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Salamander-like tail regeneration in the West African lungfish

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Salamanders, frog tadpoles and diverse lizards have the remarkable ability to regenerate tails. Palaeontological data suggest that this capacity is plesiomorphic, yet when the developmental and genetic architecture of tail regeneration arose is poorly understood. Here, we show morphological and molecular hallmarks of tetrapod tail regeneration in the West African lungfish *Protopterus annectens*, a living representative of the sister group of tetrapods. As in salamanders, lungfish tail regeneration occurs via the formation of a proliferative blastema and restores original structures, including muscle, skeleton and spinal cord. In contrast with lizards and similar to salamanders and frogs, lungfish regenerate spinal cord neurons and reconstitute dorsoventral patterning of the tail. Similar to salamander and frog tadpoles, *Shh* is required for lungfish tail regeneration. Through RNA-seq analysis of uninjured and regenerating tail blastema, we show that the genetic programme deployed during lungfish tail regeneration maintains extensive overlap with that of tetrapods, with the upregulation of genes and signalling pathways previously implicated in amphibian and lizard tail regeneration. Furthermore, the lungfish tail blastema showed marked upregulation of genes encoding post-transcriptional RNA processing components and transposon-derived genes. Our results show that the developmental processes and genetic programme of tetrapod tail regeneration were present at least near the base of the sarcopterygian clade and establish the lungfish as a valuable research system for regenerative biology.

1. Introduction

In living tetrapods, the capacity to regenerate tails is present in salamanders, frog tadpoles and some lizards. Palaeontological evidence has shown salamander-like tail regeneration in lepospondyl microsaurs, tetrapods from the early Carboniferous and early Permian [1]. Whether salamander-like tail regeneration capacity was the early condition of this group remains uncertain. Given their phylogenetic position as the extant sister group to tetrapods, lungfish might hold the key to our understanding of the evolution of regenerative capacities in tetrapods.

Lungfishes are capable of regenerating complete tails as adults. This remarkable capacity was first reported nearly 150 years ago [2] and documented in the laboratory half a century ago [3]. Since then, despite its potential as a model research system for regenerative medicine and important phylogenetic position, no reports on lungfish tail regeneration have followed, likely due to the logistical difficulties in obtaining and housing lungfish as laboratory animals.

By contrast, decades of studies have and continue to shed light on the morphological and molecular processes of tail regeneration in tetrapods. In salamanders and frog tadpoles, soon after tail amputation, a wound epithelium forms and covers the injured site [4]. The thickening of the wound epidermis gives rise to the apical epithelial cap (AEC) and, at this point, patterns of tail regeneration diverge between salamanders and frog tadpoles. In salamanders, undifferentiated cells accumulate at the amputation site, giving rise to the blastema [4]. In tadpoles, a mass of undifferentiated cells accumulates around the neural ampulla and notochord tip, forming a blastema-like cell population, termed regeneration bud [5]. In salamanders, muscle regeneration can occur via two mechanisms: the proliferation of dedifferentiated muscle cells or proliferation of Pax7+ satellite cells [6–8]. In tadpoles, muscle regeneration is achieved chiefly via the proliferation of Pax7+ satellite cells [9,10]. Ultimately, frog tadpoles and salamanders regenerate tails with high fidelity, redeploying all tissue types and anatomical patterns of their original tail.

Lizards are the only amniotes capable of tail regeneration through wound healing and blastema formation. However, instead of regenerating the spinal cord and associated skeleton, a simple ependymal tube composed of neuroglia develops enclosed by an unsegmented cartilage tube [11,12]. The new ependymal tube and tail skeleton in lizards fail to recapitulate the embryonic dorsoventral expression pattern of genes responsible for establishing roof plate, floor plate, and the lateral domains of the neural tube. Specifically, floor plate markers *Shh* and *FoxA2* in lizards are expressed along the entire regenerating ependymal tube, which consequently acquires a floor plate identity [13].

Despite the differences in the regenerative process in amphibians and amniotes, molecular studies have revealed broad similarities in the genetic programme of tail regeneration. Studies in frog tadpoles have identified a sequence of major molecular events leading to successful tail regeneration. In summary, upon injury, TGF- β signalling is required for the formation of the wound epidermis [14]. Extracellular matrix (ECM) remodelling, reactive oxygen species (ROS) signalling and inflammation are also among the first responses to tail injury [15]. Regeneration-organizing cells relocate to the wound epithelium and express Wnts and Fgfs [16]. As the regeneration bud forms and tail outgrowth occurs, additional signalling pathways are deployed, such as Bmp, Egf, Shh, Tgf- β and Notch pathways, among others [9,17,18]. Proliferating blastema cells show high expression levels of *Il11*, *Cse11* and *L1td1-like* [19] and require histone deacetylases [20] and hyaluronan, an ECM component [21] and *Il11* [22]. Likewise, during salamander tail regeneration, Wnt, Fgf and Tgf- β pathways [18,23], ion channels [24], Shh signalling [23,25], Egf, Notch and other signalling pathways [18] are required. Regenerating spinal cords also display upregulation of genes linked to immune and inflammatory response, ECM remodelling and genes encoding morphogens such as Shh, Bmps, Wnts and Fgfs [26]. Finally, morpholino-mediated knock-down of Marcks-like protein (Mlp), an extracellular protein, blocks tail regeneration [27].

The molecular profile of lizard tail regeneration has also been examined in recent years. In the common wall lizard (*Podarcis muralis*), genes exclusively upregulated in the regenerating tail include those coding for growth factors (Wnts, Shh, Bmps and Fgfs) and those within the broad categories of ECM, inflammation and immunity, metabolism and cytoskeleton [28]. Similarly, tail regeneration in geckos shows enrichment for

peptides involved in immune response, ECM remodelling, Fgfs and Bmps [29] and involves ROS signalling [30], Tgf- β signalling [31] and Fgf signalling [32]. In the green anole lizard (*Anolis carolinensis*), the regenerating tail is enriched for the gene ontology (GO) categories of the wound response, immune response, hormonal regulation and embryonic morphogenesis [33].

In sum, current molecular data have begun to reveal the broad genetic architecture of tetrapod tail regeneration, yet the evolutionary origins of this regenerative programme remain unclear. Leveraging the phylogenetic position of lungfish as an outgroup of tetrapods, we sought to characterize the morphological and genetic profile of lungfish tail regeneration. We found that lungfish tail regeneration proceeded in a salamander-like manner, with the formation of a proliferative blastemal cell population and the restoration of original tail structures including muscle, spinal cord, tail fin skeleton and proper dorsoventral patterning. Shh signalling, fundamental for amphibian tail dorsoventral patterning and regeneration, is also required for lungfish tail regeneration. RNA-seq analysis revealed that lungfish deploy a blastemal genetic programme similar to that reported in tetrapods. Interestingly, lungfish tail blastema showed the marked upregulation of transposon-derived genes and components of post-transcriptional RNA processing. Our findings suggest that salamander-like tail regeneration was present in the sarcopterygian ancestor of tetrapods and lungfish.

2. Methods

(a) Animals and surgical procedures

Thirty juvenile West African lungfish (*Protopterus annectens*) ranging from 15 to 35 cm in length were acquired through the pet trade and housed at the Universidade Federal do Para. Specimens were kept in individual 41 gallon tanks in dechlorinated tap water at 24–28°C with aeration and biological and mechanical filtration. Lungfish were anaesthetized in 0.1% MS-222 (Sigma-Aldrich). The position of amputation was determined as follows: the distance from the snout to the attachment point of the pelvic fin was measured in centimetres and divided by 1.75. The resulting number was then used as the distance from the attachment point of the pelvic fin to the point of tail amputation. Samples were labelled as ‘uninjured’ or ‘blastema’ and embedded in Tissue Tek O.C.T compound (Sakura Finetek), or stored in RNAlater (Sigma-Aldrich) at –80°C temperature for RNA extraction, frozen on dry ice for histology and *in situ* hybridization (stored at –80°C), or fixed in 4% paraformaldehyde (PFA) overnight at 4°C for section-immunohistochemistry.

(b) External morphology and histology of tail fin regeneration

Tails from animals under anaesthesia were photographed at 1 day post-amputation (dpa) and weekly to document the changes on external morphology during regeneration. For histology, frozen tissues were allowed to adjust to cryostat temperature (–20°C) for 30 min. Next, 20 μ m longitudinal sections were obtained and placed on ColorFrost Plus microscope slides (Thermo Fisher Scientific). Sections were fixed in 3% PFA for 5 min, rinsed twice in 0.1 M PBS and dehydrated in graded ethanol series (70, 95 and 100%) for 2 min each. Slides were stored at –80°C. Sections were stained with haematoxylin (Sigma-Aldrich) and eosin (Sigma-Aldrich) and imaged on an SMZ1000 stereoscope (Nikon). Tails were cleared and stained as described previously [34]. In total, histological sections were obtained from six animals, and tails from five animals were used for clearing and staining.

(c) Inhibition of Shh signalling

Cyclopamine (Selleckchem, cat. number S1146) dissolved in DMSO was added to the aquarium water at a final concentration of $1 \mu\text{g ml}^{-1}$. Control group was treated with DMSO at a final concentration of 0.1%. In both DMSO-only ($n=3$) and cyclopamine-treated ($n=3$) groups, aquarium water was changed daily with fresh cyclopamine or DMSO-only solution for six weeks. Animals were photographed and measurements of total tail length were taken weekly.

(d) Cell proliferation assay and immunohistochemistry

5-Bromo-2-deoxyuridine (BrdU) was injected intraperitoneally (80 mg per kg of body weight) into anaesthetized lungfish 24 h before tail tissue collection to observe cell proliferation. Overnight-fixed tissues were transferred to 30% sucrose, flash-frozen in OCT blocks; longitudinal and transverse sections (20 μm thickness) were obtained as described in the preceding section. Sections were permeabilized in 2 N HCl solution at 37°C for 15 min, followed by washes in 0.1 M borate buffer and in PBS Tween (0.1% Tween in 0.01 M PBS). Treatment with 0.1% trypsin at 37°C for 15 min was performed and followed by a wash in PBS Tween. Unspecific labelling was blocked with 5% normal goat serum diluted in 0.01 M PBS with 0.5% Tween and 1% bovine serum albumin for 1 h at room temperature. Next, sections were incubated with mouse anti-BrdU primary antibody (1:200, Sigma-Aldrich, cat. number B8434) in 0.01 M PBS with 1% bovine serum albumin and 0.5% Tween overnight at 4°C . On the following day, sections were incubated with the Alexa 488 conjugated goat anti-mouse secondary antibody (1:400, ThermoFisher Scientific, cat. number A-11001) for 2 h at room temperature and slides were mounted and counterstained with Fluoromount with DAPI (1:1000, ThermoFisher Scientific, cat. number 00-4959-52). For immunohistochemistry, anti- β -tubulin (mouse monoclonal, 1:500, Sigma-Aldrich, cat. number ab78078), anti-myosin heavy chain (MHC, mouse monoclonal, 1:200, Developmental Studies Hybridoma Bank, cat. number MF20), was performed using the same procedure except for HCl and trypsin treatments, followed by incubation with the Alexa Fluor 594 goat anti-mouse secondary antibody (1:1000, ThermoFisher Scientific, cat. number A-11005) and Alexa Fluor 488 phalloidin (1:1000, ThermoFisher Scientific, cat. number A-12379).

(e) Library preparation and illumina sequencing

For transcriptome sequencing, total RNA from tail tissues was extracted using TRIzol Reagent (Thermo Fisher). A two-step protocol, with the RNeasy Mini Kit (Qiagen) and DNase I treatment (Qiagen), were used to purify the RNAs and remove residual DNA. mRNA sequencing libraries were constructed using the NEXTflex® Rapid Directional qRNA-Seq™ Kit (Illumina). Lungfish reference transcriptomes and transcript abundance estimation were obtained from the sequencing of three biological replicates of blastemas at 14 dpa and three biological replicates of uninjured tail tissue, performed on an Illumina 2500 HiSeq platform with 150 bp paired-end reads. Reads from six additional runs of other regenerating tail stages were used only to help produce a comprehensive *de novo* lungfish reference transcriptome assembly.

(f) Bioinformatic analysis

The West African lungfish reference transcriptome was assembled *de novo* using Trinity-v2.9.0 with default parameters [35]. The transcriptome assembly was subjected to the EvidentialGene pipeline for greater accuracy of gene set prediction [36]. For each run, all read datasets were mapped to reference transcriptomes using CLC genomics workbench with default parameters (Qiagen). Expression data, measured by transcript count, were summed by

human homologue gene cluster (HHGC) using a custom bash script. As previously described [37], the HHGCs were defined by grouping transcripts with an e-value of 10^{-3} when compared by BLASTx against Human NCBI RefSeq database. For each HHGC, expression was calculated in transcripts per million (TPM) and mean TPM value between uninjured tails and tail blastemas were compared with a two-tailed t-test using the CLC genomic workbench with default parameters (Qiagen). A transcript or HHGC was deemed differentially expressed when its fold change is greater than 2 or less than -2 and the FDR adjusted p -value < 0.05 . A similarity matrix between samples was calculated using square root transformed TPM values for each HHGC, using Spearman's rank correlation in Morpheus software (<https://software.broadinstitute.org/morpheus>). A list of enriched GO terms and over-represented Reactome pathways was produced using WebGestalt 2019 [38]. Differentially expressed genes with false discovery rate (FDR) adjusted p -values smaller than 0.05 were ranked from highest to lowest fold change values, and the corresponding ranked list of gene symbols was used for the GO enrichment analysis. GO enriched categories or over-represented Reactome pathways were considered significant when p -values were 0.05 or less. Venn diagrams were generated using BioVenn [39]. GO enrichment and pathway over-representation analyses were performed using WebGestalt 2019 web-based tool. Protein domains were identified using HMMER v3.2.1 (<http://hmmer.org/>) against the proteome generated using TransDecoder v5.3.0 (<http://transdecoder.github.io>).

(g) In situ hybridization

Frozen sections (20 μm) from regenerating tails at 14 dpa ($n=3$) were obtained on a Leica CM1850 UV cryostat and positioned on the ColorFrost Plus microscope slides (Thermo Fisher). Sections were fixed as previously described [37] and stored at -80°C for haematoxylin and eosin staining or *in situ* hybridization. Riboprobe templates for *in situ* hybridization were produced by a two-round PCR strategy: first-round PCR produced specific fragments (400–500 bp) of selected genes, and in a second PCR a T7 promoter sequence was included at either 5' or 3' end of the fragments for the generation of templates for sense or antisense probes. The primers used were: *Col12a1* forward: 5'-GGCCGCGGTTGATGCTCCCATTGGTTAG-3' and reverse: 5'-CCCAGGGGCGAAACCCAGGAACAAGAGGTC-3', *Hmgn2* forward 5'-GGCCGCGGTTGAGCAGAACCAGCTTCATT-3' and reverse 5'-CCCAGGGGCTTAGTG GGGCAGACAATCAAC-3', *Inhbb* forward 5'-GGCCGCGGCGGTGCTTGAACCACTAAAAA and reverse 5'-CCCAGGGGCTTTG CAGAGACAGATGACGTG-3', 3'-T7 universal 5'-AGGGATCC TAATACGACTCACTATAGGGCCCGGGGC-3', 5'-T7 universal 5'-GAGAATTCTAATACGACTCACTATAGGGCCGCGG-3' (linker sequences for annealing of the 3' or 5' T7 universal primer are underlined). The riboprobes were synthesized using T7 RNA polymerase (Roche) and DIG-labelling mix (Roche). *In situ* hybridization was performed as previously described [37], using 375 ng of DIG-labelled riboprobe per slide. Slides were photographed on the Nikon Eclipse 80i microscope and the images were processed on the NIS-Element D4.10.1 program.

3. Results

(a) Establishment of a proliferative blastemal cell population during lungfish tail regeneration

We evaluated regeneration in juvenile West African lungfish upon tail amputation (figure 1a). At 7 dpa, a wound epithelium covers the amputation site, and tail outgrowth is negligible. At 14 dpa, tail outgrowth is visible at the level of the midline. At 21 dpa, the regenerating tail reaches approximately 1 cm

Figure 1. Morphological characterization of tail regeneration in the West African lungfish. (a) Progression of lungfish tail regeneration and the extent of growth up to 56 dpa. Vertical bars in the graph represent standard deviation. (b) Histological sections of regenerating lungfish tail. (c) The regeneration of skeletal elements of the tail at 60 dpa. (d) BrdU staining of proliferative cells during tail regeneration. We, wound epithelium; aec, apical epithelial cap; bl, blastema; et, endodermal tube; ptc.c, postcaudal cartilage; ns, neural spine; na, neural arch; hs, haemal spine; ha, haemal arch. Scale bars of 1 cm (a), 1 mm (b,d), 0.5 cm (c). (Online version in colour.)

in length and the skin is highly pigmented. In the following weeks, the tail continues to extend and by 56 dpa it has nearly reached its length before amputation. Histological sections showed that at 1 dpa, a 1 to 2 cell layer wound epithelium covers the amputation site (figure 1b). At 7 dpa, the wound epithelium thickens, and a mass of mesenchymal cells accumulate subjacent to it, posterior to the severed postcaudal cartilage. At 21 dpa, the regenerated post caudal cartilage bar and endodermal tube are visible. At our latest experimental endpoint (60 dpa), the postcaudal cartilage was undergoing segmentation and cartilaginous neural and haemal arches and spines were visible (figure 1c).

Next, we assessed cell proliferation during the first three weeks of tail regeneration. BrdU staining revealed that at 1 dpa, proliferating cells are mostly found in the wound epithelium. At 14 dpa, proliferating cells are found distal to the amputation plane in the region of the presumptive tail blastema. At 21 dpa, cell proliferation is observed posterior to the amputation site across the entire regenerated tail (figure 1d). Our results indicate that lungfish tail regeneration proceeds via morphological events similar to those involved in salamander tail regeneration, with the establishment of a wound epithelium, which thickens to form an AEC, the formation of a mass of proliferating blastemal cells, and restoration of original tail tissue organization.

(b) Lungfish regeneration restores muscle, spinal cord neurons and dorsoventral patterning of the original tail

To determine whether lungfish regenerating tails reestablish dorsoventral tail patterning, muscle and spinal cord neurogenesis, we examined transversal sections of lungfish uninjured and regenerating tails. In the lungfish, the notochord persists throughout its life [40]. We found that at 21 dpa, the regenerating endodermal tube is dorsally positioned relative to the notochord (figure 2a). Haematoxylin and eosin staining showed newly formed blood vessel ventral to the notochord and regenerating muscle. Phalloidin staining demarcated muscle cells in the uninjured tail and MHC immunostaining revealed muscle cells in the distal portion of the regenerating tail at 28 dpa (figure 2b). Furthermore, immunostaining for β III-tubulin revealed new spinal cord neurons forming as early as 28 dpa (figure 2c).

Shh is a key signalling molecule expressed in the floor plate of the endodermal tube in salamanders and the regenerating notochord in frog tadpoles, which is necessary for tail regeneration in both species [25,41]. To test its requirement for lungfish tail regeneration, we performed pharmacological inhibition of Shh signalling via administration of cyclopamine, an inhibitor of the Hedgehog signalling pathway. We found that in contrast with DMSO treatment (control group),

Figure 2. Establishment of dorsoventral organization and the requirement for Shh signalling during lungfish tail regeneration. (a) Histological transversal sections of uninjured and 21 dpa regenerating tail. (b) Immunostaining of DAPI and phalloidin in the uninjured tail, and DAPI and MHC in the 28 dpa regenerating tail. (c) Immunostaining of DAPI and βIII-tubulin in uninjured and 28 dpa regenerating spinal cord. (d) The effect of DMSO and cyclopamine treatment in tail regeneration. m, muscle; ptc.c, postcaudal cartilage; et, ependymal tube; bv, blood vessel. Scale bars of 1 mm (a and b, panoramic views), 0.5 mm (c, enlarged view) and 1 cm (d). Arrowheads indicate point of amputation and bars in graph represent standard deviation (d). (Online version in colour.)

continuous exposure to cyclopamine completely blocked lungfish tail regeneration, assessed at 42 dpa (figure 2d). Our results suggest that reestablishment of dorsoventral patterning, spinal cord neurogenesis and a requirement of Shh signalling might represent plesiomorphic features of tail regeneration.

(c) Differential gene expression analysis of tail blastema versus uninjured tail

To identify genes differentially expressed in the tail blastema relative to uninjured tail tissue, we produced RNA-seq libraries from uninjured tail tissues and regenerating tails at 14 dpa, a stage when proliferative blastemal cells were identified. The principal component analysis showed two distinct clusters representing uninjured and blastemal tail samples, and Spearman correlation coefficients among biological replicates were greater than 0.78, corroborating the reproducibility of RNA-seq runs (electronic supplementary material, figure S1). DGE analysis of the lungfish uninjured versus 14 dpa tail revealed 1072 upregulated genes ($FC > 2$, $FDR < 0.05$). Among the upregulated gene dataset, we identified components of the various pathways previously associated with tail regeneration in tetrapods, including the Wnt (*Wnt5a*, *Wnt5b*, *Axin2*, *Gsk3b*, *Ctnnb1*), Fgf (*Fgfr1*), Bmp (*Bmp1*, *Bmp4*, *Smad2*), Shh (*Ptch2*, *Gli2*), Notch (*Notch2*), Tgf-β (*Inhbb*, *Tgfb1*), Egf (*Vegfa*, *Megf6*, *Megf10*), ECM components and remodellers (*C1qtnf3*, *Col11a1*, *Fbn2*, *Mmp11*, *Adamts14*), Hyaluron pathway (*Hyal2*, *Has2*), immune and inflammatory response (*Il11*, *Mdk*, *Nfkbiz*) and stem cell maintenance (*Sox4*, *Sall4*, *Chd2*) (figure 3a and c). Some components of pathways previously involved in tetrapod tail regeneration were moderately upregulated ($FC > 1.5$, $FDR < 0.05$), including *Bmp2*, *Hif1a*, *Tgfb1*, *Tgfb2*, *Myc* and *Hdac7* (electronic supplementary material, table S1).

GO enrichment analysis identified categories such as mitotic cell cycle phase transition, extracellular structure organization and regulation of RNA metabolic processing

(electronic supplementary material, figure S2). Likewise, pathway enrichment analysis revealed that the lungfish tail blastema shows a high over-representation of genes in collagen biosynthesis and modifying enzymes, pathways related to mitotic cell division, and ECM organization, consistent with the major events occurring at the blastema stage of tail regeneration (figure 3b). *In situ* hybridization in 14 dpa lungfish tails of two genes encoding ECM components (*Col12a1* and *Hmcn2*) and a gene encoding a member of the TGF-β family of cytokines (*Inhbb*) showed similar expression pattern, with antisense probe signal predominantly detected in the AEC (figure 3d), and no specific signal observed in sense-control probes (electronic supplementary material, figure S3).

Il11, a gene highly upregulated and required for tail regeneration in *Xenopus* tadpoles, was also among the most highly upregulated in our dataset ($FC = 34.95$) (electronic supplementary material, table S1). The lungfish orthologue of *Mlp*, required for axolotl tail regeneration, showed moderate upregulation in lungfish tail and an FDR value just above our cut-off ($FC = 1.41$, $FDR = 0.06$). The lungfish orthologue of *Vwde*, a gene highly expressed and required for axolotl limb regeneration and associated with successful frog tadpole tail regeneration [42], was highly upregulated in lungfish, however, with an FDR value above our cut-off ($FC > 57.45$, $FDR = 0.08$). Furthermore, *Angptl2* and *Egfl6*, identified recently as tail-specific AEC factors in frog tadpoles [43], are both upregulated in our lungfish tail blastema dataset ($FC = 3.67$ and 7.62 , respectively) (electronic supplementary material, table S1). Interestingly, we also found 16 transposon-derived genes upregulated in the tail blastema (figure 3f), including *Ltd1-like*, a gene found enriched in the tail blastema of *Xenopus* tadpoles [19].

Finally, we examined a set of 10 genes recently reported to be expressed preferentially in the tail blastema of frog tadpoles relative to embryonic tail bud [19]. We found

Figure 3. Upregulated genes and over-represented pathways in lungfish tail blastema relative to uninjured tail. (a) Volcano plot showing differentially expressed genes in lungfish uninjured tail tissue and 14 dpa tail blastema (FDR < 0.05, FC > 2), selected lungfish genes up or downregulated in the blastema are noted as black dots. (b) Pathways over-represented in the tail blastema. (c) Heatmap denoting subset of upregulated genes. (d) *In situ* hybridization of genes upregulated in the blastema. (e) Area-proportional Venn diagram showing commonly upregulated genes in lungfish tail and pectoral fin blastema datasets, enriched pathways in the shared tail and pectoral fin blastema dataset, and pathways enriched exclusively on tail blastema. (f) Transposon-derived genes upregulated in the tail blastema. (g) Genes coding for serine-arginine (SR)-rich proteins upregulated in the tail blastema. Scale bars of 1 mm (panoramic views) and 0.25 mm (enlarged view). In (c), 'max' and 'min' represent maximum and minimum expression levels of each gene. (Online version in colour.)

that three out of 10 genes were *Xenopus*-specific genes, and one gene was not contained in our annotated lungfish reference transcriptome. Of the six remaining, three were upregulated in our lungfish dataset, namely *Il11* (FC = 2.77), *L1td1* (FC = 10.52), and one gene, *cd200*, was upregulated with an FDR value above our 0.05 cut-off (FC = 5.94, FDR = 0.09). Taken together, our results identify general features of a genetic programme of tail regeneration that may have been present in the last common ancestor of lungfish and tetrapods.

(d) Genes enriched in lungfish tail blastema versus pectoral fin blastema

Next, we sought to compare our tail blastema dataset to previously published data on South American lungfish pectoral fin regeneration [34]. Comparison of 1072 upregulated genes in tail blastema to the 843 genes upregulated in pectoral fin blastema revealed an overlap of 225 genes. Reactome pathway enrichment analysis showed that this overlapping dataset included genes involved in collagen metabolism,

ECM organization and mitotic cell cycle, all of which represent categories commonly found in regenerating tissues (figure 3e). Interestingly, genes exclusively enriched in tail blastema relative to pectoral fin blastema were involved in pathways related to post-transcriptional RNA processing, including transport of mature transcript to the cytoplasm, processing of capped intron-containing pre-mRNA, mRNA splicing and metabolism of RNA (figure 3e). Among the genes involved in RNA processing, there were eight members of the serine-arginine (SR)-rich splicing factors (figure 3g), all exclusively overexpressed in the tail blastema. These results suggest that post-transcriptional RNA processing may play a more significant role in tail versus pectoral fin regeneration.

4. Discussion

Here, we provided evidence of morphological and molecular hallmarks of tetrapod tail regeneration in the West African lungfish. In terms of morphology, lungfish tail regeneration was most similar to salamanders, featuring the formation of a highly proliferative wound epithelium, which thickened to form an AEC; a highly proliferative blastemal cell population; restoration of the proper dorsoventral pattern of the tail constituents; spinal cord neurogenesis; and requirement of Shh signalling. RNA-seq analysis of regenerating lungfish tail blastema revealed marked upregulation of signalling pathways previously linked to tail regeneration in tetrapods, such as Wnt, Fgf, Shh, Notch, Tgf- β and Egf, as well as ECM and inflammatory response. In addition to broad similarities, genes related to specific aspects of amphibian tail regeneration were also detected, such as the tail-specific AEC factors *Angptl2* and *Egfl6*, *Cse1l*, *L1td1-like* and *Il11*.

Like salamanders, lungfish can regenerate paired appendages in addition to tails. When we compared enriched pathways in the tail and paired-fin blastema, we found that pathways related to RNA processing were preferentially enriched in tails. Eight out of the 12 members of the SR splicing factors were found upregulated exclusively in the tail blastema. Studies in various organisms have demonstrated essential roles during development for this conserved family of RNA-binding proteins [44]. Through splicing regulation of hundreds to thousands of targets, they have been implicated in the establishment of expression programmes associated with cell identity and other cellular functions [44]. *Srsf1* (serine-arginine rich splicing factor 1), which presented a fold change of 6.94 in the tail blastema, is expressed in the central nervous system during *Xenopus* development, where it positively regulates chordin expression [45]. In addition, single-cell transcriptomic data from *Xenopus* regenerating tail shows high expression levels of *Srsf1* in spinal cord progenitors and differentiating neurons ([16], <https://marionilab.cruk.cam.ac.uk/XenopusRegeneration/>). As alternative splicing events are particularly frequent in neural tissues [46], it is possible that the greater complexity of cell types and progenitor cell dynamics related to the spinal cord regeneration may account for the increased post-transcriptional RNA processing observed in the tail blastema [47–49].

Interestingly, we also found evidence of the upregulation of transposon-derived genes. Transposable elements may have played a role in the genome expansion in the lungfish [50], the Iberian ribbed newt *Pleurodeles waltl* [51], the axolotl [52] and the coelacanth [53–55]. In *P. waltl*, the transposable element family that expanded the most was the Harbinger transposon

Figure 4. Hypothesis for the evolution of tail regeneration in sarcopterygians. Regeneration-incompetent lineages are shown in black, lineages with one or more regeneration-competent species are shown green, orange denotes *de novo* appearance of tail regeneration in Lepidosauria; green arrowhead indicates earliest occurrence of tail regeneration, black arrowhead indicates earliest loss and orange arrowhead, reemergence. Cross signifies extinct taxon. Phylogeny from Fröbisch et al., 2015 [1] and Amemiya et al., 2013 [55]. (Online version in colour.)

family, which has given rise to two vertebrate protein-coding genes, *Harb1* [56] and *Naif1* [57]. In the coelacanth, Harbinger elements accounted for 4% of the genome and were shown to possess transcriptional and enhancer activities *in vivo* [54]. In our dataset, the lungfish *Harb1* orthologue was among the highest differentially expressed transposon-derived transcripts. Future studies aimed at functionally evaluating the roles of transposon-derived genes such as *Harb1* may uncover specific roles in development and regeneration. Transposable elements and genes derived from retrotransposon have been previously implicated in regeneration in species as distantly related as sea cucumbers [58] and axolotls [59]. Future studies may help determine if transposons and transposon-derived genes simply undergo injury-induced transcriptional repression due to changes in the epigenetic landscape, or actively influence tissue regeneration [58].

Our results, together with recent palaeontological findings, provide support for tail regeneration as a plesiomorphic trait present in the common ancestor of tetrapods and lungfish (figure 4). In this scenario, tail regeneration in lizards represents a derived character state, possibly with reemergence of regenerative capacity, where tail structures fail to recapitulate the tissue organization and composition seen in the uninjured tail. It is interesting to note that the fossil microsauro *Microbrachis* shows a pattern of tail regeneration that is very similar to what is seen in modern salamanders [1,60]. These fossils are members of the stem lineage of amniotes that lived in the Upper Carboniferous, about 300 Mya [61]. Tail regeneration in these fossils has been previously discussed [62,63], and recently it was noted more definitively that the pattern of regeneration is indeed comparable to modern salamanders, in that vertebral elements are replaced, presumably with the associated spinal cord and musculature [1,60]. Moreover, vertebral centra form first in the regenerating tail of *Microbrachis*, before the associated neural arches, which is the reverse order of events seen in tail development and the same pattern seen in salamanders

[60,64]. The morphological observations in fossil microsaur suggest that at this point of evolutionary time, members of the stem lineage of amniotes were apparently still able to reactivate the positional information and tissue organization during tail regeneration. Given that the developmental pattern of lizard tail regeneration is significantly different from what is seen in amphibians and lungfish, and the phylogenetic distribution of non-regenerating species in other amniotes, we predict that tail regeneration re-evolved in lizards. Although this would seem to contradict Dollo's law of irreversibility, the reemergence of tail regeneration in lizards is not exactly a return to a former character state, as lizard tail regeneration fails to recapitulate the tissue composition and organization seen in the uninjured tail.

Our findings indicate that salamander-like tail regeneration was present in the common ancestor of lungfish and tetrapods, yet the origins of this ability may reside much earlier in evolutionary time, since amphioxus tail regeneration shares parallels with that seen in vertebrates [65]. Expanding the array of research species and searching for evidence of tail regeneration in fossil fish may help us better understand the evolutionary history of tail regeneration. Finally, our work underscores the importance of lungfish for our understanding of how tetrapod traits evolved and helps establish lungfish as an emerging model system that can inform both the history and mechanisms of regeneration.

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Ethics. All experimental procedures and animal care were conducted following the Ethics Committee for Animal Research at the Universidade Federal do Pará, under the approved protocol number 037-2015.

Data accessibility. Sequence data that support the findings of this study have been deposited in GenBank with the following BioProject accession numbers: PRJNA491932, with accession number for uninjured (SRR7880018, SRR7880019 and SRR7880016), 14 dpa blastema (SRR7880020, SRR7880021 and SRR7880024) and additional libraries of tail blastemas at 1 dpa (SRR7880017, SRR7880022 and SRR7880023) and 21 dpa (SRR7880025, SRR7880026 and SRR7880027).

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