DATASET BRIEF

Detailed tail proteomic analysis of axolotl (*Ambystoma mexicanum*) using an mRNA-seq reference database

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Salamander axolotl has been emerging as an important model for stem cell research due to its powerful regenerative capacity. Several advantages, such as the high capability of advanced tissue, organ, and appendages regeneration, promote axolotl as an ideal model system to extend our current understanding on the mechanisms of regeneration. Acknowledging the common molecular pathways between amphibians and mammals, there is a great potential to translate the messages from axolotl research to mammalian studies. However, the utilization of axolotl is hindered due to the lack of reference databases of genomic, transcriptomic, and proteomic data. Here, we introduce the proteome analysis of the axolotl tail section searched against an mRNA-seq database. We translated axolotl mRNA sequences to protein sequences and annotated these to process the LC-MS/MS data and identified 1001 nonredundant proteins. Functional classification of identified proteins was performed by gene ontology searches. The presence of some of the identified proteins was validated by in situ antibody labeling. Furthermore, we have analyzed the proteome expressional changes postamputation at three time points to evaluate the underlying mechanisms of the regeneration process. Taken together, this work expands the proteomics data of axolotl to contribute to its establishment as a fully utilized model.

Keywords:
Animal proteomics / Axolotl / Tail regeneration

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Axolotl (*Ambystoma Mexicanum*) represents an excellent model system to investigate stem cell, cancer, and regeneration due to the following remarkable features: a low tumor incidence [1, 2], exceptional regeneration capacity [3, 4], lifelong lasting neoteny, and availability of metamorphosis induction to study developmental biology [5]. Specifically, neotenic axolotl's astonishing self-repair capacity promotes this organism as an indispensable model for regeneration studies. Axolotls are capable of regenerating their extremities (forelimbs and hindlimbs) [6], tail [7], internal organs (including heart) [8], and CNS including brain [9] and spinal cord [10]. However, it is still unclear which mechanisms are involved in self-repair and regeneration. Physically lost tissues, organs, and appendages can be regenerated in a well-coordinated manner by complete restoration with full function [11].

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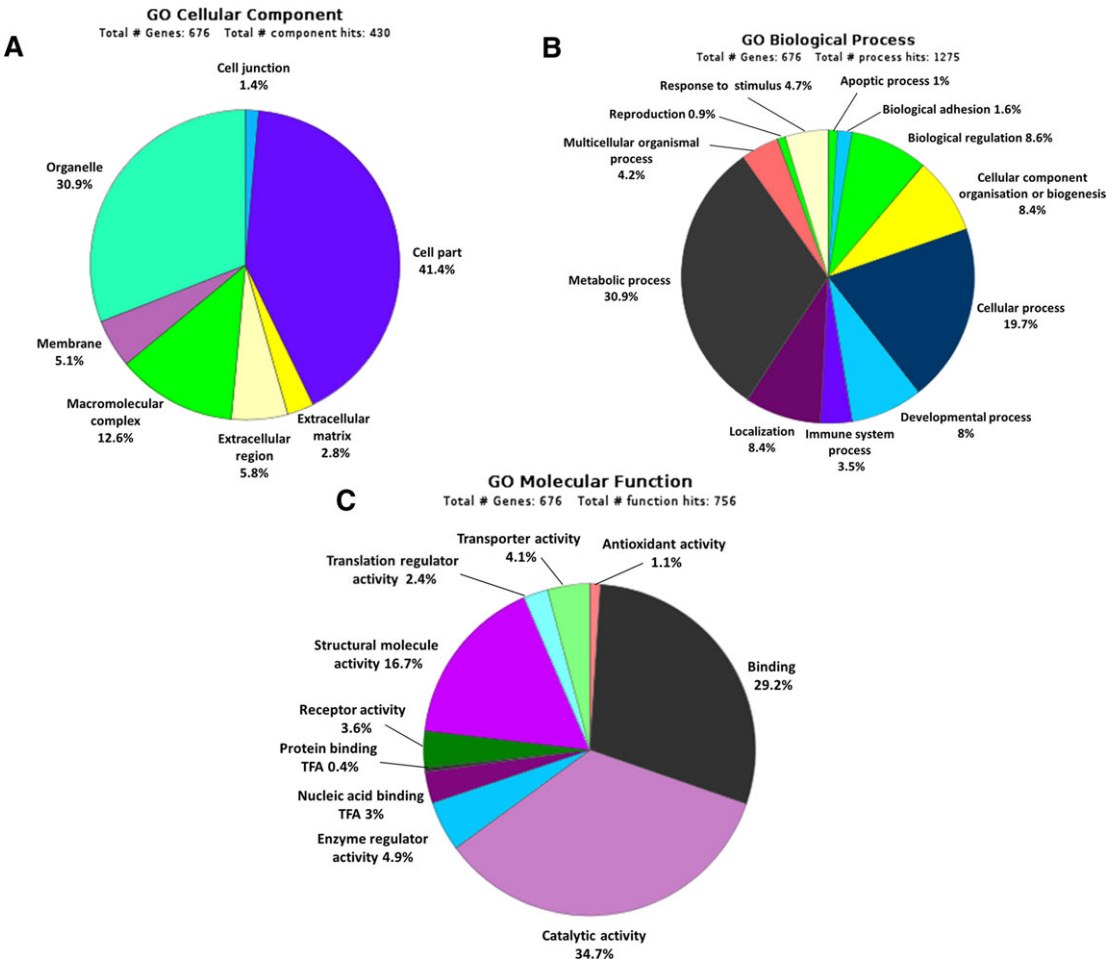

Figure 1. Classification of axolotl tail proteins by gene ontology: (A) Cellular component, (B) biological process, and (C) molecular function. TFA, transcription factor activity. Due to uncharacterized functions of some of the identified proteins, 676 of 1001 could be clustered by gene ontology.

Briefly, wound closure around cut sites is continued with dedifferentiation of somatic cells into adult stem cells to form a blastema [6, 12], and accumulation of stem cells stimulate complex tissue differentiation/restoration processes [6, 13]. To be able to utilize the fascinating regeneration capacity of axolotl, a variety of research tools should be combined to generate reference data of molecular entities. Limited database on protein profile of axolotl complicates drawing conclusions regarding qualitative and quantitative abundance of proteins during regeneration ill and developmental processes. Although recent publications about axolotl proteome [14–16] have contributed to extend the list of annotated proteins, it is still significantly low to establish a proteome database. Additionally, the current proteome data comes from the limb section of the axolotl so to improve the current knowledge about axolotl proteins for a better understanding of regeneration process; here, we present the first proteome analysis of axolotl tail and the proteome level changes at Days 1, 4, and 7 postamputation.

Axolotls used in this research were obtained from the Ambystoma Genetic Stock Center and bred in animal care facility of Istanbul Medipol University. Animals, 8–12 cm in length, were maintained in individual aquarium at ~20°C in Holtfreter's solution before sampling and anesthetized in 0.01% benzocaine (Sigma, St. Louis, MO). The tail part was amputated in the middle of cloaca and the tail tip (Supporting Information Fig. 1I). Samples were collected from approximately 1 mm proximal part of cutting site. For proteomics analyses, samples from five animals were pooled to form a set and three biological sets were prepared for each time point. To obtain samples for four time points (Days 0, 1, 4, and 7), a total of 60 animals were used. Frozen samples were powdered by using mortar and pestle for protein extraction. To define the tissue composition of samples, histological staining was applied (Supporting Information Fig. 1A–H) according to manufacturer's protocols for detailed description of the tail tissue content (Supporting Information Materials).

Table 1. Fold changes of identified proteins at Days 1, 4, and 7 postamputation to basal protein expression at Day 0

Gene ID	Protein symbol	Description	Day 1	Day 4	Day 7	Identified before[a)]
GI:148664250	HNRNPDL	Heterogeneous nuclear ribonucleoprotein D-like	−4.1	−3.6	−2.7	No
GI:11527222	HMGB2	High mobility group protein B2	−2.0	−2.2	−2.0	No
GI:116517336	FHL1	Four and a half LIM domains protein 1 isoform 2	−12.4	−18.3	−14.7	Yes
GI:148668553	HEXB	Hexosaminidase B, isoform CRA_a, partial	2.8	3.6	3.4	No
GI:148706910	ANP32E	Acidic (leucine-rich) nuclear phosphoprotein 32 family, member E, isoform CRA_b, partial	−2.2	−2.1	ND	Yes
GI:270341357	PLA2G4	Cytosolic phospholipase A2 gamma isoform 1	−2.5	−2.2	ND	No
GI:300069034	PDLİM5	PDZ and LIM domain protein 5 isoform ENH4	−4.0	−4.0	−2.7	No
GI:568908131	F13B	PREDICTED: coagulation factor XIII B chain isoform X2	2.1	3.2	3.2	No
GI:568970049	SPTBN1	PREDICTED: spectrin beta chain, non-erythrocytic 1 isoform X2	2.7	ND	ND	Yes
GI:6677799	RPS15	40S ribosomal protein S15 isoform 1	ND	−2.1	ND	No
GI:109730625	SCE1	Scel protein	ND	2.7	ND	No
GI:146325834	COL12A1	Collagen alpha-1(XII) chain	ND	2.1	ND	Yes
GI:148672100	KRT7	Keratin 7, isoform CRA_b	ND	2.8	3.5	Yes
GI:1922893	EFH	m-Calpain	ND	2.1	ND	Yes
GI:227630	SBP56	Selenium binding protein.	ND	2.1	2.6	No
GI:24528555	NTMT1	N-terminal Xaa-Pro-Lys N-methyltransferase 1	ND	2.3	ND	No
GI:255308899	RPL3	60S ribosomal protein L3	ND	−2.1	ND	Yes
GI:28174920	RPL17	Rpl17 protein, partial	ND	−6.7	ND	No
GI:46559745	HOOK3	Protein Hook homolog 3	ND	2.7	3.4	No
GI:568985493	FLNB	PREDICTED: filamin-B isoform X1	ND	2.3	3.8	No
GI:6678359	TKT	Transketolase	ND	2.3	2.3	No
GI:6680946	CIRBP	Cold-inducible RNA-binding protein	ND	−2.0	ND	No
GI:672424492	HNRNPK	Heterogeneous nuclear ribonucleoprotein K isoform 4	ND	−2.1	ND	No
GI:71059675	ARG1	Arginase-like	ND	2.1	ND	No
GI:755525909	SUGP2	PREDICTED: SURP and G-patch domain-containing protein 2 isoform X4	ND	−2.5	−2.5	No
GI:7949078	MYLPF	Myosin regulatory light chain 2, skeletal muscle isoform	ND	−2.0	−2.6	No
GI:9790161	PKP1	Plakophilin-1	ND	2.2	ND	No
GI:120407045	MATN2	Matrilin-2 precursor	ND	ND	2.8	No
GI:1360003	CBP	Nuclear poly(C)-binding protein, splicevariant E	ND	ND	2.3	No
GI:10946870	AKR1A1	Alcohol dehydrogenase [NADP(+)]	ND	ND	−2.7	No
GI:126722834	TNC	Tenascin precursor	ND	ND	−2.1	No
GI:148674494	NCOA5	Nuclear receptor coactivator 5	ND	ND	−2.2	No
GI:148701132	DPP3	Dipeptidylpeptidase 3, isoform CRA_a, partia	ND	ND	−2.0	No
GI:187956263	MYH1	Myosin, heavy polypeptide 1, skeletal muscle, adult	ND	ND	−2.6	No
GI:25992249	SFRS14	Arginine/serine-rich 14 splicing factor	ND	ND	−2.7	No
GI:3334475	PRPH	Peripherin	ND	ND	2.5	No
GI:33859624	S100A4	Protein S100-A4	ND	ND	2.4	No
GI:341940436	DDX5	Probable ATP-dependent RNA helicase DDX5	ND	ND	−2.3	No
GI:568986628	DPYSL2	PREDICTED: dihydropyrimidinase-related protein 2 isoform X1	ND	ND	2.2	No
GI:6678469	TUBA1C	Tubulin alpha-1C chain	ND	ND	2.0	No
GI:6679108	NPM	Nucleophosmin isoform 1	ND	ND	2.2	No
GI:6679587	RAB1A	ras-Related protein Rab-1A	ND	ND	2.3	No
GI:6753036	ALDH2	Aldehyde dehydrogenase, mitochondrial isoform 1 precursor	ND	ND	2.0	No
GI:83921612	TXNDP	Thioredoxin domain-containing protein 5 isoform 1 precursor	ND	ND	−4.8	Yes

a) Previously identified according to ref. [27].
ND, not detected.

A filter-aided sample preparation method was used for the generation of tryptic peptides [17]. Briefly, 50 μg protein lysate was incubated with DTT and iodoacetamide (IAA) for reduction and alkylation steps, respectively, and followed with overnight trypsinization. LC-MS/MS-based differential protein expression analysis was done following a previously published protocol [18]. In short, 200 ng tryptic peptide mixture was analyzed by nano-LC-MS/MS system (Acquity UPLC M-Class and SYNAPT G2-si HDMS; Waters, Milford, MA, USA). The analysis was performed at positive ion V mode, applying the MS and MS/MS functions over 0.7 s at mobility TOF mode. Drift time-specific collision energy was used for the fragmentation of the peptide species [19]. Tandem mass data extraction, charge state deconvolution, and

deisotoping were performed with Progenesis QI for proteomics (v.2.0, Waters) and searched against the in-house-generated database (Supporting Information Materials).

A total of 1864 peptide sequences were obtained and 1103 of them passed the identification criteria. We focused on these peptides to identify the corresponding proteins. Since there is no axolotl protein database, previously assembled axolotl mRNA sequencing data [20] was used to generate a protein database (detailed protocol in the Supporting Information Materials). Of the 1103 peptides, 1001 of them were mapped to a known eukaryotic protein; the rest was classified as either no hit or bacterial protein (Supporting Information Tables 1 and 2, and Supporting Information Materials).

Next step was the computational analysis of the identified proteins. For this purpose, the PANTHER Classification System [21] was used and mouse orthologous of all annotated proteins were retrieved to upload to the PANTHER system. To get better insights of the identified proteins, cellular component, biological process, and molecular functions analyses were done (Fig. 1) using PANTHER tool. According to cellular component analysis, most of the proteins are part of the cytosolic elements or organelles (Fig. 1A). Beside these sites, identified proteins are localized in macromolecular complex, extracellular region, membrane, extracellular matrix, and cell junction parts of the cells (Fig. 1A). Biological process analysis of proteins enables us to define important biological processes and the associated proteins (Fig. 1B). Majority of the identified proteins have a role in metabolic processes, cellular processes, or biological regulation (Fig. 1B). Localization, cellular component organization, developmental process, response to stimulus, multicellular organismal process, immune system process, biological adhesion, apoptotic process, and reproduction are described biological processes for axolotl identified proteins (Fig. 1B). Molecular activity of the axolotl's identified proteins was investigated by using molecular function analyses application of PANTHER software (Fig. 1C). Prevalent activity of proteins was found as catalytic activity, binding, or structural molecule activity (Fig. 1C). Enzyme regulator activity, transporter activity, receptor activity, nucleic acid binding transcription factor activity, translation regulator activity, antioxidant activity, and protein binding transcription factor activity are characterized as molecular functions of annotated proteins (Fig. 1C). Classification of proteins based on PANTHER tool manifests diverse biological roles, molecular activities, and localization of identified axolotl proteins.

Annotation and clustering of axolotl proteins was followed by immune-fluorescence labeling to verify the presence of the proteins in sampled tissues (Supporting Information Figs. 2 and 3). HSP 70 and HSC70 have roles in endoplasmic reticulum and mitochondrial processes, and these proteins are expressed in epidermis, skeletal muscle, and glial cells [22–24] and its expression can be detected in glial cells, nonkeratinized epidermal cells, and striated muscles (Supporting Information Fig. 2a–a″). Laminin is a glycoprotein commonly expressed in noncollagenous connective tissue and function in postnatal nervous system development and axonal regeneration [25]. In axolotl tail samples, it is labeled in white matter in spinal cord, dorsal root ganglia axons, cell membrane of epidermal layer cells, and skeletal muscle cells (Supporting Information Fig. 2b–b″). Protein phosphatase1 beta (PP1β) acts in regulation of proliferation, homeostasis, and apoptosis, and it is essential for neurofilaments [26]. PP1β expression is observed in nerve fibers and white matter in spinal cord (Supporting Information Fig. 2c–c″) of axolotl tail sample. Tubulin is stained in nerve fibers, axonal extension of spinal cord, skeletal muscle cells, epidermal leydig cells, and basal membrane of the epidermis (Supporting Information Fig. 2d–d″). Alfa smooth muscle actin is labeled in artery and vein walls and skeletal muscle cells (Supporting Information Fig. 2e–e″). No staining with negative control by leaving primary antibodies out exhibits specificity of the immune labeling (Supporting Information Fig. S3). Verification of identified proteins in sampled tissues by immune staining provides evidence for the quality of proteomics results. The generated protein database was used for analyzing the protein expressional changes after amputation and discovering the statistically significant alterations triggered to initiate the regeneration process. We have decided to follow the process starting at an early stage at Day 1 and compared it to the alterations at later stages at Days 4 and 7.

Label-free protein expression analysis at Day 1 compared to Day 0 yielded nine statistically significant protein changes of which six were downregulated and three were upregulated, as shown in Table 1 and Supporting Information Table 3. The identified proteins play a role in biological regulation, developmental processes, immune system process, and cellular component organization or biogenesis. These were the pathways triggered at the early stages of regeneration. At Day 4 postamputation, there were 25 statistically significant protein alterations with 13 downregulation and 12 upregulation (Table 1 and Supporting Information Table 3) and at Day 7, there were 31 statistically significant protein level changes with 13 downregulation and 18 upregulation (Table 1 and Supporting Information Table 3). As shown in Table 1, some of the identified proteins cannot be quantified in all time points. Many proteins, which play a role in developmental, metabolic, and cellular processes, were identified to be statistically significantly altered, some of which were demonstrated in a previous study [27]. Some of the most interesting proteins identified are PDZ and LIM domain protein 5, Matrilin-2, tenascin, and periferin have been shown to take part in regulation of dendritic spine morphogenesis, peripheral nerve regeneration, neuronal regeneration, and filament cytoskeleton organization, respectively. Further studies on the identified proteins will shed light on the genesis and progression of regeneration process.

Considering the axolotl's evolutionary proximity to mammals than invertebrates and zebrafish, and functional conservation of proteins among animals, axolotl represents a great promise as a vertebrate model. Extensive usage of axolotl as a model organism can provide useful information to

understand unknown mechanisms of several processes such as regeneration and avoidance of tumor formation. However, its broad utilization is still limited due to inadequate "omics" databases. Here, in this study, we present the first report on tail proteome of axolotl by combining a preassembled mRNA sequence dataset and high-throughput proteomics. Our results extend the current protein profile of axolotl significantly. Furthermore, immunohistochemistry results provide experimental evidence of presence of the proteins on tissues obtained from proteomics analyses. Also, for the first time, we report the protein expressional changes of the tail section postamputation by label-free LC-MS/MS analysis. We believe that we have generated a broadened proteome database for axolotl research to be used for in-depth regeneration analysis.

The authors have declared no conflict of interest.

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