Tail regeneration in the Tokay Gecko (Gekko gecko)

Nurhidayat, L.

Chapter 3. Transcriptomic Study of Tail Regeneration in The Tokay Gecko

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This chapter is part of: Nurhidayat, L., Benes, V., Blom, S., Gomes, I., Firdausi, N., de Bakker, M.A.G., Spaink, H.P., Richardson. M.K. Tokay gecko tail regeneration involves temporally collinear expression of HOXC genes and early expression of satellite cell markers. *BMC Biology*. Under revision. (2024).

Abstract

Regeneration is the replacement of missing or lost tissue with a functional copy. It is an important survival strategy used by animals to deal with injuries. Some lizards are well-known for the phenomenon of tail autotomy — the spontaneous shedding of part of the tail when the animal is attacked by a predator — followed by regeneration of the missing piece of the tail. Transcriptomic comparisons of multiple stages of tail regeneration stages are needed to give more a comprehensive picture of tail regeneration in this species. In addition, comparing tail regeneration in an adult lizard with the process of normal tail development is also important to determine whether adult regeneration involves the same mechanisms as embryonic tail development. We have performed mRNA sequencing (bulk transcriptomics) at 0, 4, 8, 16, 20, 28 days post autotomy (dpa) in the tokay gecko (a lizard) and compared the results with one stage of tail development in the embryonic tailbud of the same species. We found a distinctive molecular signature at each stage of tail regeneration. The early-regeneration (0–8 dpa) transcriptome was dominated by immune-related genes and genes associated with wound epithelium formation and bone remodeling. The blastema formation phase involves mass up-regulation of genes associated with developmental pathways. Finally, the 28 dpa transcriptome is characterized by maturation and functional development of skeletal muscle. The transcriptome profile of the regenerating adult tail is quite different from that of the embryonic tailbud.

Introduction

Regeneration is the replacement of missing or lost tissue with a functional copy. It is important as a one of the survival strategies used by animals to deal with injuries (Bely and Nyberg, 2010; Gurtner et al., 2008). Regeneration is seen at various levels of biological organization, ranging from the cell (as in muscle cell and hepatocytes) to the whole body (as in Hydra and Planaria), and can take place at different stages of the animal's development (Bely and Nyberg, 2010). In all species, regeneration always involves a particular programmed cell-proliferation and differentiation after the injury (Ricci and Srivastava, 2018; Tanaka and Reddien, 2011).

Regeneration typically requires a significant population of stem cells (Ricci and Srivastava, 2018; Tanaka and Reddien, 2011). Species differences in the availability of stem cells might partly explain the diverse range of regenerative capacity in different species. Regenerative capacity in different species is also influenced by factors such as the characteristics of stem cells, potentials for dedifferentiation and transdifferentiation, the expression of genes associated with regeneration, the presence of epigenetic regulators, and immune system responses (Zhao et al., 2016).

Comparing regeneration in different species is not only interesting to fundamental biology, but it may also one day have translation applications in regenerative medicine and tissue engineering (Daponte et al., 2021; Stocum, 2006; Zhao et al., 2016). Regenerative medicine involves the application of regeneration science to the treatment of disease, and aims to develop treatments for traumatic injuries, heart disease, degenerative diseases, cancers and others diseases for which treatment options are limited (Ahmed et al., 2014; Oviedo and Beane, 2009).

One group of model species commonly used in regeneration studies are the lizards (Lacertilia) a group of cold-blooded 'reptiles'. This group includes the geckos (Gekkonidae). Lizards are well-known for the phenomenon of tail autotomy whereby the tail breaks off at pre-existing fracture planes when grabbed by a predator. This presumably distracts the predator because the broken-off tail fragment remains twitching for some time (Bae et al., 2014). Autotomy ability in lizards is generally accompanied by the ability to regenerate the missing tail piece.

Tail regeneration in lizards results in the development of a new, complex structure (the replacement tail) from a group of proliferating cells on the tail stump called the blastema. It is blastema formation and differentiation, among other things, that make the lizard a useful model for studying tissue regeneration (Bely and Nyberg, 2010; Hill et al., 2012; Lozito and Tuan, 2015; Russell et al., 2015).

Transcriptomic studies have been done on tail regeneration in lizards including the Common Wall Lizard, *Podarcis muralis* (Vitulo et al., 2017), the green anole, *Anolis*

carolinensis (Hutchins et al., 2014), and the Common House Gecko *Hemidactylus frenatus* (Nagumantri et al., 2021) (nomenclature from Pubmed Taxonomy, www.ncbi.nlm.nih.gov/taxonomy/). However, there is still a need for transcriptomic comparisons of more extensive series of tail regeneration stages (this Chapter). In addition, we have compared tail regeneration in an adult lizard with the process of normal tail development.

Materials and methods

Ethics statement

The tokay gecko (*Gekko gecko*) is listed as 'least concern' in the International Union for Conservation of Nature *Red List* (IUCN, https://www.iucnredlist.org/species/195309/2378260) and also listed in Appendix II in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES; https://cites.org/eng/taxonomy/term/3919). In Indonesia, the tokay gecko is not a protected species. All animals were collected in Yogyakarta, Indonesia under a license issued by the Ministry of Environment and Forestry, the Republic of Indonesia (permit number SK.83/KSDAE/SET/KSA.2/5/2021 signed on 7 May 2021) and a recommendation letter issued by The Indonesian Institute of Sciences (Lembaga Ilmu Pengetahuan Indonesia/LIPI; Recommendation Number: B- 2158 /IV/KS.01.04/3/2021 signed on 19 March 2021). The transport of samples from Indonesia to the Netherlands was conducted under CITES permit numbers 05717/IV/SATS-LN/2021 and 09160/IV/SATS-LN/2022 issued by the Ministry of Environment and Forestry, the Republic of Indonesia.

All experimental and surgical procedures needed for sample collection were done at the Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia with the approval from The Ethical Committee of the Integrated Laboratory for Research and Testing (Laboratorium Penelitian dan Pengujian Terpadu/LPPT) Universitas Gadjah Mada (Ethical Clearance number: 00014/04/LPPT/IV/2021 signed on 30 April 2021). Tail autotomy was performed under ketamine anesthesia.

Animal collection and maintenance

Thirty-nine adult tokay geckos were captured in the vicinity of the Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia. They were acclimatized for two weeks, two males with one female, in a stainless-steel terrarium and fed various species of live crickets and sometimes cockroaches, twice a day. They were maintained at ambient temperature and sprayed once a day with water. The geckos were also basked under natural sunlight once every two days for 1 h in the period 09:00–11:00. Twenty-one were used for the harvesting of regeneration blastemas under anesthesia. Of these, three were euthanized for the harvesting of internal organs. The remaining 18 geckos were bred to produce eggs for embryo sampling.

Experimentation and sample collection

Regenerated tail samples

Twenty-one adult tokay geckos were anaesthetized with intramuscular ketamine injection (50–75 mg/Kg body weight) administered in the thigh prior to the autotomy procedure. Autotomy was performed by gently restraining the gecko manually, while leaving the tail accessible. Using the thumb and index finger, a firm pinch was applied to the required autotomous region (every segment after the 5th caudal vertebra) of the tail causing the gecko to spontaneously shed its tail. The wound was painted with Betadine® iodine antiseptic solution but no dressing was applied. After the autotomy procedure was performed, the geckos were allowed to regenerate a new tail. The geckos were distributed into experimental groups based on the number of days post-autotomy, each consisting of three geckos. After ketamine-anesthesia, the tails of geckos in each group were re-autotomized 2 cm cranially from the previous autotomy site at 0, 4, 8, 16, 20, 28 days post-autotomy (dpa). The regenerated tail samples were washed and further handled in phosphate-buffered saline (PBS, 4 °C) and subsequently fixed with cold RNA*later*® stabilization solution (Invitrogen).

Embryo samples

Eighteen adult tokay geckos were grouped and bred as described above. The terraria were checked daily for the presence of gecko eggs. The eggs were left in the terrarium for the required number of days of incubation at ambient temperature. The embryos were harvested into cold PBS and subsequently fixed with RNA*later*® (4 °C).

Organ samples

Three adult tokay gecko were euthanized by an overdose of intramuscular ketamine (150 mg/Kg body weight, intramuscular injection). The geckos were then dissected to harvest internal organs, namely: pancreas, liver, brain, heart, skeletal muscle, whole skin, kidneys and lungs. These eight organ samples were cut up in cold PBS and placed in RNA*later*® (4 °C).

RNA isolation and cleaning

The samples were taken from RNA*later*® solution with sterile forceps and rinsed with sterile PBS. They were then put in 1.5 mL Eppendorf tubes together with 1 mL Trizol and two steel balls (⌀ 4 mm). The tubes were then shaken with a TissueLyser II (Qiagen) at 30/s for 1–2 min. The resulting tissue homogenates were triturated with a hypodermic needle (21G = 0.8mm) attached to a 1 mL syringe until no tissue clumps were visible. The homogenates were then incubated for 30 min and subsequently centrifuged for 10 min at 12,000 RCF (relative centrifugal force).

The supernatants were transferred into new tubes and 0.2 mL of chloroform added to each. The mixture in the tubes were incubated for 2 to 3 min after 11 s of vigorous hand shaking and then centrifuged for 15 min at 16,000 RCF. The colorless aqueous phase of each mixture was transferred to a fresh tube, and 0.7 mL of propan-2-ol (isopropyl alcohol, isopropanol) added to each tube. After leaving for 10 min at room temperature, the samples were centrifuged at 16,000 RCF for 15 min. The supernatants were discarded, and the RNA pellet washed with 1 mL of 75% ethanol. The pellet with 75% ethanol was centrifuged again for 5 min at 7,500 RCF. The supernatants were discarded, and the RNA pellets allowed to dry for 5–10 min. The dry RNA pellets were dissolved in 50 µL of RNase-free water and incubated at 55° C for 10 min. The total RNA of each sample was then cleaned with Qiagen RNeasy Mini Kit (Cat. No. 74104) according to the manufacturer's protocols.

Library preparation and transcriptome sequencing

The library preparation and transcriptome sequencing were done by the European Molecular Biology Laboratory (EMBL) Heidelberg, Germany with in-house protocols. The sequencing platform used was Illumina NextSeq 2000 with a read length of 2×100 bp.

Transcriptome data analysis

Transcriptome data analysis was carried out within the computing resources of the Academic Leiden Interdisciplinary Cluster Environment (ALICE) provided by Leiden University. The transcriptomic analysis workflow is shown in Figure 3-1.

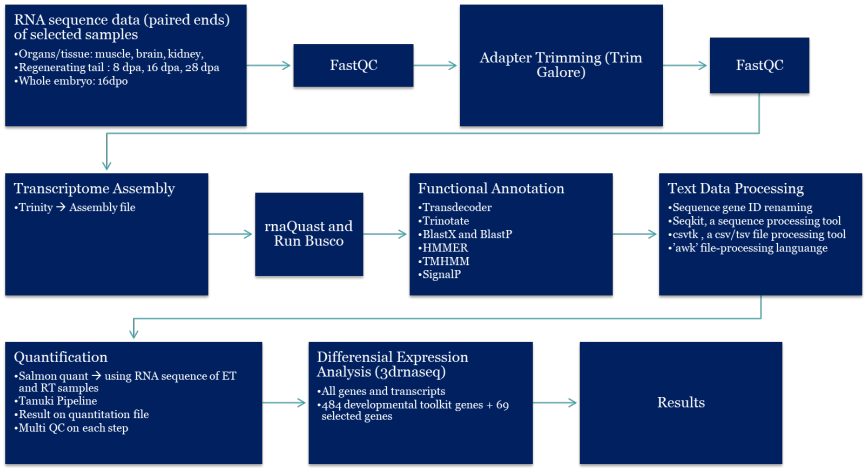

Figure 3-1. **Transcriptomic analysis workflow for analyzing tail regeneration in the tokay gecko adult tail and the embryonic tailbud**. *Key*: ET, embryonic tailbud; QC, quality control; RT, regenerating adult tail.

The sequencing data (paired-end reads, fastq files) were examined with FastQC (https://github.com/s-andrews/FastQC) to check the sequence quality. The sequence adapters were subsequently cut using Trim Galore (https://github.com/FelixKrueger/TrimGalore) before transcriptome assembly. Later on, the transcriptome assembly was done using Trinity RNA-Seq *de novo* assembler (Grabherr et al., 2011; Haas et al., 2013). Finally, the transcriptome assembly results were then evaluated using rnaQUAST (Bushmanova et al., 2016) with the *Gekko japonicus* genome as a reference, and BUSCO (Manni et al., 2021a; Manni et al., 2021b), and FastQC.

Functional annotation was initiated by predicting coding region within the Trinity assembly result using Transdecoder (https://github.com/TransDecoder/TransDecoder). The predicted protein fasta files were then search against databases (NCBI, pFAM, Swissprot) using the Trinotate functional annotation pipeline (Bryant et al., 2017). The tools made use of NCBI Blast+, HMMER (Potter et al., 2018), TMHMM (Krogh et al., 2001), and SignalP (Almagro Armenteros et al., 2019). After functional annotation was completed, the transcripts of both regenerated and embryonic tail samples were quantified using Salmon 1.6.0 (Patro et al., 2017) with Tanuki pipeline (https://github.com/RxLoutre/tanuki.git). The quantification files were then analyzed with differential expression analysis with 3DRNAseq (Guo et al., 2021) using limma (R package) pipeline for differential expression comparison (Ritchie et al., 2015). Differential gene/transcript expression analyses were conducted using $\log_2$ fold change (L_2FC) calculations based on contrasted experimental groups. Statistical significance of expression changes was assessed through t-tests, with p-values adjusted via the Benjamini-Hochberg method to account for multiple testing and control the false discovery rate (FDR). Genes/transcripts were considered significantly differentially expressed in a contrast group if they met the criteria of adjusted p-value < 0.01 and $L_2FC \geq 1$. Furthermore, Gene Ontology and Kegg Pathways were analyzed using Gene Set Enrichment Analysis (Subramanian et al., 2005) with clusterProfiler 4.0 (Wu et al., 2021). Some interesting pathways were visualized based on the transcriptome data using Pathvisio (Kutmon et al., 2015).

Results

For quality control, see Figure S 3-1.

A distinctive molecular signature at each stage of tail regeneration

We conducted mRNA-seq of the regenerating tail at different stages, namely 0, 4, 8, 16, 20, 28 days post autotomy (dpa; *N*=3 per stage; Figure 3-2). Filtering of low-expressed transcripts [counts per million (CPM) cut-off = 1 and minimum samples to CPM cut-off = 9] resulted in 34,472 expressed transcripts from 12,190 expressed genes.

Differential gene expression analysis was performed by comparing each stage with the previous adjacent stage (for example, stage 4 with stage 0, stage 8 with stage 4 and so on). The stagewise pattern of differentially expressed genes is shown in Figure 3-3A , Figure 3-3C, and Figure 3-5A.

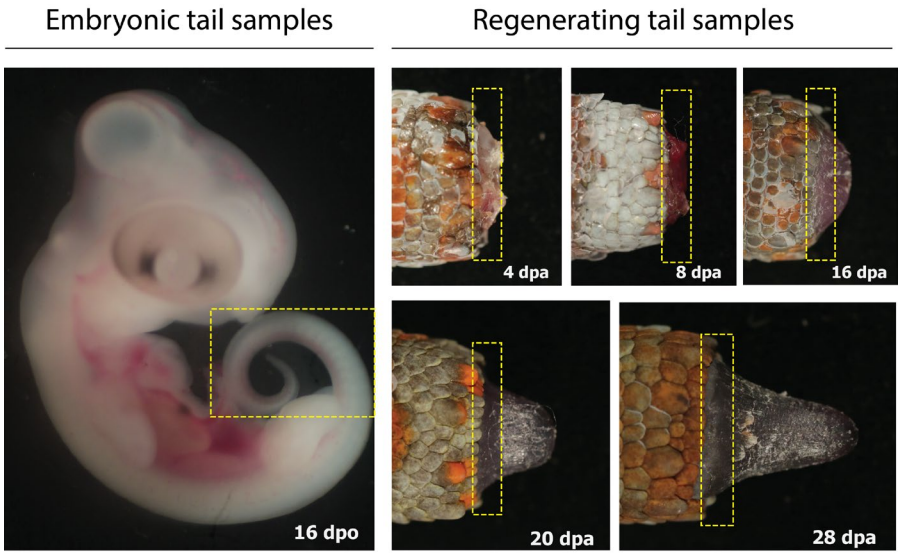

Figure 3-2. **Gecko samples used for transcriptome analysis** (indicated by boxed areas)

As can be seen, the most dramatic change in the transcriptome is seen at the beginning of regeneration, between 0 and 4 dpa. The next major change is at the blastema formation stage, between 8 and 16 dpa. Of these stages, the 0–4 dpa regenerating tail has relatively more uniquely regulated genes, namely 1,241/2,565 (Figure 3-3C). This compares with only 355/1,462 uniquely genes in the 8–16 dpa regenerating tail. At the other stages (4–8 dpa, and the stages after 16 dpa) the number of uniquely regulated genes is much less (Figure 3-3C).

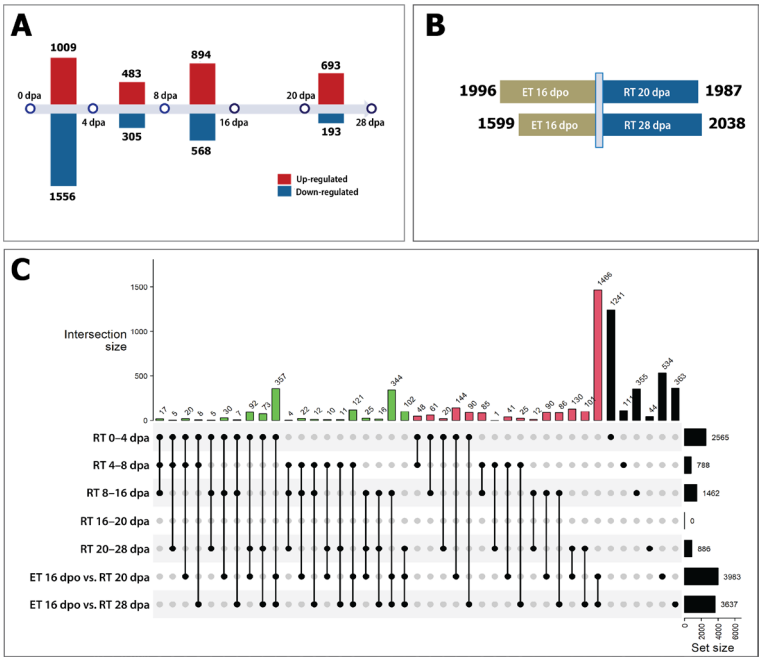

Figure 3-3. **Transcriptome analysis shows a distinctive gene expression profile at each stage of regenerating tail and embryonic tail.** (A) Number of up- and down-regulated genes during tail regeneration stages. Differential gene expression analysis by stage-wise comparison. Three biological replicates each stage. Significant differential expression (DE) genes with adjusted p-value < 0.01 and L2FC≥1. (B) Number of genes regulated in regenerating tail *vs.* embryonic tail. Three biological replicates each stage. Significant differential expression (DE) genes with adjusted p-value < 0.01 and L2FC≥1. ET, embryonic tail; RT, regenerating tail. (C) UpSet of cross-state showing the comparison between different stages of regeneration and tail development. ET, embryonic tail; RT, regenerating tail.

We characterized gene ontology (GO), with a focus on biological processes, and analyzed KEGG pathways using gene set enrichment analysis (GSEA) of all genes expressed at each stage (Figure 3-4). Immune system-related GO terms are enriched at 4 dpa, while muscle activity-related and muscle development-related GO terms are depleted (Figure 3-4A). Bone remodeling-related and epidermis development-related GO terms are also enriched at this stage. In the 8 dpa regenerating tail, only collagen fibril organization and cartilage development are enriched, while epidermis development is depleted.

At 16 dpa, many developmental processes-related GO terms are enriched (e.g. pattern specification process, embryonic morphogenesis, *Wnt* Signaling pathway, cell fate specification, response to BMP, somite development.). By contrast, immune system-related GO terms are depleted. At 20 dpa, muscle development-related GO are becoming enriched together with extracellular matrix organization, while the

developmental-processes-related GO terms are maintained. Finally, at 28 dpa, the muscle-activity-related and muscle development-related GO terms, that were suppressed at 4 dpa, are now enriched.

The KEGG pathway analysis gives a similar pattern to the GO enrichment analysis (Figure 3-4B). Thus, immune system-related pathways are enriched at 4 dpa, as are osteoclast differentiation, TNF signaling pathways, pathways in cancer and steroid biosynthesis. The enrichment of pathways in cancer at this early stage draw our attention so we made a pathway visualization based on our data (Figure S 3-3). It showed some pathways that initiate proliferation are upregulated, such as PI3K-AKT, MAPK, and cell cycle pathways, sustain angiogenesis such as VEGF and HIF-1 signaling, and evading apoptosis potentially via apoptosis inhibitor upregulation.

At 8 dpa, some immune system-related pathways, such as NFκB and IL-17 signaling pathways, together with cancer pathway and TNF signaling pathway are depleted in the regenerating tail. The only two enriched KEGG pathways at this stage are complement and coagulation cascades and ECM receptor interaction. At 16 dpa, pathways related to stem cells and cancer development are enriched, together with melanogenesis and axon guidance, while immune-system-related pathways are depleted. Later on, only cardiac muscle contraction pathway that is enriched in 20 dpa of regenerated tail while maintaining pathways that are enriched in the previous stage. Finally, the further downregulation of immune-system-related pathways is still happening in the 28 dpa regenerating tail.

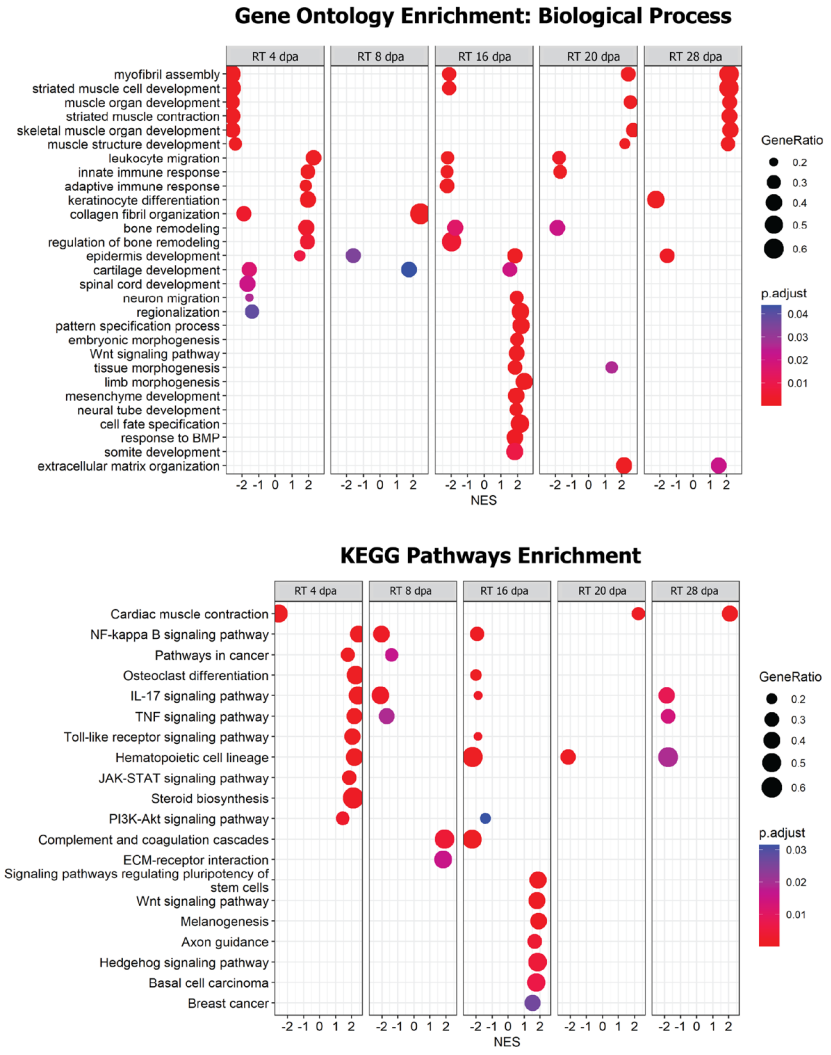

Figure 3-4. **Gene Set Enrichment Analysis reveals biological processes and pathways enriched across stages of tail regeneration**. (A) Biological processes enrichment at different stages of tail regeneration. ET, embryonic tail; RT, regenerating tail. (B) Pathway enrichment at different stages of tail regeneration. RT, regenerating tail. Note that each stage of regeneration has its own signature pathway enrichment.

The regenerating adult tail has a different transcriptomic profile to the embryonic tail

In order to determine whether adult regeneration involves the same mechanisms as embryonic tail development, we performed mRNA-seq on the embryonic tail (*N*=3) at 16 days post oviposition (dpo; Figure 3-2; stage based on (Noro et al., 2009). The heatmap of differentially expressed genes (whose differential expression was

statistically significant) shows that the embryonic tail has a different expression profile from the regenerating adult tail (Figure 3-5A). Notably, genes in clusters 6 and 9 of the heatmap (Figure 3-5A) are strongly expressed in the embryonic tail but not in any stages of the adult regenerating tail.

We then looked at the so-called developmental toolkit genes (developmental patterning genes). These genes encode transcription factors, signaling molecules, transmembrane receptors and other proteins that play a crucial role in embryonic pattern formation. They constitute 484 genes according to (Binda et al., 2024). We found 236/484 of these developmental patterning genes among the significant differentially expressed genes in all comparisons (Figure 3-5B; see for example clusters 3 and 5 of the heatmap in Figure 3-5B).

Then, to focus only on comparable stages of histogenesis, we compared the embryonic tail transcriptome with that of 20 and 28 dpa regenerating tails only. We chose this narrower comparison because the embryonic tail and regenerating tail at these stages all show early stages of tissue differentiation. The comparison generated 3,983 genes that are differentially expressed in embryonic tail *vs.* regenerating tail at 20 dpa (Figure 3-3B). At least 1,996 of these genes are significantly enriched in the 16 dpo embryonic tail whereas 1,987 genes are significantly enriched in the 20 dpa regenerated tail. A similar outcome is also seen in the comparison of the embryonic tail with the 28 dpa regenerating tail. Thus, in that comparison, there are 3,637 genes that are significantly different in expression, of which 1,599 genes are significantly enriched in the embryonic tail, and 2,038 genes that are significantly enriched in the 28 dpo regenerating tail (Figure 3-3B). These two comparisons share 1,446 differentially expressed genes (Figure 3-3C). Of these, 534 genes are uniquely regulated in the embryonic tail *vs.* the 20 dpa regenerating tail, and 363 are in the embryonic tail *vs.* the 28 dpa regenerating tail (Figure 3-3C). In summary, these data show major differences between embryonic tail and regenerating adult tail transcriptomes.

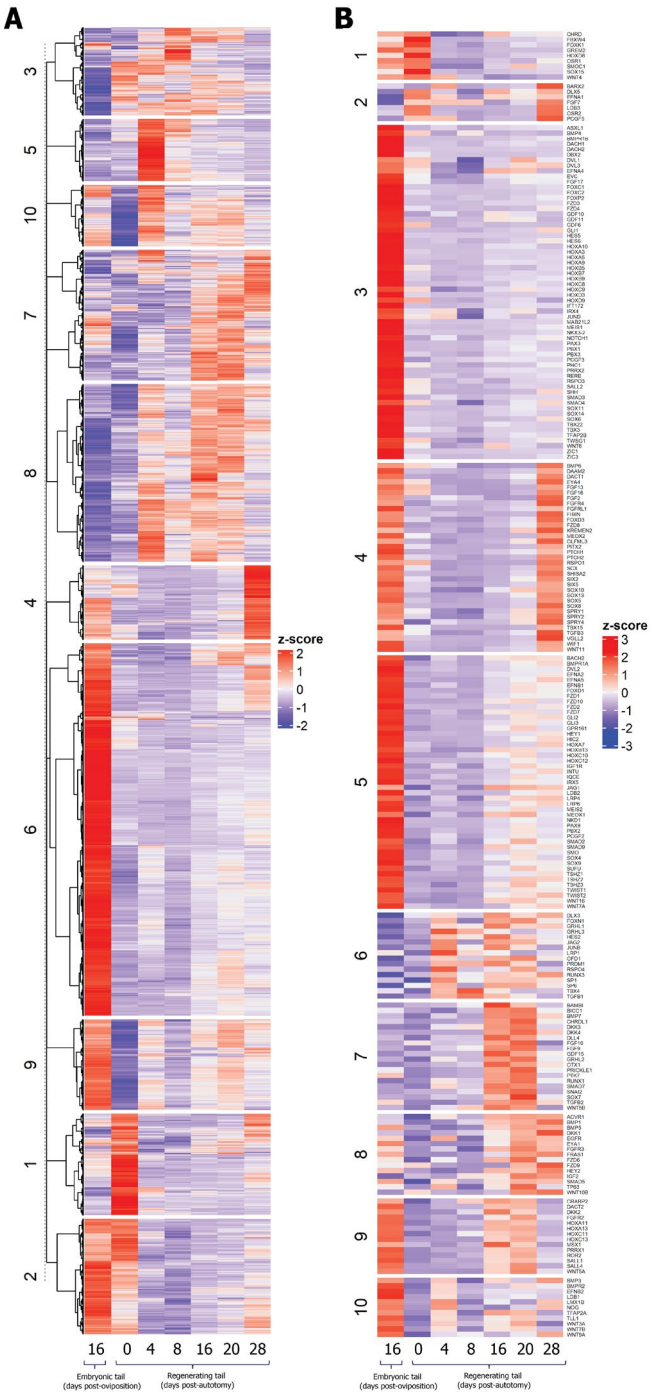

Figure 3-5. **Heatmaps showing the different transcriptome profiles of regenerating and embryonic tails in the tokay gecko**. 6,817 significant differentially expressed genes of whole transcriptome (A) and 236 significant differentially expressed genes of toolkit genes only (B) in embryonic and regenerating tails of the tokay gecko. Significant differential expression (DE) genes with adjusted p-value <0.01 and $L_2FC \geq 1$.

We also characterized gene ontology (GO) and KEGG pathways using gene set enrichment analysis (GSEA) for both comparisons of the embryonic tail and the adult regenerating tail (Figure 3-6). For GO enrichment analysis, both comparisons have a similar result whereby the embryonic tail is dominated by developmental process-related GO terms, while the adult regenerating tails, of both stages, are dominated by immune system-related GO terms and epidermis development-related GO terms (Figure 3-6A). Major differences in GO terms included, in the embryonic tail *vs.* 20 dpa regenerating tail, a comparable enrichment for genes associated with spinal cord development, anterior/posterior pattern specification, and embryonic pattern specification. By contrast, the same GO terms are dominant in the embryonic tail when compared with the 28 dpa regenerating tail. Some GO terms related to tissue morphogenesis or axonogenesis are equally enriched in embryonic tail *versus* 28 dpa regenerating tail. By contrast, these same GO terms are dominant in the embryonic tail if compared to the 20 dpa regenerating tail. Moreover, muscle structure development-related, and extracellular matrix organization GO terms, are dominant in the 28 dpa regenerating tail compared to the embryonic tail.

The KEGG pathway analysis gives a similar pattern to the GO enrichment analysis. Thus, the embryonic tail is relatively enriched in developmental process-related pathways while the regenerating tail is enriched in immune system-related pathways (Figure 3-6B). The term pathways in cancer is dominant in the 20 dpa regenerating tail *vs.* the embryonic tail (Figure 3-6B). Finally, the term signaling pathways regulating pluripotency of stem cells is dominant in the embryonic tail *vs.* the 20 dpa regenerating tail comparison, while this term is equally enriched in embryonic tail *vs.* 28 dpa regenerating tail (Figure 3-6B).

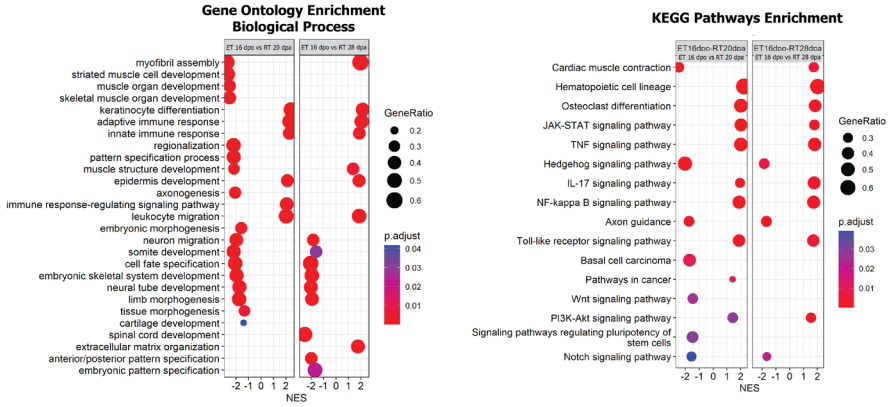

Figure 3-6. **Gene Set Enrichment Analysis reveals biological processes and pathways enriched in regenerating tail and embryonic tail comparison.** (A) Biological process enrichment in regenerating tail vs. embryonic tail. ET, embryonic tail; (B) Pathway enrichment in regenerating tail *vs.* embryonic tail.

In-depth expression profiling on organogenesis related genes

We further explored the expression profile of genes involved in selected tissues in the regenerating tail to see how gene expression in tail regeneration compares with that in tail development in the embryo.

One of the sets of genes we are interested in comparing between regeneration and development are the HOX genes, a family of transcription factors that play a key role in embryonic axial patterning (Di-Poi et al., 2010; Holzman et al., 2018; Kawanishi et al., 2003; McGinnis and Krumlauf, 1992; Spitz et al., 2001; van den Akker et al., 2001) and, potentially, in adult tissue regeneration (Gersch et al., 2005; Rux and Wellik, 2017). Because the tail is an axial structure, we analyzed the expression profile of HOX genes in the transcriptomes of regeneration blastemas and, for comparison, the embryonic tail. Genes from all HOX clusters were expressed in the embryonic tail (Figure 3-7). However, using a stage-wise comparison among regenerating tail blastemas the only HOX genes that were statistically significantly upregulated at any stage were *hoxa5*, *hoxa13*, *hoxb9*, *hoxb13* and *hoxc13* (Figure 3-7). Even though there was minimal evidence in the bulk transcriptomes of any statistically significant upregulation of hox genes during regeneration (Figure S 3-2), visual inspection of the graphs (Figure 3-7) appeared to show that the posterior HOXC genes *hoxc10*–13 might be enriched in the regenerating tail.

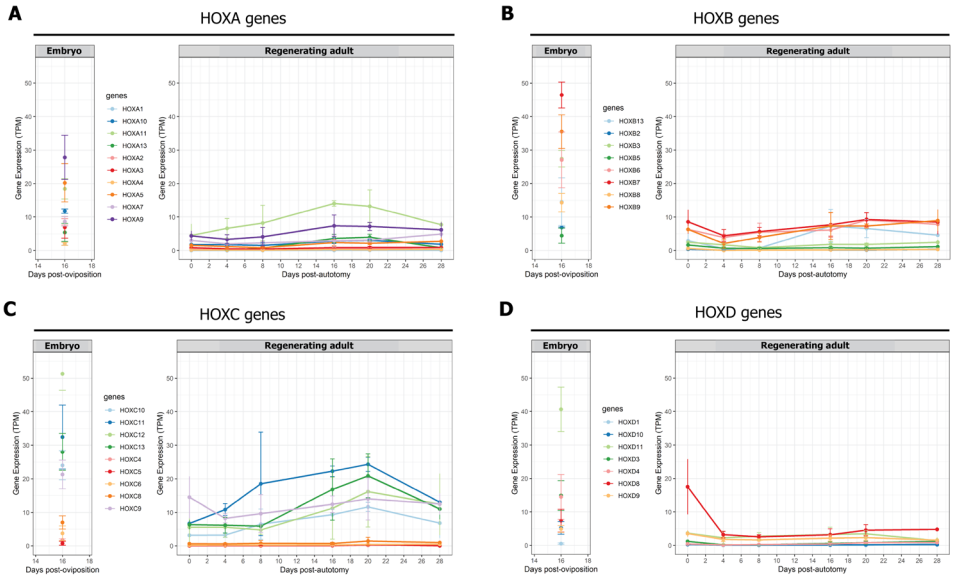

Figure 3-7. **HOXC genes are more active than the other HOX genes clusters during tail regeneration in the gecko**. (A) Expression profile of HOXA genes in the embryonic tail (left) and during adult tail regeneration (right). (B) Expression profile of HOXB genes in the embryonic tail (left) and during adult tail regeneration (right). (C) Expression profile of HOXC genes in the embryonic tail (left) and during adult tail regeneration (right). (D) Expression profile of HOXD genes in the embryonic tail (left) and during adult tail regeneration (right). Note that HOX transcript abundances in the embryonic tails are generally higher than that of the regenerating tails.

The axial skeleton of the normal tail of the gecko is made up of segmentally arranged vertebrae (Chapter 2). In the regenerating gecko tail, however, the vertebrae are replaced by a continuous, unsegmented 'cartilage tube' (Chapter 2; (Lozito and Tuan, 2015)). Therefore, we also examine the typical chondrogenesis-related genes *shh*, *sox9*, *wnt5a*, *wnt3*, *bmp1* and *bmp3* to test the hypothesis that genes associated with chondrogenesis in the development of vertebrae in the embryo, are not expressed in the regenerating tail. We also include *pax1* since it mediates the notochordal signal required for differentiation of the ventral part of the vertebrae (Koseki et al., 1993).

Our transcriptome data show that, in general, the chondrogenesis-related genes had low transcript abundance in the early regenerating tail compared to the embryonic tail (Figure 3-8). Exceptions were *wnt3a* and *bmp3* which had expression levels during early regeneration comparable to the embryonic tail. At later stages of adult tail regeneration, only *wnt5a* and *bmp1* showed equal or higher transcript abundance than the embryonic tail. *Pax1*, a gene critical for the development of the ventral vertebrae in the embryo, has a very low level of expression at all stages.

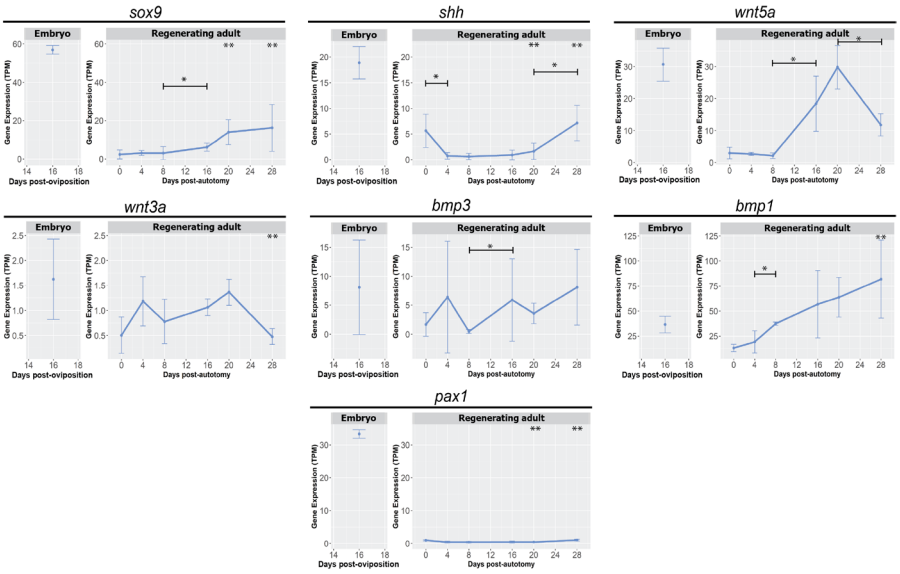

Figure 3-8. **Expression profiles of chondrogenesis genes in the embryonic tail (left) and during adult tail regeneration (right)**. One asterisk (*) shows significant differential expression in stage-wise comparison. Two asterisk (**) shows significant differential expression compared to embryonic tail 16 dpo. Significant differential expression (DE) genes with adjusted p-value < 0.01 and $L_2FC \geq 1$.

The skeletal muscles of the regenerating tail are formed segmentally, as a longitudinal series of myomeres separated by fibro-connective tissue myosepta. We wanted to examine whether muscle development in the regenerating tail resembles the development of axial muscles in the embryo. To do this, we looked at the transcript profiles of genes related to muscle development, namely: *pax3*, *pax7*, *myod1*, *myog*, *ckm*, and *tnc*. Furthermore, we hypothesized that the segmentation of the regenerating muscles was based on the activity of genes that are normally involved in embryonic somite segmentation. To test this hypothesis, we looked at the expression of *lfng* and *hes7*, because they are known to be involved in somite segmentation (Bessho et al., 2001; Chen et al., 2005; Kageyama et al., 2012; Okubo et al., 2012) . We also looked at paralogs of *hes7*, namely *hes1*, *hes2*, *hes5*, and *hes6* to see if they have acquired a function in regeneration (by neofunctionalization).

If we examine Figure 3-9A, we can see that *pax3* were significantly upregulated at 20 dpa. *Pax7*, *myod1*, *myog* and *ckm* all showed a significant increase in transcript abundance at stage 20–28 dpa. At 28 dpa, *myog*, *ckm*, and *myod1* were all significantly upregulated. All of the somite segmentation genes (Figure 3-9) except *hes2* showed higher expression in the embryo tail than in the regenerating tail. The other major finding was that *lfng* was significantly upregulated at 28 dpa.

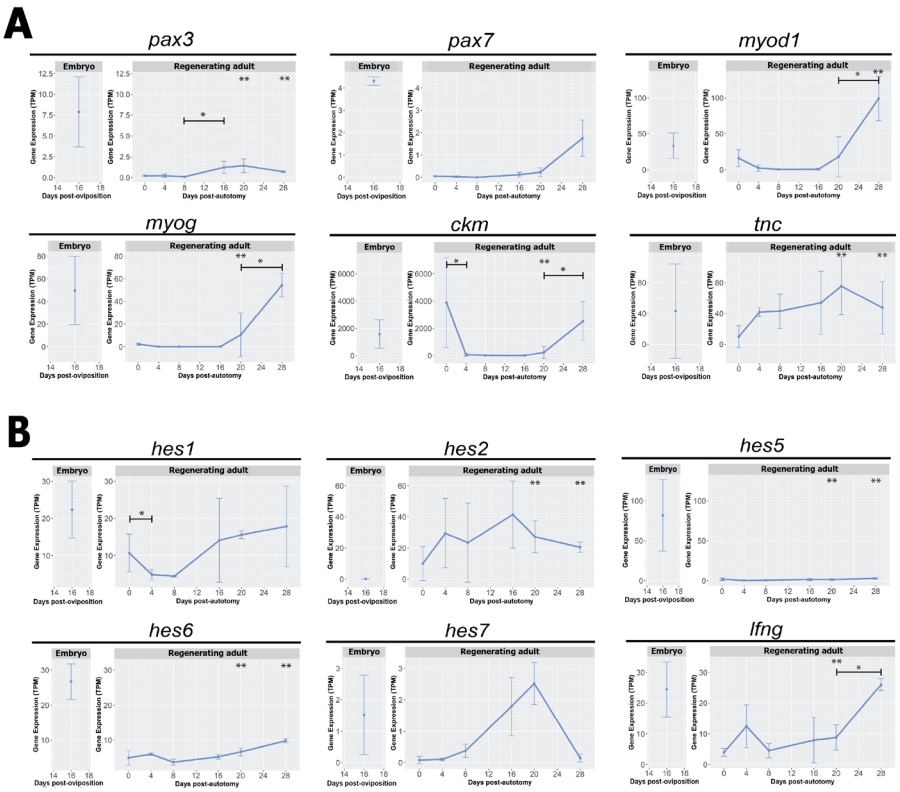

Figure 3-9. Expression profiles of myogenesis genes (A) and somite segmentation genes (B) in the embryonic tail (left) and during adult tail regeneration (right). One asterisk (*) shows significant differential expression in stage-wise comparison. Two asterisk (**) shows significant differential expression compared to embryonic tail 16 dpo. Significant differential expression (DE) genes with adjusted p-value < 0.01 and $L_2FC \geq 1$.

We also examined the transcript abundance of these axonogenesis-related genes in the transcriptomes. *Gap43* and *sema3a* showed statistically significant downregulation when compared to the embryonic tail transcriptome. *Gap43* was significantly downregulated between 0 and 4 dpa and its expression is significantly lower in the adult regenerating tail than in the embryo tail. *Sema3a* expression was significantly lower in the regenerating tail at all stages, than in the embryonic tail (Figure 3-10). Visual inspection of the graphs indicates that all of them, except *nefh,* had much lower expression in the regenerating tail than in the embryonic tail (Figure 3-10).

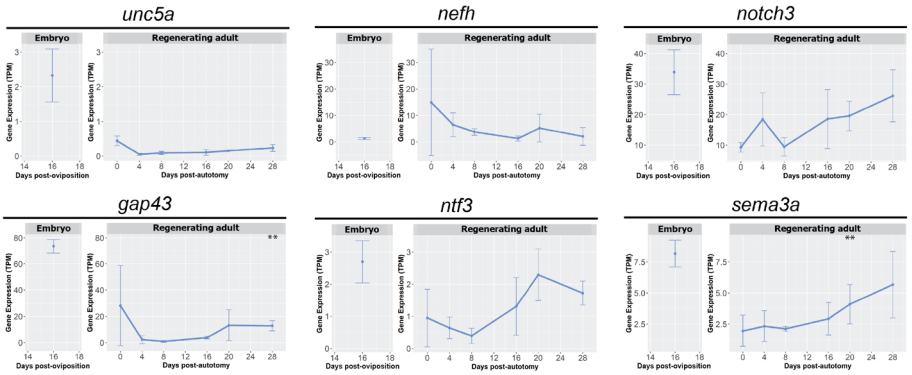

Figure 3-10. **Expression profile of axonogenesis genes in the embryonic tail (left) and during adult tail regeneration (right)**. One asterisk (*) shows significant differential expression in stage-wise comparison. Two asterisk (**) shows significant differential expression compared to embryonic tail 16 dpo. Significant differential expression (DE) genes with adjusted p-value < 0.01 and $L_2FC \geq 1$.

Discussion

Our analysis of mRNA-seq data from regenerating tails at different stages revealed a unique molecular signature in each stage of gecko tail regeneration which we have summarized graphically in (Figure 3-11). We found a much higher number of differentially-expressed genes at the initial stage (0–8 dpa) of tail regeneration compared to what is seen in tail regeneration in the Common Wall Gecko (*Hemidactylus frenatus*) (Nagumantri et al., 2021).

The early-regeneration transcriptome was dominated by immune-related genes and genes associated with wound epithelium formation and bone remodeling. During the early stages of regeneration, immune-related genes play a vital role in both protecting against infection and initiating blastema formation (Londono et al., 2020; Tsai et al., 2019; Vitulo et al., 2017). Additionally, platelets in blood clots release chemokines that aid in recruiting inflammatory cells like neutrophils and macrophages to wound sites (Darby et al., 2014). Macrophages are known to have an important role in the regeneration of many species, for instance, salamanders , zebrafish (Laplace-Builhé et al., 2021), and lizards (Vonk et al., 2023), and spiny mouse (Simkin et al., 2017). Macrophages contribute significantly to tissue regeneration by clearing debris from the wound site by phagocytosis, and by facilitating the shift from inflammation to regeneration. They are able to change their phenotype, which is essential for tissue remodeling and functional recovery (Wynn and Vannella, 2016). Thus, macrophages have functionality for potential guidance and modulation to shift from inflammation to regeneration (Mescher et al., 2015; Oishi and Manabe, 2018; Wynn and Vannella, 2016).

Our data also indicate the enrichment of NF-kappa B signaling and JAK-STAT signaling pathways at this early stage. Both pathways are known for their involvement in inflammatory responses (Hu et al., 2021; Liu et al., 2017). However, they also have some roles in tissue regeneration. NF-kappa B signaling has a role in bone regeneration (Mishra et al., 2020), while JAK-STAT signaling has a role in muscle regeneration (Price et al., 2014; Tierney et al., 2014). This indicates that the initial immune response is likely essential for triggering the regenerative cascade in tokay gecko tails.

Another group of genes enriched in the early stages (4–8 dpa) are the bone remodeling genes. These genes are essential for bone regeneration (Dimitriou et al., 2011; Dirckx et al., 2013), which in the case of tokay gecko tail regeneration is the cartilage tube formation. Epidermis development-related genes are also enriched at this stage, which is consistent with the formation of the wound epidermis, a critical structure for successful regeneration (Alibardi, 2012). The wound epidermis forms early after amputation and provides signals to underlying cells to start the regeneration process. Specifically, the wound epidermis produces factors including FGFs that stimulate blastema formation and outgrowth (Alibardi, 2012).

In addition, we found that cancer pathways are enriched in this stage. These cancer-related pathways might indicate the high proliferative capacity and avoidance of apoptosis that characterize the regeneration blastema (Figure S 3-3). Other workers have suggested similarities between the blastema and cancers (Alibardi, 2022; Oviedo and Beane, 2009). Further investigation into the relationship between regeneration and cancer-related processes could yield valuable insights, as both involve rapid cell division and tissue growth, despite being regulated through distinct mechanisms. It might also shed light on how animals capable of body-axis regeneration like zebrafish, salamander, and lizards evolved the ability to regenerate complex structures while avoiding the cancer-like uncontrolled growth (Alibardi, 2019; Greco et al., 2023; Oviedo and Beane, 2009; Sarig and Tzahor, 2017).

Contrasting with the early inflammatory and proliferative phase, we observed a shift towards more developmental and patterning processes during the blastema formation stage (16-20 dpa). The blastema, a mass of proliferating progenitor cells, is the key structure that drives regenerative outgrowth. The regeneration blastema in the tokay gecko tail is much larger than the blastema in the regenerating zebrafish caudal fin (Pfefferli and Jazwinska, 2015) and the regenerating axolotl limb (Gerber et al., 2018; Kragl et al., 2009). We find that the onset of blastema formation involves mass up-regulation of genes associated with developmental pathways, and down-regulation of immune-related genes. Because of this transcriptomic profile, we believe that 16–20 dpa is the dominant phase of pattern formation.

For example, genes in the WNT signaling pathway are enriched at this stage. This pathway is not only involved in developmental patterning (Komiya and Habas, 2008) but is also essential for tissue regeneration by maintaining stem/progenitor cell fate and coordinating regenerative outgrowth (Clevers et al., 2014; Wang et al., 2007). In the regenerating lizard tail, Wnt signaling pathways are crucial for blastema formation by promoting cell proliferation and regulating proximo-distal patterning (Alibardi, 2020).

Pathway visualizations of our data suggest that Wnt signaling pathways in the regeneration tail are more involved in regulating tissue patterning and remodeling processes, rather than primarily inducing pluripotency (Figure S 3-4, Figure S 3-5). This modelling predicts that the blastema in the tokay gecko is not the result of any kind of dedifferentiation. Instead, it arises from lineage-specific progenitor cells originating from diverse tissues surrounding the wound site, which coordinate to regenerate the complex structure in a regulated manner (discussed also by (Clevers et al., 2014). This is further supported by the enrichment of muscle activity-related and muscle development gene ontology terms during the blastema formation stage, indicating that muscle differentiation and reorganization occur concurrently with the patterning processes.

We also found that Hedgehog signaling pathways were enriched in these stages (pathway visualization in Figure S 3-6). Hedgehog signaling is critical for many developmental processes in vertebrates (Varjosalo and Taipale, 2008). This pathway, in response to BMP signaling, is essential in initiating chondrogenesis in the regenerating lizard tail (Lozito and Tuan, 2015; Lozito and Tuan, 2016; Vonk et al., 2023). Hedgehog signaling is also known to interact with Wnt and Notch signaling to pattern vertebrate body axes and somites (Lewis et al., 2009). Thus, the simultaneous activation of Wnt, Notch, and Hedgehog pathways in the regenerating tokay gecko tail suggests the redeployment of a complex network of conserved developmental regulators to repattern the lost structure (Hikasa and Sokol, 2013).

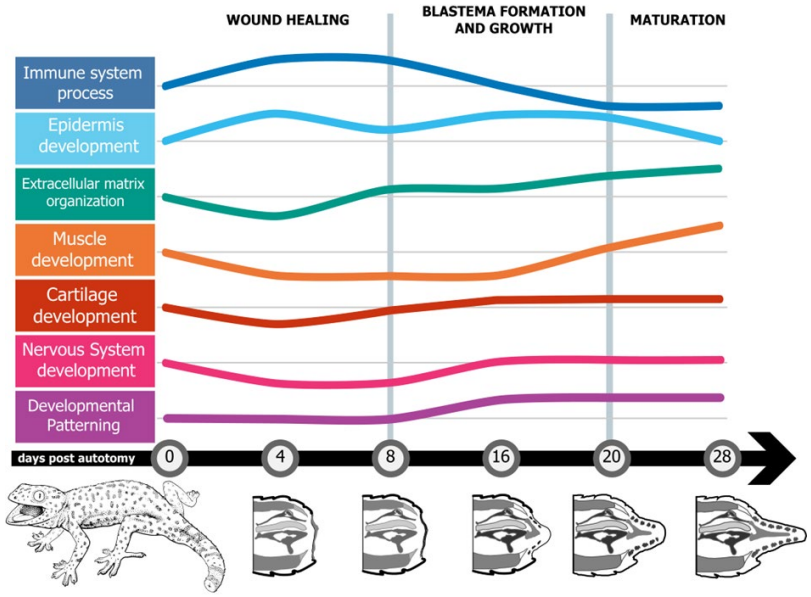

Figure 3-11. **Schematic overview of biological process enrichment in the transcriptome at selected stages of tail regeneration.** The height of each line represents (schematically) the relative enrichment or the process. The greyscale drawings at the bottom represent the histological structure of the regenerating tail at that stage.

Tail regeneration of a lizard involves the development of different tissues or organs, including skeletal muscle, cartilage tube, skin, nerve fibers and others (Di-Poi et al., 2010; Holzman et al., 2018; Kawanishi et al., 2003; McGinnis and Krumlauf, 1992; Spitz et al., 2001; van den Akker et al., 2001). Organ development involves a highly regulated process governed by specific gene regulatory networks (GRNs) and signaling pathways (Zaret, 2002). These pathways ensure proper cell differentiation, tissue formation, and organogenesis through precise control of gene expression. Some other vertebrates such as salamanders and zebrafish are known to reactivate embryonic patterning mechanisms in the blastema during regeneration (Gerber et al., 2018; Pfefferli and Jazwinska, 2015; Randal Voss et al., 2018; Thummel et al., 2007; Wouter et al., 2024).

Interestingly, we find that the gene expression profile of the regenerating tail does not fully recapitulate that of embryonic tail development. When we compared the transcriptome of the adult regenerating tail and the embryonic tail, we found that the regenerating tail shares a limited subset of differentially expressed genes and pathways with the embryonic tail. The regenerating tail is dominated in its early stages by the upregulation of immune system genes, while the embryonic tail is dominated by classical developmental regulatory genes (Figure 3-12).

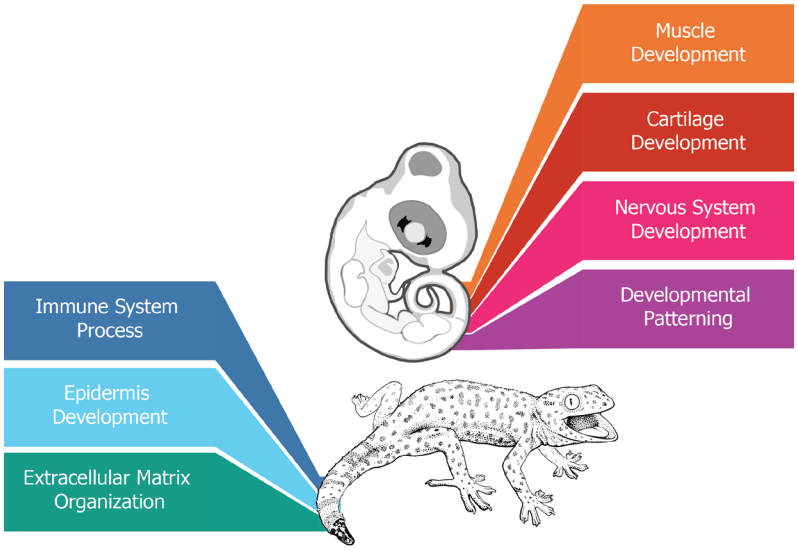

Figure 3-12. **Tail regeneration is not simply a recapitulation of embryonic development.** Schematic illustration highlighting the major differences in the dominant pathways in the regenerating tail vs. the embryonic tail.

The regenerated lizard tail is a functional replica of the original but shows some anatomical differences (Di-Poi et al., 2010; Holzman et al., 2018; Kawanishi et al., 2003; McGinnis and Krumlauf, 1992; Spitz et al., 2001; van den Akker et al., 2001). For instance, the axial skeleton of the normal tail of the gecko is made up of segmentally arranged vertebrae. In the regenerating gecko tail, however, the vertebrae are replaced by a continuous, unsegmented 'cartilage tube' (Lozito and Tuan, 2015). The other major difference is in the nervous system which in the regenerating tail only has the ependymal tube and nerve innervation instead of a complete spinal cord and nerve extensions characteristic of the original tail (Alibardi, 2014; McLean and Vickaryous, 2011). These anatomical differences indicate that the regeneration process only partially recapitulates the embryonic development of the tail and likely relies on distinct mechanisms to reform the lost structures.

Our histological analysis in Chapter 2 revealed that the regeneration blastema in the tokay gecko tail lacks a distinct apical growth zone that might function as a developmental field. As we will show in Chapter 5, the expression profile of the pre-blastema is more consistent with a heterogeneous mix of connective tissue and cells of the muscle lineage. These differences between the gecko blastema and, say, an embryonic tailbud or an axolotl regeneration blastema, may explain why the gene expression profile of the regenerating tokay gecko tail does not fully recapitulate that of embryonic tail development. In contrast, during axolotl limb regeneration, an apical growth zone forms within the blastema, and this growth zone exhibits gene expression

patterns similar to embryonic limb development (Bryant et al., 2002; Gardiner et al., 1995; Gerber et al., 2018; Kragl et al., 2009; McCusker et al., 2015; Muneoka and Bryant, 1982; Muneoka and Bryant, 1984). Similarly, the blastema in zebrafish tail regeneration retains similarities with the embryonic tail bud (Pfefferli and Jazwinska, 2015). Consequently, both axolotl limb and zebrafish tail regeneration are able to reform structures closely resembling the original limb and tail, respectively, through major reactivation of embryonic developmental programs.

In summary, our data demonstrate that the regenerating tail of the tokay gecko does not completely recapitulate the embryonic tail development program. Instead, it activates a limited set of developmental pathways, such as Wnt and Hedgehog, along with genes involved in cell proliferation, immune response, and tissue remodeling to reform only some of the lost structures, and some of those structures (including the scales) are anatomically distinct from those of the original tail. In Chapter 5, we will further discuss the spatial and tissue-specific expression of these organogenesis related genes.

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Supplementary Information

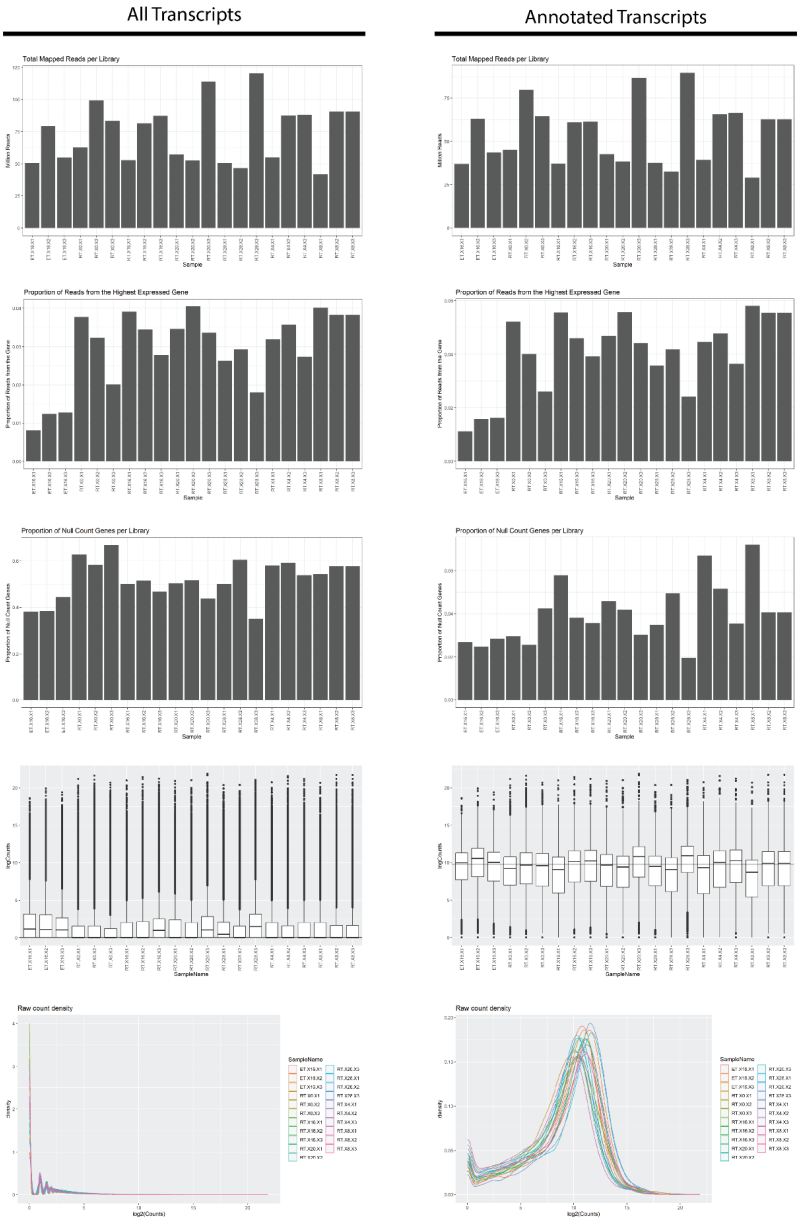

Figure S 3-1. **Quality control results of transcriptome analysis in this chapter.** Note that using only annotated transcript for further analysis give more distinguishable sample comparison.

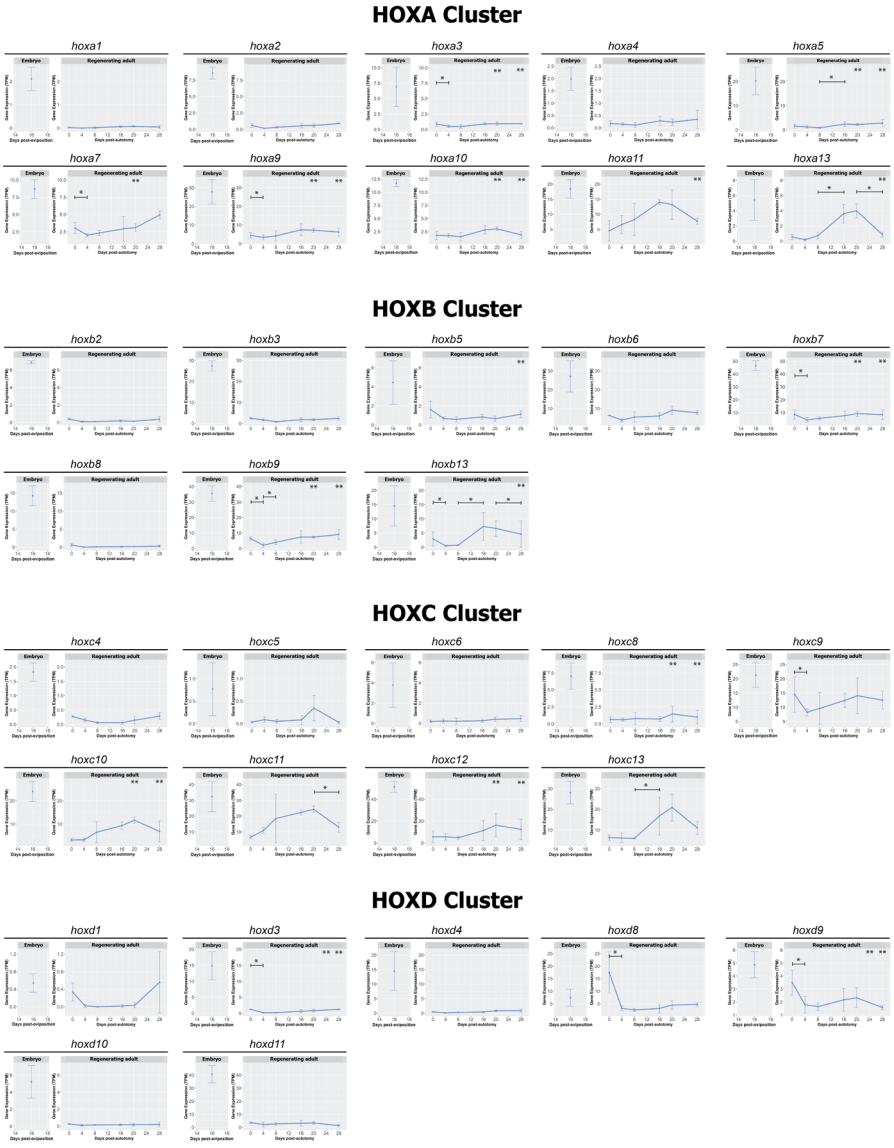

Figure S 3-2**. The transcriptome profiles of HOX genes in the embryonic and the regenerating tail of tokay gecko (*G. gecko*).**

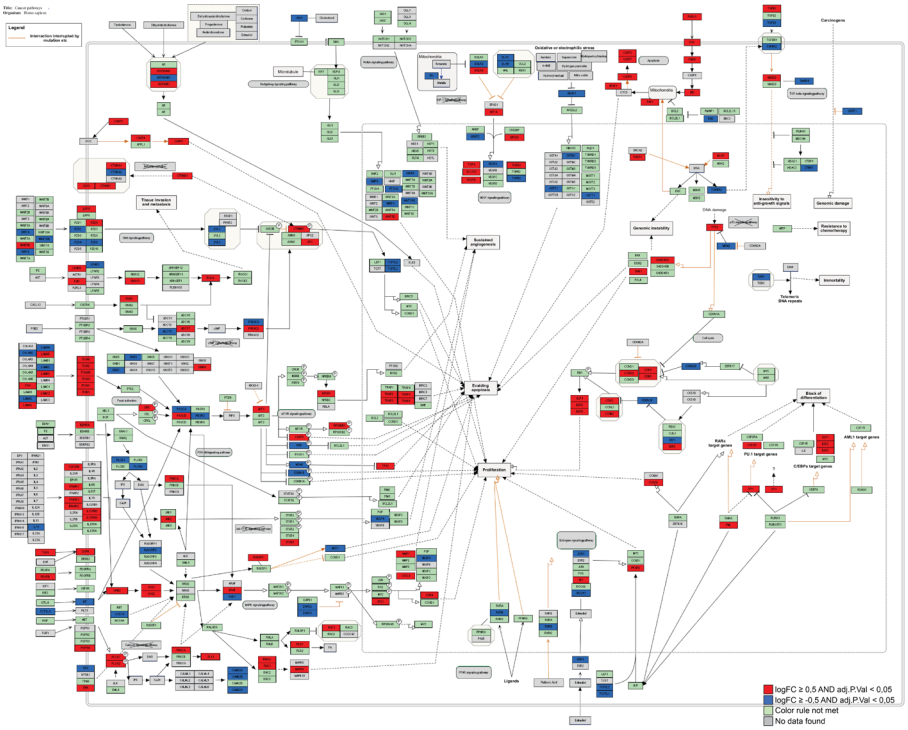

Figure S 3-3. **Pathway visualization of Cancer pathways in the 4 dpa regenerating tail of the tokay gecko**. The pathway visualization was made using Pathvisio based on differential gene expression in the 4 dpa *vs*. 0 dpa comparison.

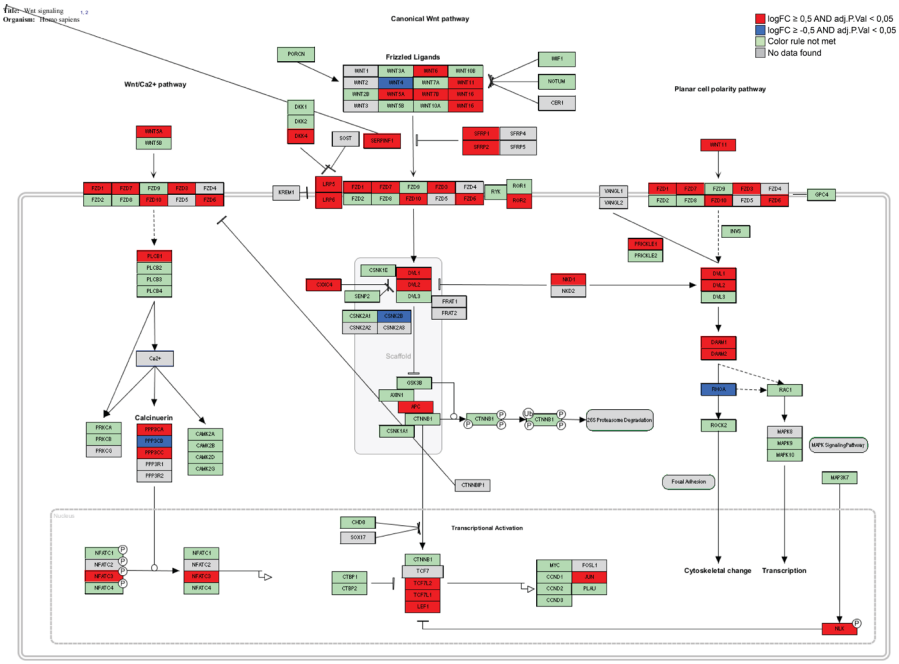

Figure S 3-4. **Pathway visualization of Wnt signaling pathways in the 16 dpa regenerating tail of the tokay gecko.** The pathway visualization was made using Pathvisio based on differential gene expression in the 16 dpa *vs.* 8 dpa comparison.

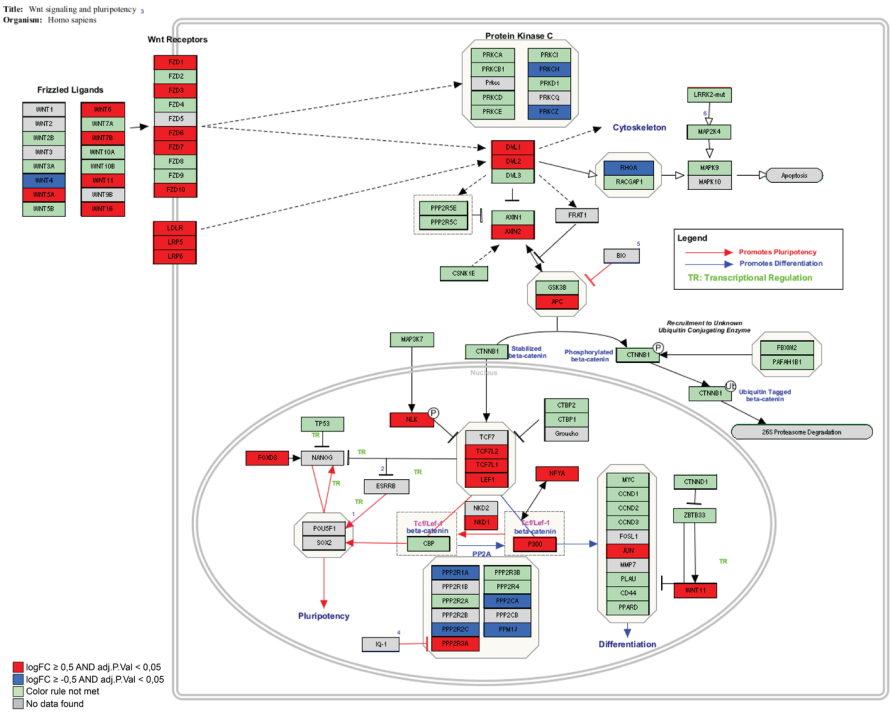

Figure S 3-5. **Pathway visualization of Wnt signaling and pluripotency pathways in the 16 dpa regenerating tail of the tokay gecko.** The pathway visualization was made using Pathvisio based on differential gene expression in the 16 dpa *vs.* 8 dpa comparison.

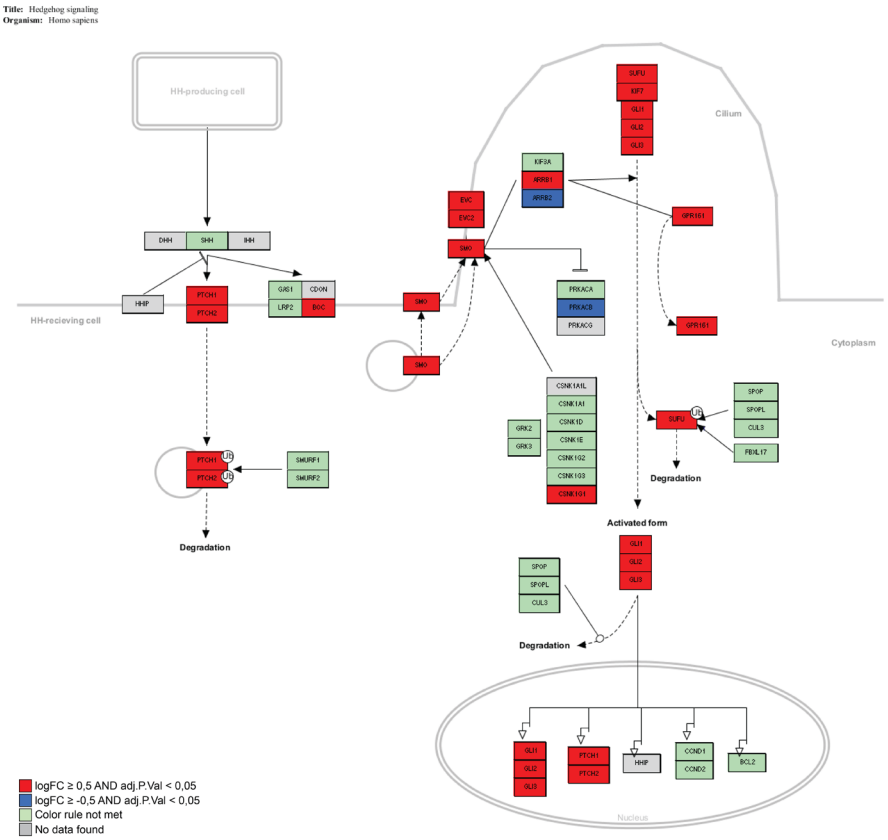

Figure S 3-6. **Pathway visualization of hedgehog signaling pathways in the 16 dpa regenerating tail of the tokay gecko**. The pathway visualization was made using Pathvisio based on differential gene expression in the 16 dpa *vs.* 8 dpa comparison.