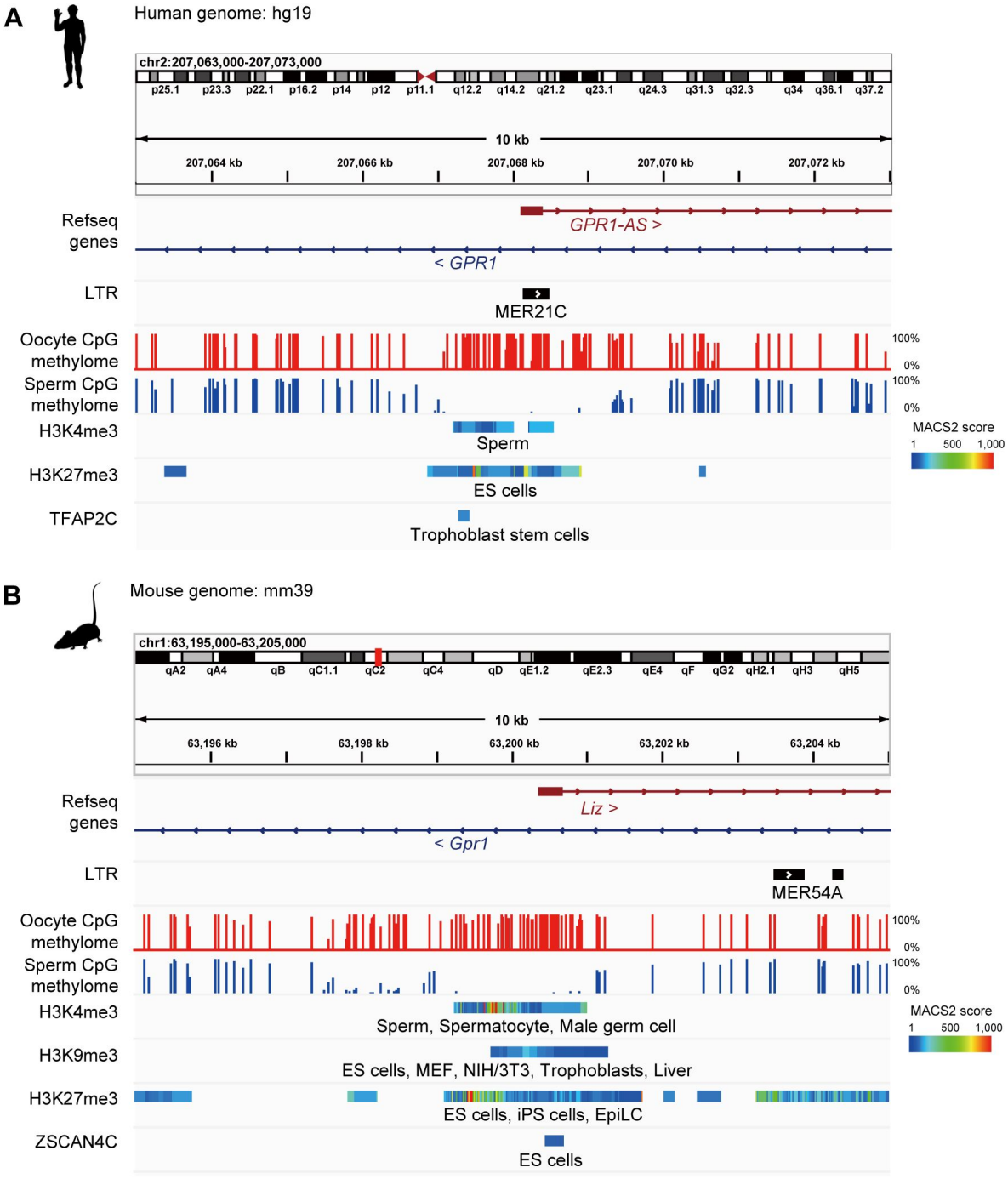


Figure 7 – figure supplement 1.

Interspecies epigenomic comparisons between human *GPR1-AS* and mouse *Liz*

IGV screenshots of the first exon of *GPR1-AS*/*Liz* in human (A) and mouse (B) showing DNA methylation, enrichment of post-translational histone modifications (H3K4me3, H3K9me3, and H3K27me3) and transcription factor binding sites (TFAP2C and ZSCAN4C) from ChIP-Atlas in various tissues. DNA methylomes from oocyte and sperm from mouse and human were published previously (Brind'Amour et al. 2018). Animal silhouettes were obtained from *PhyloPic* (mouse silhouette by *Katy Lawler*, available under a *CC BY 4.0* license).

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Additional information

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NGS data processing: S.K., K.T., T.T

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Additional files

Supplemental files 1,2,3

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Reviewer #1 (Public review):

Summary:

The study tests the conservation of imprinting of the ZBDF2 locus across mammals. ZDBF2 is known to be imprinted in mouse, human and rat. The locus has a unique mechanism of imprinting: although imprinting is conferred by a germline DMR methylated in oocytes, the DMR is upstream to ZDBF2 (at GPR1) and monoallelic methylation of the gDMR does not persist beyond early developmental stages. Instead, a lncRNA (GPR1-AS, also known as Liz in mouse) initiating at the gDMR is expressed transiently in embryos and sets up a secondary DMR (by mechanisms not fully elucidated) that then confers monoallelic expression of ZDBF2 in somatic tissues.

In this study, the authors first interrogate existing placental RNA-seq datasets from multiple mammalian species, and detect GPR1-AS1 candidate transcripts in human, baboon, macaque and mouse, but not in about a dozen other mammals. Because of the varying depth, quality and nature of these RNA-seq libraries, the ability to definitely detect the GPR1-AS1 lncRNA is not guaranteed; therefore, they generate their own deep, directional RNA-seq data from tissues/embryos from five species, finding evidence of GPR1-AS in rabbit, chimpanzee, but not bovine, pig or opossum. From these surveys, the authors conclude that the lncRNA is present only in Euarchontoglires mammals. To test the association between GPR1-AS and ZDBF2 imprinting, they perform RT-PCR and sequencing in tissue from wallabies and cattle, finding biallelic expression of ZDBF2 in these species that also lack a detected GPR1-AS transcript. From inspection of the genomic location of the GPR1-AS first exon, the authors identify an overlap with a solo LTR of the MER21C retrotransposon family in those species in which the lncRNA is observed, except for some rodents, including mouse. However, they do detect a degree of homology (46%) to the MER21C consensus at the first exon on Liz in mouse. Finally, the authors explore public RNA-seq datasets to show that GPR1-AS is expression transiently during human preimplantation development, an expression dynamic that would be consistent with the induction of monoallelic methylation of a somatic DMR at ZDBF2 and consequent monoallelic expression.

Strengths:

The analysis uncovers a novel mechanism by which a retrotransposon-derived LTR may be involved in genomic imprinting.
The genomic analysis is very well executed.
New directional and deeply-sequenced RNA-seq datasets from placenta or trophectoderm of five mammalian species and marsupial embryos, which will be of value to the community.

Weaknesses:

Although the genomic analysis is very strong, the study remains entirely correlative. All of the data are descriptive, and much of the analysis is performed on RNA-seq and other datasets from the public domain; a small amount of primary data is generated by the authors. Evidence that the residual LTR in mouse is functionally relevant for Liz lncRNA expression is lacking.

Comments on revision:

The authors have responded very constructively to all points raised by me and the other reviewers. For example, the authors have gone to further, extensive efforts in seeking to identify an LTR at the mouse Liz locus - which is not found - but additional multiple genome alignments provide evidence for sequence conservation consistent with retention of a functional relic of the MER21C in rodent genomes. Moreover, they demonstrate the promoter activity of this mouse sequence region in transfections. They have also demonstrated

imprinted expression of ZDBF2 in two additional species - rabbit and rhesus macaque - consistent with their model.

https://doi.org/10.7554/eLife.94502.2.sa3

Reviewer #2 (Public review):

Summary:

This work concerns the evolution of ZDBF2 imprinting in mammalian species via initiation of GPR1 antisense (AS) transcription from a lineage-specific long-terminal repeat (LTR) retrotransposon. It extends previous work describing the mechanism of ZDBF2 imprinting in mice and humans by demonstrating conservation of GPR1-AS transcripts in rabbits and non-human primates. By identifying the origin of GPR1-AS transcription as the LTR MER21C, the authors claim to account for how imprinting evolved in these species but not in those lacking the MER21C insertion. This illustrates the principle of LTR co-option as a means of evolving new gene regulatory mechanisms, specifically to achieve parent-of-origin allele specific expression (imprinting). Examples of this phenomenon have been described previously, but usually involve initiation of transcription during gametogenesis rather than post-fertilization, as in this work. The findings of this paper are therefore relevant to biologists studying imprinted genes or interested more generally in the evolution of gene regulatory mechanisms.

Strengths:

(1) The authors convincingly demonstrate the existence of GPR1-AS orthologs in specific mammalian lineages using high quality RNA-seq libraries collected from diverse mammalian species.

(2) The authors demonstrate imprinting of the ZDBF2 locus in rabbits and Rhesus macaques using allele-specific expression analysis. The transcription of GPR1-AS orthologs therefore correlates with imprinting of the ZDBF2 locus.

Weaknesses:

(1) Experimental evidence directly linking GPR1-AS transcription to ZDBF2 imprinting in rabbits and non-human primates is lacking. Consideration should be given to the challenges associated with studying non-model species and manipulating repeat sequences. Further, this mechanism is established in humans and mice, so the authors' model is arguably sufficiently supported merely by the existence of GPR1-AS orthologs in other mammalian lineages.

https://doi.org/10.7554/eLife.94502.2.sa2

Reviewer #3 (Public review):

Kobayashi et al identify MER21C as a common promoter of GPR1-AS/Liz in Euarchontoglires, which establishes a somatic DMR that controls ZFDB2 imprinting. In mice, MER21C appears to have diverged significantly from its primate counterparts and is no longer annotated as such.

The authors used high-quality cross-species RNA-seq data to characterise GPR1-AS-like transcripts, which included generating new data in five different species. The association between MER21C/B elements and the promoter of GPR1-AS in most species is clear and convincing. The expression pattern of MER21C/B elements overall further supports their role in enabling correct temporal expression of GPR1-AS during embryonic development.

In the revised version of the manuscript the authors provided additional support for the common evolutionary origin of the GPR1-AS/Liz promoter between primates and rodents. They also showed a more extensive concordance between the presence of GPR1-AS-like transcripts and ZDBF2 imprinting.

Altogether, these findings robustly support the conclusions of the paper, shedding light into the events underlying the evolution of imprinting at the ZDBF2 locus.

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Author response:

The following is the authors' response to the original reviews

> ***Recommendations For The Authors:***
>
> ***Reviewer #1 (Recommendations For The Authors):***
>
> *Recommendations Analysis:*
>
> *(1) Given that a MER21B/C LTR was not immediately identified at the start site of the Liz lncRNA in the mouse, and its match is only 46%, this raises the question of whether an analogous LTR would be identified at the homologous location in other species on deeper analysis. The authors need to argue that what has been conserved in the LTR alone in mouse is the essential element conferring the ability to initiate transcription of Liz. A transient reporter assay might be sufficient to do this.*

We believe that the 46% identity between the first exon of mouse Liz and the consensus sequence of MER21C is so weak that its traces as MER21C are too attenuated to be detected by standard in silico analyses, such as homology searches. For instance, when pairwise alignments are performed between the first exon of mouse Liz and the consensus sequences of solo-LTRs other than MER21C, MER21C does not emerge as the most similar sequence (Figure 5 – figure supplement 1). This is in stark contrast to similar analyses involving the first exon of human and rabbit GPR1AS (which overlaps with MER21C), where MER21C is identified as the most similar sequence. [pages: 26, 31-32]

The positions of these LTRs were initially annotated using RepeatMasker. To ensure robust analysis, we performed additional searches with RepeatMasker under more sensitive conditions, adjusting search engines (e.g., RMblast to HMMER or Cross-match) and sensitivity settings. Nevertheless, MER21C or closely related LTRs were still undetectable in mouse, rat, and hamster (Figure 4 – figure supplement 1). However, a multiple genome alignment generated by Cactus/UCSC revealed a syntenic region corresponding to the first exon of human GPR1-AS, overlapping with LTR21C, in the genomes of mice, as well as rats and hamsters (Figure 4 – figure supplement 2). Although RepeatMasker did not annotate MER21C at the GPR1 locus in these species, homologous regions were observed across all selected Euarchontoglires. Due to the limitations of the Cactus alignment track in delineating precise homologous boundaries across species, extracting sequences for evolutionary tree construction was not feasible. Nevertheless, these findings support the hypothesis that the first exon of GPR1-AS (Liz in mice) originated from a MER21C insertion in the common ancestor of Euarchontoglires. [pages: 21, 24-25]

A combination of traditional annotation of repetitive elements using RepeatMasker and the reconstruction of ancestral genomes through multiple genome alignment can reveal highly degenerated LTR relics. This approach is likely to point to significant future directions for research. This point is further elaborated in the discussion section. [page 42]

Furthermore, in response to the reviewer's suggestion, we investigated the promoter activity of the GPR1-AS and Liz first exons, which are hypothesized to have originated from the same MER21C insertion. Using a dual reporter assay, we demonstrated that the first exon of mouse Liz exhibits promoter activity in a human cell line comparable to that of the human GPR1-AS promoter. Thus, despite the relatively low sequence similarity between the Liz first exon and the MER21C consensus sequence (46% as determined by pairwise alignment, Figure 5 – figure supplement 2), the promoter activity remains functionally conserved. We further discuss the potential functional motifs within the putative MER21C LTR-derived sequences in Figure 4B-D. Taken together, these findings suggest that despite a high level of degeneracy of the promoter region in rodents, including mice, the most parsimonious explanation for the origin of this regulatory element in rodents is the presence of the same LTR relic detectable in humans/primates, which is essential for robust transcription initiation of Liz and GPR1-AS, respectively. [pages: 27, 32]

> *(2) Imprinting will depend on an initiating mechanism in the germline, in addition to events in the embryo that induce the secondary DMR at ZDBF2. The authors should therefore examine as far as possible the presence of a gDMR in the species with/without GPR1-AS1 and ZDBF2 imprinting. Whole-genome bisulphite sequencing data from oocytes and sperm should exist for some of the relevant species (e.g., pig, cow: Ivanova et al. 2020 PMID: 32393379; Lu et al. 2012 PMID: 34818044).*

As the reviewer noted, the presence of a gDMR is essential for the establishment of imprinting. Following another reviewer's suggestion, we have now demonstrated that the ZDBF2 gene in rhesus monkeys is also subject to imprinting (see Figure 3C-D). We also acquired whole genome bisulfite sequencing data for rhesus monkey sperm and oocytes, identified DMRs between them, and discovered an oocyte-specifically methylated gDMR in the first exon of GPR1-AS (which overlaps with MER21C)(Figure 3 – figure supplement 1A). This finding is consistent with observations in humans and mice. Conversely, we obtained similar sequencing data for porcine and bovine sperm and oocytes and conducted the same analysis (Figure 3 – figure supplement 1A,B). However, we did not detect any oocyte-specific methylated gDMRs in the GPR1 intragenic region (where GPR1AS is transcribed from an intron of GPR1) in these species of the Laurasiatheria superorder. These results support the hypothesis that ZDBF2 is not imprinted in lineages outside the Euarchontoglires, the superorder which includes both rodents and primates. We have included these important DMR results as a supplement to Figure 3. [pages 16-21]

> *Presentation:*
>
> *(1) The first section of the Introduction would benefit from the inclusion of some additional general references on genomic imprinting.*

We have added two review articles, Tucci et al. (2019) and Kobayashi (2021), as references in the first section of the Introduction. [page 5]

> *(2) Introduction statement: "....nearly 200 imprinted genes have been identified in mice and humans. However, less than half of these genes overlapped in both species." This was the conclusion of one study (Tucci et al. 2016), so it would be better to provide a caveat to the statement "However, one comparative analysis suggested that fewer than half of these genes overlapped in both species".*
>
> *The point being that the actual number of imprinted genes is still a matter of debate (see Edwards et al. 2023 PMID: 36916665), and the extent of overlap will depend on the strength of the evidence for each gene in the human and mouse imprinted gene lists. So,*

> *it is very difficult to put an accurate figure on the extent of overlap - but the authors' point is valid that there are species- or lineage-specific imprinted genes.*

We have revised this sentence following reviewer #1's suggestion. [page 5]

> *(3) Introduction statement: "The establishment of species-specific imprinting.....can be driven by various evolutionary events, including.....differences in the function of DNA methyltransferases". I am not aware that this has been described as an evolutionary event causing species-specific imprinting - without supporting evidence, I recommend to remove this suggestion.*

We thank the reviewer for this comment and realize that we should have been more explicit here. We were referring to DNMT3C, a rodent-specific member of the DNMT3 family, which is responsible for the paternal methylation imprinting of Rasgrf1 in mice (Barau et al., Science, 2016), in association with the piRNA pathway and targeting of a specific retrotransposon within the DMR (Watanabe et al. Science, 2011). The Rasgrf1 gene is imprinted in mice but not considered imprinted in humans (though some conflicting data exist). While it is likely that the emergence of DNMT3C was a pre-requisite to the establishment of Rasgrf1 imprinting in evolutionary terms, clear evidence is lacking. Following the reviewer's suggestion, we have removed the phrase "differences in the function of DNA methyltransferases" from the text. However, we have reintroduced this point in the Introduction section as a potential mechanism that may contribute to the establishment of species-specific imprinted genes, alongside the roles of ZNF445 and ZFP57, which regulate the maintenance of imprinting with partially divided roles between humans and mice. [page 6]

> *(4) It would be very useful for readers to have a schema of the Gpr1/Zdbf2 locus that indicates the locations of the germline and somatic DMRs and their relationship to the Liz transcript.*
>
> *(5) There is a summary figure amongst the Supplementary Figures (Suppl. Fig. 7) - it would be beneficial to readers to have this summary figure in the main text rather than the supplement.*

Following reviewer #1's suggestion, we have moved the regulatory system schema at the Gpr1/Zdbf2 locus, originally shown in Supplementary Figure 7, to the main text as Figure 7. In addition, in response to comment 4, we have revised the figure to explicitly depict the relationship between the Liz transcript and the establishment of the somatic DMR (sDMR), enhancing the clarity of the regulatory interactions at this locus. [page 38]

> *(6) With a focus of the study on LTRs as cis-regulatory elements having been co-opted in genomic imprinting mechanisms - whether in the female germline (as in Bogutz et al. 2019) or in the current study as an activating element post-fertilisation - it is a real omission that the authors do not to refer to the role of tissue-specific LTRs as the candidate regulatory elements in non-canonical imprinting (see Hanna et al. 2019 PMID: 31665063). Please include in Introduction and/or Discussion.*

We added a sentence explaining canonical and non-canonical imprinting and the cases where LTRs act as regulatory elements in non-canonical imprinting, with reference to the study of Hanna et al., as suggested. [page 6]

> *(7) Discussion statement: "Two paternally expressed imprinted genes, PEG10/SIRH1 and PEG11/RTL1/SIRH2 have been identified in mammals. They encode GAG-POL proteins of sushi-ichi LTR retrotransposons and are essential for mammalian placenta formation and maintenance."*

> *These sentences should be combined: "Two paternally expressed imprinted genes, PEG10/SIRH1, and PEG11/RTL1/SIRH2, that encode GAG-POL proteins of sushi-ichi LTR retrotransposons have been identified in mammals and are essential for mammalian placenta formation and maintenance."*

We have revised this sentence according to reviewer #1's suggestion. [page 41]

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *When showing assembled GPR1-AS transcripts via genome browser tracks, it would be valuable to add normalized counts of reads mapping to each strand, in order to more convincingly demonstrate the existence of these transcripts. I ask for this because in my experience Stringtie will assemble transcripts that are only marginally supported by reads.*

In response to Reviewer #2's suggestion, FPKM and TPM values for all StringTiepredicted GPR1-AS-like transcripts are now included in Figure 6. Each of these transcripts has a TPM value greater than 1, supporting their validity. [pages: 35]

> ***Reviewer #3 (Recommendations For The Authors):***
>
> *(1) The tree in Figure 5A is one of the main arguments supporting the divergence of the mouse Liz promoter from a common MER21C element, but this contains only a handful of species, making it difficult to appreciate the full extent of its evolution. Presumably its faster mutation rate in mouse would also be supported by other closely related rodents, which would solidify the conclusion that the Liz promoter is derived from an ancient MER21C insertion. So my suggestion is to expand this tree substantially to other species, comparing sequences syntenic to the GPR1-AS/Liz promoter.*
>
> *(2) It may also be worth trying different TE/LTR annotation tools and/or running Repeatmasker with different parameters, to see if an MER21C element is detected in mouse using a more sensitive approach.*

In response to this suggestion, we performed computational analyses with RepeatMasker under various settings (e.g., switching search engines from RMblast to HMMER or Crossmatch, adjusting speed/sensitivity settings from default to slow). Despite these modifications, a MER21C element was not detected near the mouse Liz promoter. However, a multiple genome alignment track generated by Cactus/UCSC revealed a syntenic region, corresponding to the first exon of human GPR1-AS, which overlaps with LTR21C, also present in the genomes of mouse, rat, and hamster (Figure 4 – figure supplement 1). While RepeatMasker did not identify MER21C at the GPR1 locus in these species, homologous regions were observed across all selected Euarchontoglires. Although the Cactus alignment track does not delineate the exact boundaries of homologous regions across species (relative to humans) and thus precludes extracting each homologous sequence to construct an evolutionary tree, these findings support the hypothesis that the first exon of GPR1-AS (referred to as Liz in mice) originated from an ancient MER21C insertion in the common ancestor of Euarchontoglires. [pages: 21, 24-25]

> *(3) According to Dfam, MER21C is not common to all eutherians, but specific to Boroeutheria, whilst MER21B is presumably specific to Euarchontoglires. To clarify MER21C/B evolution, it would be useful to show the number of elements present in select species from each group (including an outgroup).*
>
> *(7) In Figure 4 it is hard to distinguish between red and purple.*

Initially, we referenced Repbase (e.g., MER21C: Origin/Eutheria), but, as Reviewer #3 noted, Dfam should be the primary reference. We have now included the copy numbers of MER21C and MER21B for each genome in Figure 4, providing a clearer understanding of their evolutionary appearance (MER21C appears specific to Boroeutheria, while MER21B is specific to Euarchontoglires). Additionally, we adjusted the MER21B position color from purple to dark purple to improve visibility. Furthermore, we have also underlined the copy number of MER21C or MER21B located within the GPR1 region in each species. For example, in the Treeshrew genome, the LTR overlapping with GPR1-AS is annotated as MER21B, so we underlined the copy number of MER21B (2,305). These changes now clearly indicate whether homologous sequences to the first exon of GPR1-AS are annotated as MER21C or MER21B in each genome. [page 22]

> *(4) Could the imprinting status of ZDBF2 not be determined in chimpanzees and rabbits? Or is it already known? Either way, a clarification would be useful to further support the concordance between GPR1-AS-like transcripts and ZDBF2 imprinting.*

The imprinting status of ZDBF2 had not previously been reported in chimpanzees, rhesus macaques, or rabbits, where GPR1-AS-like transcripts were identified. Therefore, we conducted allele-specific expression analysis of ZDBF2 using blood samples from rhesus macaques and rabbits. As expected, paternal-allele-specific expression of ZDBF2 was observed in both species, consistent with findings in humans and mice. These results have been added to Figure 3. Although we did not analyze the imprinting status in chimpanzees, we believe the existing data sufficiently support our hypothesis. [pages: 16, 19-20]

> *(5) The authors briefly discuss the role of KRAB-ZFPs in controlling TE expression. An interesting addition would be to analyse the expression of the main KRAB-ZFP that binds to MER21C (ZFP789, according to data from PMID 28273063). This could be linked to the temporal control of MER21C expression.*

In response to Reviewer #3's suggestion, we focused on the expression pattern of ZNF789 (noted by the reviewer as ZFP789), the primary KRAB-ZFP known to bind MER21C, as identified by Didier Trono's group (PMID 28273063). Strikingly, our analysis reveals that ZNF789 is specifically downregulated at the 4-cell stage, which aligns with the timing of MER21C reactivation. While it remains to be determined whether this downregulation directly influences MER21C reactivation or the initiation of GPR1-AS expression, this finding is significant and consistent with our model. We have incorporated this information in Figure 5 – figure supplement 3. [pages: 33]

> *(6) The sentence "Liz directs DNA methylation at the somatic DMR, which competes with ZDBF2 to repress the paternal allele" (introduction) was confusing to me.*

This sentence has been revised to be more accurate as follows; Liz transcription counteracts the H3K27me3-mediated repression of Zdbf2 by promoting the deposition of antagonistic DNA methylation at the secondary DMR. [page 7]

> *(8) In Figure 5 I take it that 'consensus motif' refers to ELF1/2? Maybe change the legend.*

To clarify potential confusion around the term 'consensus motif,' which may have been mistaken for 'consensus MER21C' (the consensus sequence of MER21C-LTR from the Dfam database), we have revised the figure legend. We now refer to the motif as the "common motif," indicating the sequence common to all MER21C-derived sequences and overlapping with the first exon of GPR1-AS. [page 29]

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