Letter

Discoveries

Title

Monotreme-specific conserved proteins derived from retroviral reverse transcriptase

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Abstract

Endogenous retroviruses (ERVs) have played an essential role in the evolution of mammals. Many ERV-derived genes are reported in the therians that are involved in the placental development. However, the contribution of the ERV-derived genes in monotremes, which are oviparous mammals, remains to be uncovered. Here, we conducted a comprehensive search for possible ERV-derived genes in platypus and echidna genomes and identified three reverse transcriptase-like genes, named "*RTOM1*, *2*, and *3*." They were found to be clustered in the *GRIP2* intron. Phylogenetic analysis revealed that *RTOM1*, *2*, and *3* are strongly conserved between these species, and they were generated by tandem duplications before the divergence of platypus and echidna. The *RTOM* transcripts were specifically expressed in the testis, suggesting the physiological importance of *RTOM* genes. This is the first study reporting monotreme-specific *de novo* gene candidates derived from ERVs, which provides new insights into the unique evolution of monotremes.

Endogenous retroviruses (ERVs) are remnants of retroviral genomes found in the host genomes. ERVs are retroviruses that infect the host germline cells and are integrated into the host genome (Johnson 2019). Young ERVs retain their viral open reading frames (ORFs), but gradually lose their intact ORFs due to the accumulation of mutations. However, proteins expressed from ERVs sometimes evolve as functional genes in the host (Ueda et al. 2020). A typical example is the syncytin genes, ERV-derived fusogenic genes, which are expressed in the human placenta (Mi et al. 2000; Blond et al. 2000; Blaise et al. 2003) and are required for mouse placenta formation (Dupressoir et al. 2009; Dupressoir et al. 2011). Syncytin genes have been independently acquired from different ERVs in different mammalian lineages, which is a representative example of the convergent evolution (Imakawa et al. 2015). In addition, other ERV-derived genes have also been found to be expressed in the placenta. For example, *HEMO* encoding a secreted envelope protein (Heidmann et al. 2017) as well as *gagV1* and *pre-gagV1* genes (Boso et al. 2021) are highly expressed in the human placenta. However, it is unknown whether ERV-derived genes are co-opted in monotremes that are egg-laying mammals. Comparative studies for the detection of ERV-derived genes have been conducted in mammalian genomes, including the platypus (Nakagawa and Takahashi 2016; Wang and Han 2020). For monotremes, however, only the genome sequence of one species, the platypus, was available (OANA5). and the quality was limited (Warren et al. 2008). Recently, high-quality monotreme genomes of platypus (mOrnAna1.p.v1) and echidna (mTacAcu1.pri) were sequenced using long-read sequencing technology (Zhou et al. 2021), making it possible to search for conserved ERV genes in monotremes. Here, we performed a comparative analysis of the genomes of these two monotremes and found three ERV-derived genes that are specific to the monotreme lineage.

To comprehensively search for ERV genes in monotremes, we extracted ORFs from the genomes of platypus and echidna. The amino acid sequences obtained by the virtual translation of these ORFs were used as queries for the sequence search. We used the hidden Markov model (HMM) of the retroviral genes in the Gypsy Database 2.0 (GyDB) (Llorens et al. 2011) as the subject of the sequence search (supplementary table S1). We identified ORFs similar to *gag*, *pro*, *pol*, and *env* genes (fig. 1A). These ORFs are presumed to be a mixture of (1) ORFs that are evolutionarily conserved and (2) ORFs of young transposons that retain their ORFs. To exclude young ERV ORFs, we performed the clustering analysis based on the amino acid sequence identity. Since young ERVs are thought to be included in large clusters due to their mutual similarity to each other, we removed sequences that belonged to large clusters consisting of more than 10 sequences. Next, using the platypus ORFs as queries, and the echidna ORFs as the subjects, we conducted a sequence similarity search using BLASTp. We obtained nine ORF pairs with high amino acid similarity and the same synteny between platypus and echidna (supplementary table S2). For six pairs among these, we found respective homologs in the human genome, indicating that they were either ERV genes acquired in the common mammalian ancestor or host genes with high similarity to ERVs. Indeed, one of the six genes is *ASPRV1* that is a known ERV-derived protease gene acquired in the common ancestor of mammals and is responsible for skin maintenance (Matsui et al. 2011). The remaining three genes were not found in the human genome. They were located tandemly in the intron of the *GRIP2* gene in the opposite direction (fig. 1B). All three ORFs showed high similarity to the reverse transcriptase (RT) of spumaretrovirus in GyDB (supplementary table S3). Therefore, we designated these genes as *RTOM* [RT-like ORF

in Monotreme], and three genes were named as *RTOM1*, *RTOM2*, and *RTOM3* in order of their location from the 5′ direction (fig. 1B). The *RTOM* coding sequences were searched in the genomes of 6 mammals, 2 birds, 8 reptilians, and 2 amphibians (supplementary table S4); however, significant hits were not obtained other than in platypus and echidna (BLASTn: E-value < 1E-5). Therefore, the *RTOM* genes could be monotreme-specific and by originated more than 55 million years ago, the divergence time of platypus and echidna (Zhou et al. 2021).

We found that the gene structures of *RTOM* genes in the platypus genome were annotated in the RefSeq database (fig. 2A). *RTOM1*, *2*, and *3* genes of platypus contained two introns in the 5′ UTR, and the entire *RTOM* ORFs are expressed as mRNA excluding a second splicing variant of *RTOM3* that partially lost its ORF (fig. 2A). In echidna, *RTOM2* and *RTOM3* gene structures were annotated in the RefSeq transcripts; however, *RTOM1* was not annotated. By conducting transcriptome assemblies of RNA-seq data of echidna tissues (supplementary table S5), we reconstructed the *RTOM1* transcript (fig. 2B; supplementary data S1). As a result, all echidna *RTOM* transcripts have two introns in the 5′ UTR, which was similar to observations for platypus. According to the alignment of the six amino acid sequences of platypus and echidna *RTOM* genes, RTOM2 lacks a region shared by RTOM1 and RTOM3, but the C-terminal region was conserved among the RTOM proteins without insertion or deletion (fig. 2C). To investigate the tissue-specific expression of *RTOM* genes, we analyzed the RNA-seq data of platypus and echidna (supplementary table S5). In platypus, *RTOM1*, *2*, and *3* were all highly expressed in the testis (fig. 2D). *GRIP2* was expressed not only in the testis but also in the brain, and its expression level was lower than that of the *RTOM* genes. This suggests that the

104 *RTOM* expression was not a result of the *GRIP2* expression. We further investigated the
105 mapped reads using Interactive Genome Viewer (Thorvaldsdóttir et al. 2013) and found
106 that *RTOM3* showed a splicing variant with an intron in the coding region, as shown in
107 the RefSeq transcript (supplementary fig S1). In echidna, we found that all *RTOM*
108 transcripts were specifically expressed in the testis, similar to platypus. Expression of
109 *GRIP2* in echidna testis was also relatively low, strengthening the idea that the *RTOM*
110 expression is independent of *GRIP2* expression (fig. 2E). Given the higher expression
111 level of *RTOM2* in both platypus and echidna, this gene may play a central role of the
112 RTOM proteins. It is still possible that the relative expression levels of three genes may
113 change according to tissues and developmental stages that were not examined in this study.
114
115 To obtain insights into the viral origin of the *RTOM* genes, we performed a BLASTp
116 search of the amino acid sequence of platypus RTOM1 against the NCBI virus database.
117 We found that retrovirus Pol proteins from various distinct lineages, namely
118 gammaretrovirus, deltaretrovirus, epsilonretrovirus, and spumaretrovirus, are similar to
119 the RTOM1 proteins (BLASTp: E-value < 1E-20). In all hits, the retroviral Pol proteins
120 showed high similarity to the latter half of RTOM1 (about 370-607aa). Domain search
121 against the Pfam database (Mistry et al. 2021) in the HMMER web service (Finn et al.
122 2011) revealed that the latter half of RTOM1 and RTOM3 contain RT domains (fig. 3A;
123 supplementary fig. S2). A phylogenetic tree was constructed from the RT regions of the
124 RTOM proteins and the retroviral Pol proteins (fig. 3B). The RTOM proteins appear to
125 be more related to class III retroviruses, including spumaviruses or spumavirus-related
126 MuERV-L (Llorens et al. 2009). The tree topology suggested that RTOM1 emerged at
127 first, and RTOM2 and RTOM3 were then generated by tandem gene duplications before

the divergence of platypus and echidna (fig. 3C). In the non-RT region of RTOM1 (approximately 1-369aa), no significant hits for retroviruses were obtained (fig. 3A). We performed a BLASTp search for all non-redundant proteins in the GenBank database for the non-RT region of RTOM1; however, no similar proteins were found except for RTOM2 and 3 (E-value < 0.05). Therefore, the non-RT region of the *RTOM* genes dons not seem to be derived from ERV genes or conserved host genes. Considering the structural divergence of the non-RT region, such as deletion of RTOM2 and splicing variant of platypus RTOM3 (fig. 2C), the RT region is a core domain of the RTOM proteins, and the non-RT region may provide functional modifications specific to each *RTOM* protein.

During the 187-million-years history after diverging from monotremes (Zhou et al. 2021), therians have acquired many ERV genes and evolved their unique features, especially the placenta (Imakawa and Nakagawa 2017). Our work revealed that monotremes also domesticated ERV genes. The functional inference of the RTOM proteins is difficult as co-opted RT genes, such as RTOMs, have not been reported in other vertebrates to the best of our knowledge. One possibility is that RTOM proteins may function as restrictive factors against ERVs and retrotransposons. For example, gag-derived *Fv1* (Best et al. 1996) and env-derived *Fv4* (Ikeda and Sugimura 1989) inhibit retroviral infection in mice. It is possible that the RT domains in the *RTOM* genes compete with retrotransposition as antagonists. Another possibility is that RTOM proteins are involved in physiological functions unique to monotremes. In future studies, it would be important to clarify which cells, viz. germ cells or somatic cells, in testis express the *RTOM* genes. Further studies pertaining to *RTOM1*, *2*, and *3* in platypus and echidna will expand our understanding of

ERV co-option during the evolution of mammals.

Materials and Methods

Identification of conserved ERV genes

The platypus genome (mOrnAna1.p.v1, GCF_004115215.1) and the echidna genome (mTacAcu1.pri, GCF_015852505.1) were used for the ERV gene screening (please see fig. 1). The 240-nt ORF flanked by stop codons were retrieved using the getorf program in the European Molecular Biology Open Software Suite (Rice et al. 2000). For HMM-based sequence search, hmmscan was used (Expected threshold: 1E-5) in HMMER3 v3.2.2 (Eddy 2011). ORFs were clustered using CD-HIT v4.8.1 (Li and Godzik 2006) with 50% amino acid identity. The sequence search for platypus ORFs against echidna ORFs was conducted using BLASTp v2.10.0+ with an e-value < 1E-50 (Camacho et al. 2009). Hits with a bitscore > 400 were retrieved and checked the synteny was checked using the UCSC genome browser (https://genome.ucsc.edu/index.html).

Expression analysis

RNA-seq data of platypus (20 samples from 6 tissues) (Marin et al. 2017) and echidna (11 samples from 7 tissues) (Zhou et al. 2021) were used (supplementary file S5). Low-quality reads were trimmed and filtered using fastp v0.19.5 with default options (Chen et al. 2018). The filtered reads were mapped to the each reference genome using HISAT2 v2.1.0 (Pertea et al. 2016). Based on the 11 RNA-seq sequencing data mapped on the echidna genome, we obtained the echidna *RTOM1* transcript by conducting transcriptome assembly using Stringtie2 v2.1.6 with "--merge" option (Kovaka et al. 2019). We added the coordinates of the echidna *RTOM1* transcript (supplementary data S1) to the RefSeq

gene coordinates. We then calculated the expression levels for 20 platypus and 11 echidna RNA-seq samples using the Stringtie2 program with default options (Kovaka et al. 2019).

Phylogenetic analysis

Representative retroviral Pol amino acid sequences were retrieved from the GyDB collection (https://gydb.org/index.php/Alignment?alignment=POL_retroviridae_Biology_Direct_41_2009&format=txt) (Llorens et al. 2009). A multiple alignment was generated using MAFFT v7.487 (Katoh and Standley 2013), and poorly aligned regions were removed using trimAl v1.4.rev15 (Capella-Gutiérrez et al. 2009). A phylogenetic tree was constructed using IQ-TREE2 v2.0.8 (Minh et al. 2020) with 1000 replicates of ultrafast-bootstrap (Hoang et al. 2018). The tree was visualized using FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

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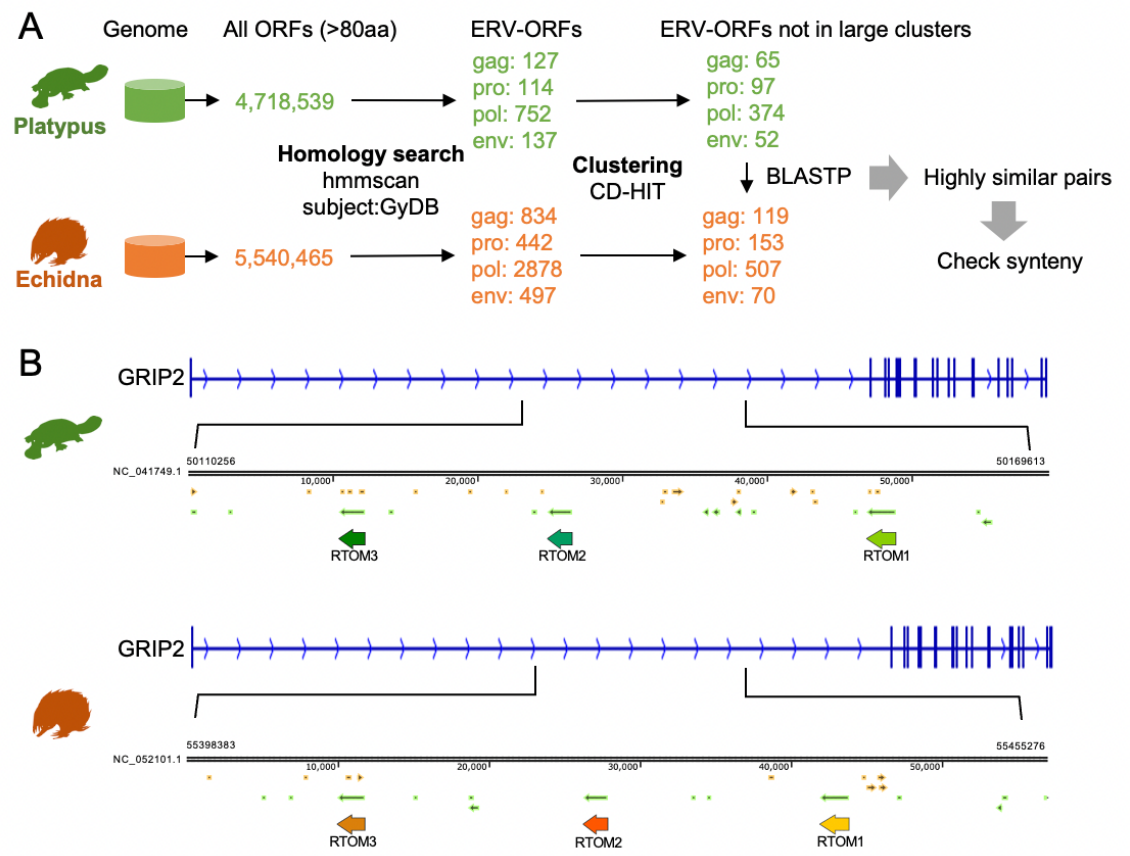
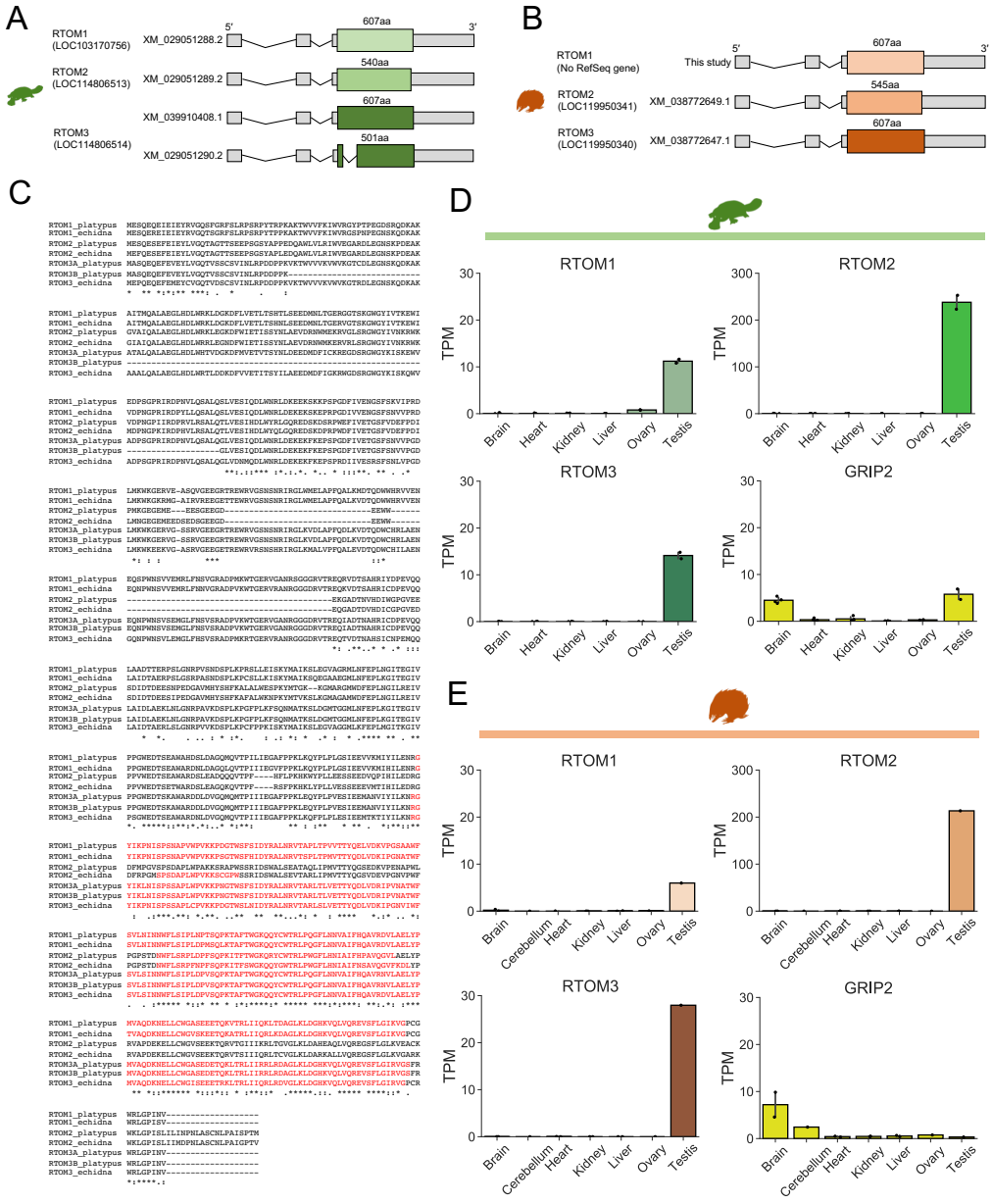
297 **Figure legends**



298

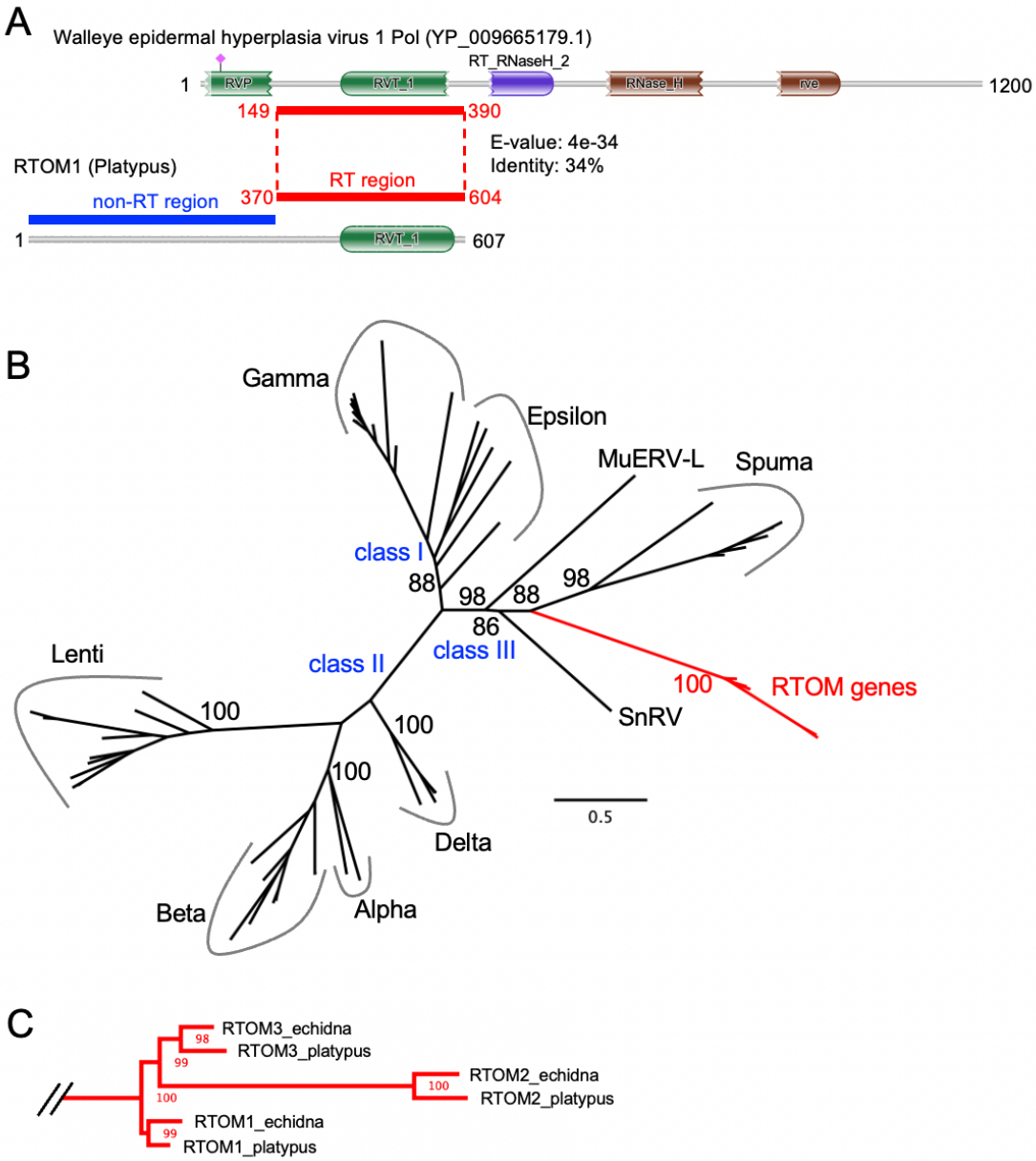
299 FIG. 1. Identification of RTOM1, 2, and 3. (A) Schematic representation of the in silico
300 screening for conserved ERV-derived genes in platypus and echidna. (B) Genomic
301 context of RTOM1, 2, and 3.



302

303 FIG. 2. Expression of *RTOM1*, *2*, and *3*. (A) Schematic representation of the RefSeq
304 transcripts of the *RTOM* genes in platypus. (B) Schematic representation of the
305 reconstructed *RTOM1* transcript and RefSeq transcripts of the *RTOM2* and *3* genes in
306 echidna. (C) Multiple alignment of the amino acid sequences of RTOM proteins. The
307 amino acid sequence of echidna RTOM1 was obtained from the genomic ORF.
308 "RTOM3A_plasypus" and "RTOM3B_platypus" are protein isoforms derived from

309 "XM_039910408.1" and "XM_029051290.2," respectively. The regions showing
310 similarity to the HMM of spumaretrovirus RT domain in GyDB are indicated in red. (D
311 and E) Tissue-specific expression of *RTOM* genes and *GRIP2* in (D) platypus and (E)
312 echidna. Normalized expression levels are presented as transcript per million (TPM).
313



314

315 FIG. 3. Evolution of RTOM1, 2, and 3. (A) Comparison between platypus RTOM1 and
316 retroviral Pol protein. Walleye epidermal hyperplasia virus 1 is represented as an example.
317 A region showing similarity to the Pol protein by BLASTp was designated as “RT region.”
318 A region that did not show similarity to any retroviral genes was designated as “non-RT
319 region.” (B) A phylogenetic tree constructed from the amino acid sequences of RT regions
320 of the six RTOM proteins and the retroviral Pol proteins in GyDB. The multiple alignment

is available in supplementary data S2. Ultrafast-bootstrap values obtained from 1000 times replication are shown in major branches. (C) Detailed representation of the clade of the RTOM genes in (B).

Supplementary Materials

Supplementary fig. S1. Screenshots of Interactive Genomic Viewer of RNA-seq reads on the RTOM genes.

Supplementary fig. S2. Protein domains in RTOM1, 2, and 3.

Supplementary table S1. The HMM profiles in GyDB used in this study.

Supplementary table S2. ERV-like ORFs shared between platypus and echidna

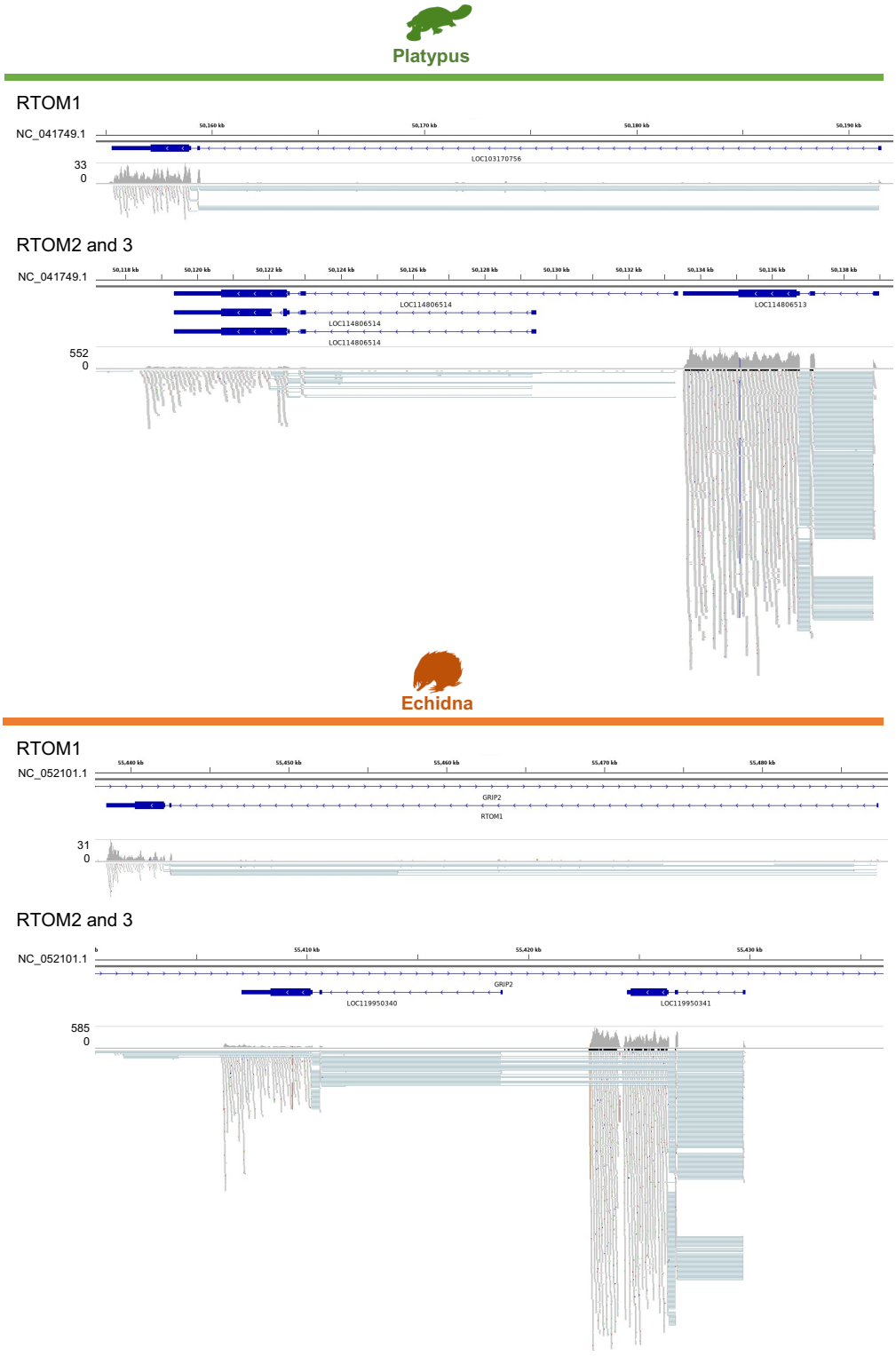
Supplementary table S3. The GyDB HMMs hit to the RTOM genes

Supplementary table S4. Species and genomes used for genes similar to the RTOM genes.

Supplementary table S5. RNA-seq data used in this study.

Supplementary data S1. Nucleotide sequence of the echidna *RTOM1* transcript.

Supplementary data S2. Alignment of representative retroviral *pol* genes and the *RTOM* genes.



Supplementally FIG. S1. Screenshots of Interactive Genomic Viewer of RNA-seq reads on the *RTOM* genes. The transcript tracks in blue lines display the coordinates from the

341 RefSeq GTF files. Thick blue lines indicate the coding sequences. Since there is no
342 corresponding RefSeq transcript for echidna *RTOM1*, its gene coordinate was manually
343 added from assembled transcripts in this study (Materials and Methods).
344

Supplementally FIG. S2. Protein domains in RTOM1, 2, and 3. The domain search was conducted using hmmscan in HMMER web server with default options (https://www.ebi.ac.uk/Tools/hmmer/search/hmmscan).