Proteomic identification and expression of oral apparatus constituents in cell regeneration of giant ciliate *Stentor coeruleus* (strain WHEL)

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Abstract

Background The giant ciliate *Stentor coeruleus* is a suitable model organism for studying morphogenesis and regeneration at the single-cell level. The anterior end of the cell is dominated by thousands of cilia organized into a ciliated band of membranelles, known as the oral apparatus (OA). This feeding organelle can be induced to shed by urea treatment. The OA then regenerates via a series of defined morphological events and the cell resumes normal feeding activity. These defined events provide an ideal opportunity to identify the protein constituents of OA and their expression patterns in OA regeneration. **Results** We identified OA constituents in *S. coeruleus* by mass spectrometry. A total of 882 OA-associated proteins were identified; the homologs of 181 of these are known OA constituents in other organisms. The expression pattern of OA-associated genes during regeneration was investigated using single-cell transcriptome sequencing. The expression of most OA-associated genes was high during regeneration, indicating their stable expression after OA shedding. We also identified OA-associated differentially expressed genes that may be involved in regulating OA reconstruction. This information enabled us to hypothesize the architecture of OA of *S. coeruleus* WHEL. **Conclusions** We have identified protein components of the OA and determined the temporal changes in their expression during OA regeneration in *S. coeruleus* WHEL. Although our results are preliminary, they form the basis of further studies into the molecular mechanisms of OA development in *S. coeruleus*. In addition, as *S. coeruleus* is an important model organism for studying morphogenesis, our findings may help to improve the understanding of OA formation in ciliates.

Background

Stentor is considered a good system for studying regeneration in protozoa because of its large size and amenability to surgical manipulation [1]. In particular, it can be studied on regeneration as a single cell (structural unit of life) without the need to consider the influence of surrounding cells [2]. In 1885, Gruber firstly found *Stentor* can be cut into three pieces, each of which could develop into new individuality, and he described in detail the regeneration process of a trisected *Stentor* cell [3,4]. Several years later, Balbiani reported a series of fundamental biological processes in the same organism, including wound healing, holdfast development, and nuclear behavior during regeneration [5,6].

In *Stentor*, the process of regeneration includes (i) regeneration of the oral apparatus (OA) and its requirements; (ii) regeneration of the holdfast; and (iii) reconstitution of the normal shape [1]. Of these, OA regeneration is the most attractive and amazing regeneration paradigm. The OA is dominated structure on the anterior end of the cell, consisting of thousands of centrioles and cilia [7] organized into a ciliated band of membranelles. The membranelar band encircles the broad end of the cell body and terminates inside the buccal cavity or gullet [8,9]. However, the protein constituents of OA and their spatiotemporal expression patterns in OA regeneration are unknown.

Stentor coeruleus is a very large ciliate that measures 0.5–2 mm when fully extended. Each cell ciliate is blue to blue-green in color and contains a macronucleus that resembles a string of beads. *S. coeruleus* is

a filter feeder that uses its OA to sweep other living cells into its mouth. The feeding apparatus comprises a frontal field, a cytostome and cytopharynx region, and the ciliated membranellar band. Sucrose or urea shock can cause the OA to shed [10]. Thereafter, a new OA regenerates within 8 h via a series of well-characterized morphological stages [11]. These defined stages provide an ideal opportunity to identify the protein constituents of OA and their expression patterns in OA regeneration.

Here, we identified the protein constituents of OA in *S. coeruleus* using mass spectrometry and investigated the expression features of OA-associated genes using single-cell RNA-sequencing (RNA-Seq). The results provide new insights to the molecular basis of OA in *S. coeruleus*.

Results

OA-associated proteins of S. coeruleus identified by mass spectrometry

A total of 882 proteins were identified in extracts from the shed OA of *S. coeruleus* WHEL (note that some may be partial protein sequences because of incomplete transcript assembly). We divided these into three groups: (1) components of different organelles; (2) known OA proteins; and (3) other proteins.

The OA proteome contained 264 proteins associated with components of organelles including mitochondria, ribosomes, vesicles, endoplasmic reticulum, Golgi apparatus, peroxisomes, and centrosomes (Supplementary Table 1). Of these, 149 proteins (57%) were classified as mitochondrion-associated proteins based on in silico annotation. Although we cannot fully exclude the possibility of contamination, the high number of mitochondrial components identified by mass spectrometry data indicates that there may be a lot of mitochondria associated with the OA. In flagellated protozoa or mammalian sperm, mitochondria are reported to be concentrated around the base of the flagella [20]. Hence, it is possible that mitochondria located near the base of the cilia produce the ATP required to drive ciliary movement in the OA of *S. coeruleus* WHEL [21].

The second group of proteins were homologs of known OA-associated proteins reported in other organisms: this group included molecular chaperone, calmodulin (CaM), adenosine triphosphatase (ATPase), microtubule, guanosine triphosphatase (GTPase), serine/threonine-protein kinase, microfilament, vacuolar protein sorting 4 (VPS4), translation elongation factor 1- α (EF-1 α), and intermediate filament (Table 1). Microtubules, microfilaments, and intermediate filaments are associated not only with the eukaryotic cytoskeleton but also with cilia or basal bodies. Ciliary axoneme is a microtubule-based cytoskeleton inside cilia. In *Tetrahymena*, OA membranelles are connected by microtubules, passing from one membranelle to another, establishing connections between the proximal ends of basal bodies. Near each basal body is a small fibrous ring, the structure of which is maintained by the microfilaments alone [22]. In total we found 35 associated proteins function as aforementioned proteins that were identified in the OA of *S. coeruleus* WHEL. Therefore, it is speculated that the *Stentor* OA as a cortical feeding structure is composed of ciliated and non-ciliated basal bodies interconnected by a framework of microtubules and filaments, similar to in *Tetrahymena* [23]. In addition, different classes of molecular chaperones have been reported in the cilia of diverse organisms: heat shock protein (Hsp)

70 and Hsp90 have been detected in the cilia [24] and Hsp60 in the basal bodies [25] of *Tetrahymena*, and Hsp40 (containing a DnaJ domain) and the TCP-1/CCT α subunit of the cytosolic chaperonin CCT (chaperonin containing TCP-1) in cilia have been identified in sea urchin embryos [26,27]. We identified a broad group of 40 molecular chaperones in the OA in *S. coeruleus* WHEL. Of these, Hsp82 (a member of the Hsp90 family) and one or more of Hsp70 family members were associated with both soluble tubulin and tubulin assembled into microtubules in the cilia and cell cortex of *Tetrahymena* [24]. Hsp60, a mitochondrial chaperone involved in folding of mitochondrial proteins, binds to citrate synthase/14nm filaments (intermediate filaments) and is believed to help form the OA in protozoa [25]. Moreover, Hsp40 is a component of the radial spoke complex in sperm flagella of the ascidian *Ciona intestinalis*, where it may be involved in interactions between the radial spoke and central microtubules [28,29]. TCP-1/CCT α has also been detected in centrosomes, indicating that it may assist in microtubule nucleation [30]. These results demonstrate that chaperones are widely distributed ciliary and flagellar components [27]. In addition, Ca²⁺/CaM signaling is reported to be closely associated with ciliary motility [31]. Further studies showed that CaM is localized to axonemal microtubules and that EF-1 α is a Ca²⁺/CaM-binding protein that regulates the actin cytoskeleton in cilia [25,32,33]. Serine/threonine-protein kinase 1 is hypothesized to control the growth of cilia in *Tetrahymena*. In ciliates, the cytostome engages in phagocytosis, which is used as a means of feeding and provides part or all of their nourishment. In phagocytosis, vesicle formation is essential for consuming food via the OA and for transporting proteins between organelles. The VPS4 protein contains highly conserved AAA-ATPase domains; its homologs have been implicated in vesicle formation in yeast and may play a similar role in *Tetrahymena* [34]. Notably, all of these proteins were shown to localize to the OA in *Tetrahymena* [31,34,35], and CaM, EF-1 α , VPS4, and serine/threonine-protein kinase were identified as known OA proteins in the OA of *S. coeruleus* WHEL. Some proteins likely to be associated with the phagosome and vesicular transport were also identified in the OA of *S. coeruleus* WHEL. These include three subunits of the vacuolar ATPase and members of the Rab family of small GTPases in *Tetrahymena thermophila* [36]. Rab proteins are also associated with ER-to-Golgi transportation [37]. Our findings indicate that these protein components might be conserved elements of the OA of ciliates.

Proteins in the third group (neither organellar or known homologs of OA constituents) contained transmembrane helix or structural motifs or were involved in proteolysis, transcription/translation, signaling, amino acid metabolism, fatty acid metabolism, glycolysis/gluconeogenesis, or ubiquitination (Table 2). Phobius [38] or TMHMM [39] software predicted a total of 33 membrane proteins (i.e. containing at least one transmembrane helix) which were annotated as enzymes, transports, and channel proteins. These might either interact with or form part of OA membranes. We also identified 75 proteins containing structural motifs, especially Armadillo, HEAT, leucine-rich repeat, and WD40 repeat, which are known to be involved in protein-protein interactions. Thus, these proteins may be structural components of the OA. In addition, we identified seven ubiquitination related proteins. The same structural motif-containing proteins and ubiquitin family proteins have also been identified in the OA and are associated with the basal body or centriole in *Tetrahymena* [40].

Overall expression patterns of OA-associated proteins during regeneration

The OA can be induced to shed by urea shock [10], after which a new OA regenerates over a time course of 9 h via a series of well-characterized morphological stages (detailed in Fig. 1a) [1,11]. After approximately 1.5 h, a cortical primordium appears as a simple zone of basal bodies growth (Fig. 1a, 1.5h). The primordium then lengthens and forms new ciliary membranelles that grow from a mass of basal bodies in the primordial region (Fig. 1a, 3h–6h). Finally, the entire structure migrates to the anterior end of the cell, considering the position previously held by the shocked organelles (Fig. 1a, 8h–9h). Commensurate with the final cortical changes, the macronucleus coalesced into two large nodes (Fig. 1a, 6h) and then re-nodulates to complete the OA regeneration (Fig. 1a, 8h) [13].

To analyze the associated changes in gene expression, single-cell RNA-Seq was performed at 0.5, 1.5, 3, 4, 6, 8, and 9 h after urea shock. Compared with the control cell, 3223 transcript fragments were upregulated and 3061 were downregulated (with at least four fold change (FC) in expression in at least one time point) during regeneration. The results of GO enrichment analysis were similar to those previously reported in a single-cell RNA-Seq study of another *S. coeruleus* strain Pranidhi Sood and colleagues; Supplementary Table 2), indicating that our single-cell RNA-Seq data is reliable.

As a previous single-cell RNA-Seq study of *S. coeruleus* gave comprehensive results for differentially expressed genes (DEGs) in regeneration [11], our main focus was on determining changes in the expression of the genes encoding OA-associated proteins that we had identified by mass spectrometry. We first found that the expression level was generally higher for OA proteins than for non-OA proteins (Fig. 1b). This result indicates that OA-associated proteins play a vital role in OA regeneration. Using the 4FC cut-off, differential expressed OA proteins were identified at each time point during the regeneration process. We found that only a small fraction of OA proteins was up- or downregulated at each time point: the expression of >83% OA-associated genes were unchanged compared with the control cell (Fig. 1c). Together with the high level of expression of OA-associated proteins, this result indicates that most OA-associated genes are stably expressed, similar to housekeeping genes.

Differentially expressed OA-associated proteins during regeneration

The expression of 232 (29%) OA-associated genes changed in at least one time point during regeneration: 44 were upregulated and 188 were downregulated. The expression profiles of all 232 DEGs were clustered by calculating the Z-score of fragments per kilo base of transcript sequence per million base pairs sequenced (FPKM) (Fig. 2). In general, three groups of OA-associated DEGs were identified.

Group 1 contains 44 upregulated genes that showed maximum expression at a single stage of OA regeneration (Table 3): 13 genes were maximally expressed at 0.5 h, eight at 1.5 h, two at 3 h, five at 4 h, nine at 6 h, two at 8 h, and five at 9 h. Several genes that may be important in OA regeneration were found. Expression of cilia/flagella-associated protein 20 (CFA20; SCOERU6382613) peaked at 3 h. CFA20

is a cilium/flagellum-specific protein involved in axonemal structure organization and motility in *Paramecium* [41] and *Chlamydomonas* [42], and functions in regulating cilia size and morphology [43]. Expression of this gene corresponded with a major morphologically feature: appearance of the first cilia. Two Gas2-related domain (SCOERU6109121, SCOERU5478902) containing proteins with microtubule-binding and stabilizing activity [44] and five CaM proteins (SCOERU5237302, SCOERU2832411, SCOERU5679902, SCOERU2839501, SCOERU5228401) with ciliary motility activity [31] reached a peak of expression at the 6–8 h timepoint of OA formation. The selective stabilization of microtubules has been proposed as the basis of cytoplasmic asymmetry during morphogenesis [45]. In most cases, post-translationally modified tubulin is found to accumulate within relatively stable microtubules in eukaryotes. Tubulin polyglycylation is the final post-translationally modification to take place during *Drosophila* spermatogenesis and its occurrence corresponds to the end of spermatozoan maturation, and may function in axoneme motility [46]. Cilia can bend as the microtubules slide past one another. The arrangement of cilia permits their coordinated movement in response to cytoplasmic signals [47]. Based on these reports, we speculate that group 1 genes may be involved in ciliary maturity and migration in the rebuilt OA.

Group 2 contains 40 genes (Table 4) that were significantly downregulated during the initial stages of OA regeneration but increased expression during the ciliary biogenesis stage of OA regeneration (from 3 h to 6 h). Upregulation of these genes during this stage suggests that they are vital for cilia growth and membranellar band formation in the OA. Some promising candidates that may have important roles in this process were identified. Five HSP genes were identified: three Hsp70 (SCOERU6199801, SCOERU6807002, SCOERU5535901) and two Hsp90 (SCOERU6611202, SCOERU6824901) genes. Homologs of these HSP genes were reported to be involved in microtubule assembly and were associated with actin family forming microfilaments [24]. Four TCP-1/CCT α genes (SCOERU6653011, SCOERU6653002, SCOERU6525301, SCOERU6273502) were identified that may be involved in microtubule nucleation [30]. We also identified a CaM (SCOERU6088901) associated that may be involved in centriole replication and EF-1 α (SCOERU6661701), which regulates the actin cytoskeleton in cilia [25,32,33]. In addition, three tubulin genes (SCOERU6841701, SCOERU6841712, SCOERU6507601), two actin genes (SCOERU6773301, SCOERU6773311), and two intermediate filament genes (SCOERU6558001, SCOERU6230702), which are components of cytoskeletal filaments, were also identified.

Group 3 contains 148 genes that were downregulated at crucial stages in ciliary biogenesis during OA regeneration (from 3 h to 6 h). These mainly encode enzymes related to metabolism, mitochondria, and proteolysis. This result suggests that the energy for OA formation is provided by reducing the metabolic process associated with normal growth.

Discussions

In this study, we investigated the OA proteome of *S. coeruleus* WHEL and identified 882 OA-associated proteins. This result helps us to understand the OA structure. We also investigated the expression

patterns of OA-associated genes using single-cell RNA-Seq and found that most OA-associated genes do not show significant changes in expression during regeneration. It means that removal of OA does not interrupt the expression of these genes, indicates that most of the materials required to rebuild the OA are already available, which ensures its rapid reconstruction after injury and the rapid resumption of feeding activity. We also identified a large number of DEGs during regeneration. In particular, some DEGs that are upregulated during OA formation are potential candidate regulators of OA regeneration.

The ciliate *S. coeruleus* consists of a complex oral band composed of thousands of long cilia organized into structures called membranelles. These form the membranelar band, which can be induced to shed by urea treatment (Fig. 3a). In 1955 Fauré-Fremiet and Rouiller discovered that each membranelle is composed of two short, parallel rows of the ciliary basal body, which are connected to each other laterally and give off ciliary rootlets into the cell interior. The whole basal structure of the membranelle extends for about 20 µm into the endoplasm (Fig. 3b) [48,1]. Our findings suggest that *S. coeruleus* WHEL has a similar OA that includes cilium and subcellular structures (Fig. 3c). As show in figure 3c, the centrosome is a primary organelle that servers as the main microtubule organizing center in the animal cells, as well as a regulator of cell progression [49]. In addition, centrioles, which are core centrosomal components, have another distinct function as basal bodies. Centriole biogenesis is required for formation of the basal body. After the basal body is formed, it migrates to the cell surface and docks onto the actin-rich cortex. Next, the basal body nucleates an outgrowth of axonemal microtubules, which protrude beneath an extension of the membrane to give rise to the cilium. After this, continued elongation of the cilium requires the selective import and transport of ciliary proteins to the tip mediated by intraflagellar transport (IFT) proteins, such as IFT20 and IFT54. These two proteins are involved in vesicle transport from the Golgi apparatus to cilia [50,51]. The movement of IFT proteins along microtubules is catalyzed by dynein and kinesin motor proteins. Ciliogenesis also requires the transport of membrane proteins to construct the ciliary membrane [52]. The ribosomal, endoplasmic reticulum, vesicular, and Golgi apparatus proteins identified in the OA proteome might also be associated with forming the OA structure. The main function of mitochondria and peroxisomes is to produce the energy required for cellular metabolism. Based on our findings, we propose that the cilium is an indispensable structure, and underneath subcellular structures are essential for OA maintenance and regeneration in *S. coeruleus*.

Conclusions

In this study, we have identified protein components of the OA and determined the temporal changes in their expression during OA regeneration in *S. coeruleus* WHEL. This information enabled us to hypothesize the architecture of OA of *S. coeruleus* WHEL. Although our results are preliminary, they form the basis of further studies into the molecular mechanisms of OA development in *S. coeruleus*. In addition, as *S. coeruleus* is an important model organism for studying morphogenesis, our findings may help to improve the understanding of OA formation in ciliates.

Methods

Cell isolation, cultivation and species identification

S. coeruleus cells were isolated from freshwater samples taken from East Lake (N30°32'52"; E114°21'7"), Wuhan, China in October 2014. Cells were maintained in culture at 20°C in Modified *Stentor* Medium (MSM) in the dark [2], supplementing the *Chlorogonium elongatum* in moderation as living prey. The *S. coeruleus* 18S small subunit (SSU) ribosomal RNA genes were PCR amplified using the universal ciliate primer pair. The gene shared 99% sequence identity with the 18S SSU rRNA gene sequence of *S. coeruleus* isolate WM001, previously reported by Slabodnick *et al.* (GenBank accession: MPUH01002597) [12]. However, the assembled transcriptomes using RNA-Seq data show relatively low sequence identity to the genome of *S. coeruleus* isolate WM001 (GenBank accession: MPUH000000000). These results suggest that the strain isolated in this study was not identical to the WM001 strain, the genome of which has been sequenced; thus, we named the isolated strain *S. coeruleus* WHEL.

Induction of regeneration and sample collection

For sample collection, 30ml cultures of *S. coeruleus* WHEL cells were grown at 20°C to obtain about 300 cells. All experiments were performed using cells from cultures that had been starved for several days because observation of macronuclear nodes is facilitated by the absence of food vacuoles in the cytoplasm. Before urea shock, a single cell was collected as a control cell (Fig. 4a). To induce regeneration, cells were incubated for 1 min in a 4% urea solution (shown in Fig. 4b) [10]. This treatment caused the membranellar bands to be sloughed from the cells, followed by replacement of the entire complement of feeding organelles [13]. After urea treatment, cells were washed in cold MSM, centrifuged at 3,200rpm for 30 s at 4°C, and resuspended in fresh MSM. On the one hand, we picked up a large number of shedding OA distinguished from the cortex subfractions by mouth pipette using a Zeiss anatomical lens (Fig. 4c). On the other hand, we collected populations of somatic cells (without OA) and placed them in normal culture conditions for OA regeneration (Fig. 4d). Regeneration proceeds through a series of characteristic morphological changes involving both the cell cortex and macronucleus. With the development of their morphology, we isolated a single cell from the cell populations at each of the following time in regeneration process: 0.5, 1.5, 3, 4, 6, 8, and 9 h. In total, eight cells were isolated per treatment: one control cell and seven cells at different times of the regeneration process. Each cell was placed in a 0.2 ml thin-walled PCR tube containing 2 µl of cell lysis buffer (0.2% (vol/vol) Triton X-100 and 2 U/µl RNase inhibitor). The OA and cell samples were snap frozen and stored at -80°C until use.

Single-cell RNA-Seq

Total eight single-cells were amplified using Smart-Seq2 (SMARTer Ultra Low RNA kit (Art. No. 634936, Clontech, USA) (for Illumina sequencing; insert size about 350bp) [14]. Illumina sequencing libraries were constructed according to the manufacturer's protocols. Paired-end (150bp x 2) sequencing was performed for all the samples using Illumina HiSeq4000 sequencer.

Transcriptome assembly and functional annotation

Raw single-cell RNA-Seq reads were filtered by the `fastq_quality_filter` (-q 20, -p 80) in the FASTX-Toolkit [15]. We first *de novo* assembled a reference transcriptome by merging all eight sets of RNA-Seq data using Trinity [16] with default parameters. The assembled transcript fragments served as reference transcriptome sequences. Predicted protein sequences (note that some were partial sequences of a protein) were obtained by translating the six possible reading frames of the assembled transcripts using the TransDecoder [16]. All predicted (or partial) protein sequences were integrated into a database that was used for protein identification from mass spectrometry data. We used InterProScan–5.19–58.0 [17] for functional annotation of the protein sequences.

Analysis of differentially expressed genes

Filtered reads were mapped back to the assembled transcriptome, and the read count for each gene was obtained using the RSEM output which integrated in Trinity with default parameters [16]. We chose the number of FPKM to represent the gene expression abundance. DEGs were defined as those with an FDR $p\text{-value} \leq 0.001$ and $\log_2^{\text{FC}} \geq 2$ expression (4-FC). Gene ontology (GO) enrichment analysis provided all of the GO terms that were significantly enriched in DEGs using BinGO [18], and we filtered the DEGs that corresponded to biological functions.

Protein extraction

OAs were harvested, washed three times with cold ultra-pure water (Milli-Q, Millipore), mixed with the NuPAGE LDS sample buffer (Invitrogen, USA), and sonicated at 70°C for 10min. Protein were then separated by NuPAGE gel electrophoresis, followed by silver staining.

Mass spectrometry

Stained gels were cut into particles, washed three times with ultra-pure water, and destained with 600µl Silver Out buffer at room temperature, with shaking. Proteins were then digested overnight with 1µg trypsin at 37°C. After trypsin digestion, the peptides were desalted by passing through a Self-packed RP C₁₈ solid phase extraction column, acidified by adding 15µl loading buffers (0.1% formic acid and 3% acetonitrile), and analyzed by LC-MS/MS (ekspert nanoLC; AB Sciex TripleTOF 5600-plus, USA).

Protein identification and quantification

ProteinPilot software version 5.0.1 [19] was used to identify and quantify OA proteins. For protein identification and quantification, unique peptides were used to search the predicted protein sequence database (derived from the assembled transcriptome) with additional filters: for protein identification, a

significance threshold of $p \leq 0.05$ (95% confidence) was used; and for protein quantitation, unique peptides were set at ≥ 1 .

Abbreviations

OA: oral apparatus; RNA-Seq: RNA-sequencing; CaM: calmodulin; ATPase: adenosine triphosphatase; GTPase: guanosine triphosphatase; VPS4: vacuolar protein sorting 4; EF-1 α : translation elongation factor 1- α ; Hsp: heat shock protein; FC: fold change; DEGs: differentially expressed genes; FPKM: fragments per kilo base of transcript sequence per million base pairs sequenced; IFT: intraflagellar transport; MSM: Modified *Stentor* Medium; SSU: small subunit; GO: gene ontology.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

WM, JX, CJ, WY and WW conceived the work. WW, JX wrote the manuscript. All authors have approved the final version of the manuscript.

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Tables

Table 1. Classification of oral apparatus associated proteins in *Stentor coeruleus* WHEL: proteins previously identified in the oral apparatus of other species.

Category	Number of genes
Molecular chaperone	40
Calmodulin	37
ATPase	24
Microtubule	22
GTPase	18
Serine/threonine-protein kinase	13
Microfilament	11
Vacuolar protein sorting 4	9
Translation elongation factor 1-α	5
Intermediate filament	2

Table 2. Classification of proteins associated with the oral apparatus of *Stentor coeruleus* WHEL: other proteins.

Category	Number of genes
Structural motif	75
Proteolysis related proteins	49
Membrane protein	33
Transcription/translation	32
Amino acid metabolism	27
Signaling	24
Fatty acid metabolism	23
Glycolysis/gluconeogenesis	21
P-loop containing nucleoside triphosphate hydrolase	15
Ubiquitination	7
Other	131

Supplementary Table 2. Significantly enriched gene ontology classification of transcript fragments upregulated in at least one time point during oral apparatus regeneration in *Stentor coeruleus* WHEL.

GO classification	Number of genes
Protein amino acid phosphorylation	41
Translation	39
Microtubule-based process	25
Protein folding	18
DNA integration	14
Microtubule-based movement	12
Regulation of cell cycle	11
Cell cycle	10
Negative regulation of biological process	10
DNA replication	8
Negative regulation of cellular process	7
Mitosis	6
DNA recombination	5
Regulation of cell growth	4
Negative regulation of angiogenesis	3
Protein refolding	3
Regulation of exit from mitosis	3
Reproductive process	3
Response to heat	3
S-adenosylmethionine biosynthetic process	3
Platelet activation	2
Post-chaperonin tubulin folding pathway	2
Sensory perception of sound	2
tRNA 3'-terminal CCA addition	2
Tubulin complex assembly	2
Skeletal muscle fiber development	2

Table 3. Functional annotation of 44 upregulated differentially expressed genes encoding oral apparatus associated proteins expressed during regeneration in *Stentor coeruleus* WHEL.

Time since shock (hour)	Protein ID	InterProScan annotations	Log ₂ ^{fold} change
0.5	SCOERU2943621	NA	5.09
	SCOERU7006301	NA	7.02
	SCOERU2877401	NA	5.50
	SCOERU6679132	IPR012336:Thioredoxin-like fold	6.32
	SCOERU6943501	IPR004345:TB2/DP1/HVA22-related protein	6.90
	SCOERU2877402	NA	5.44
	SCOERU5764801	IPR001451:Hexapeptide repeat	5.79
	SCOERU2795601	IPR000793:ATPase, F1/V1/A1 complex, alpha/beta subunit, C-terminal	5.71
	SCOERU5105811	IPR004000:Actin family	4.53
	SCOERU2812101	IPR003172:MD-2-related lipid-recognition domain	4.75
	SCOERU6988901	NA	6.35
	SCOERU5749901	IPR001806:Small GTPase superfamily	5.10
	SCOERU3786411	IPR016161:Aldehyde/histidinol dehydrogenase	5.67
1.5	SCOERU2968301	IPR020546:ATPase, F1 complex, delta/epsilon subunit, N-terminal	3.88
	SCOERU7061601	NA	5.60
	SCOERU7009101	NA	4.98
	SCOERU5436301	IPR000297:Peptidyl-prolyl cis-trans isomerase, PpiC-type	7.07
	SCOERU6787901	IPR000308:14-3-3 protein	5.39
	SCOERU2806701	IPR019956:Ubiquitin	5.37
	SCOERU3667401	IPR003000:Sirtuin family	3.42
	SCOERU5130801	IPR013766:Thioredoxin domain	3.56
3	SCOERU6382613	IPR007714:Cilia/flagella-associated protein 20/WDR90/C3orf67	3.72
	SCOERU6954101	IPR019734:Tetratricopeptide repeat	3.98
4	SCOERU2800911	NA	3.85
	SCOERU4638001	NA	5.20
	SCOERU6593301	IPR004000:Actin family	7.01
	SCOERU7014001	NA	4.05
	SCOERU6797711	IPR001916:Glycoside hydrolase, family 22	4.09
6	SCOERU6902301	IPR005788:Disulphide isomerase	4.34
	SCOERU6935001	NA	4.23
	SCOERU5237302	IPR011992:EF-hand domain pair	4.86
	SCOERU5212701	IPR001404:Heat shock protein Hsp90 family	5.72
	SCOERU6079601	NA	4.06
	SCOERU2832411	IPR011992:EF-hand domain pair	4.25
	SCOERU6109121	IPR003108:Gas2-related domain	4.81
	SCOERU5478902	IPR003108:Gas2-related domain	4.63
8	SCOERU5679902	IPR011992:EF-hand domain pair	5.68
	SCOERU2839501	IPR011992:EF-hand domain pair	5.72
9	SCOERU5228401	IPR011992:EF-hand domain pair	4.14
	SCOERU6948601	NA	3.87
	SCOERU6991801	NA	4.61
	SCOERU5131001	IPR005018:DOMON domain	3.61
	SCOERU2821601	IPR002913:START domain	3.54
9	SCOERU7013301	IPR004045:Glutathione S-transferase, N-terminal	5.00

NA, no annotation information.

Table 4. Functional annotation of 40 downregulated differentially expressed genes encoding oral apparatus associated proteins expressed during regeneration in *Stentor coeruleus* WHEL.

Time since shock (hour)	Protein ID	InterProScan annotations	Log ₂ fold change
0.5	SCOERU6558001	IPR001664:Intermediate filament protein	-14.50
	SCOERU6199801	IPR013126:Heat shock protein 70 family	-16.13
	SCOERU6230702	IPR001664:Intermediate filament protein	-16.16
	SCOERU6653011	IPR002423:Chaperonin Cpn60/TCP-1 family	-15.11
	SCOERU6653002	IPR002423:Chaperonin Cpn60/TCP-1 family	-14.27
	SCOERU3340101	IPR006140:D-isomer specific 2-hydroxyacid dehydrogenase,	-13.26
	SCOERU6177802	IPR009014:Transketolase C-terminal/Pyruvate-ferredoxin o	-13.72
	SCOERU6611202	IPR001404:Heat shock protein Hsp90 family	-7.17
	SCOERU5662301	IPR004541:Translation elongation factor EFTu/EF1A, bacterial/organelle	-13.69
	SCOERU6825012	IPR014001:Helicase superfamily 1/2, ATP-binding domain	-4.10
	SCOERU6773301	IPR004000:Actin family	-5.23
	SCOERU6467901	IPR000308:14-3-3 protein	-14.57
	SCOERU6490301	IPR018108:Mitochondrial substrate/solute carrier	-5.54
	SCOERU6807002	IPR013126:Heat shock protein 70 family	-3.46
	SCOERU6654401	IPR001557:L-lactate/malate dehydrogenase	-15.06
	SCOERU6525301	IPR002423:Chaperonin Cpn60/TCP-1 family	-5.49
	SCOERU6348101	IPR001680:WD40 repeat	-4.24
	SCOERU6661701	IPR004539:Translation elongation factor EF1A, eukaryotic/archaeal	-4.55
	SCOERU6773311	IPR004000:Actin family	-4.83
	SCOERU6508701	IPR001806:Small GTPase superfamily	-5.16
	SCOERU6188202	IPR000652:Triosephosphate isomerase	-14.55
	SCOERU6728001	IPR005788:Disulphide isomerase	-14.02
	SCOERU5219902	IPR000741:Fructose-bisphosphate aldolase, class-I	-14.41
	SCOERU6177301	IPR020828:Glyceraldehyde 3-phosphate dehydrogenase, NAD(	-6.13
	SCOERU6841701	IPR000217:Tubulin	-3.79
	SCOERU6823801	IPR000504:RNA recognition motif domain	-13.80
	SCOERU6214201	IPR005952:Phosphoglycerate mutase 1	-14.06
	SCOERU3306301	IPR001697:Pyruvate kinase	-6.73
1.5	SCOERU3671001	IPR018108:Mitochondrial substrate/solute carrier	-2.77
	SCOERU5535901	IPR013126:Heat shock protein 70 family	-6.48
	SCOERU6824901	IPR001404:Heat shock protein Hsp90 family	-7.31
	SCOERU6273502	IPR002423:Chaperonin Cpn60/TCP-1 family	-13.77
	SCOERU6088901	IPR011992:EF-hand domain pair	-13.29
	SCOERU6914701	IPR001148:Alpha carbonic anhydrase	-2.77
	SCOERU6506211	IPR005722:ATPase, F1 complex, beta subunit	-14.94
	SCOERU6805001	IPR000308:14-3-3 protein	-4.97
	SCOERU6455701	IPR004045:Glutathione S-transferase, N-terminal	-7.35
	SCOERU6841712	IPR000217:Tubulin	-17.00
	SCOERU6507601	IPR000217:Tubulin	-6.94
	SCOERU6628602	IPR001557:L-lactate/malate dehydrogenase	-14.81

Figures

Figure 1

Overall expression patterns of OA-associated proteins during regeneration in *Stentor coeruleus* WHEL. (a) Morphological events during oral apparatus regeneration in *Stentor coeruleus* WHEL (based on Sood et al., 2017). Time (in hours) since urea shock is on the upper left. 0h, the normal cell shape before urea shock. 0.5h, after the OA is shed, the anterior end of the cell is healed. The event marks the start of regeneration. 1.5h, basal bodies have begun to form at the locus of the OA primordium, appearing as a rift across fine striping near area of widest stripes. 3h, the first cilia of the membranellar band are visible in the OA primordium. 4h, the new membranellar band elongates and extends along the OA primordium. At this stage, the nodes of the macronucleus begin to condense. 6h, the new OA is completely formed and the macronucleus has fully coalesced. 8h, the new OA is fully regenerated. The membranellar band has completely wrapped around the anterior end of the cell and the macronucleus has re-nodulated. 9h, The cell resumes the normal shape and feeding activity. (b) Comparison of the expression levels of oral apparatus associated genes, ribosomal genes and other transcript fragments during regeneration in

Stentor coeruleus WHEL. Category of genes at each stage are indicated (left to right: other transcript fragments, oral apparatus genes, ribosomal genes). Relative expression level was calculated using the log2FPKM method. The lines of the whiskers in the box represent median lines. (c) Stacked histogram showing the proportion of oral apparatus associated genes with unchanged and changed expression at each stage of regeneration in Stentor coeruleus WHEL. The same of 809 proteins were analyzed at each stage. $P < 0.0001$, calculated using the chi-squared test.

Figure 2

Gene expression profiles of 232 differentially expressed genes encoding oral apparatus associated proteins during regeneration in in Stentor coeruleus WHEL. Genes were clustered into three groups (indicated by the colored bar on the y-axis). Time (in hours) since urea shock is shown on the x-axis. Group 1 contains all upregulated genes with maximum expression at a single stage of regeneration. Group 2 includes genes that were repressed at the initial stages of regeneration but subsequently increased expression during ciliary biogenesis. Group 3 includes genes that showed rarely changes at the initial stages of regeneration but subsequently downregulated during ciliary biogenesis. (The Z-score is calculated per row).

Figure 3

Hypothetical architecture of oral apparatus in *Stentor coeruleus* WHEL. (a) The membranelle band is shed during urea shock (based on Sood et al., 2017). (b) Structure of the membranelle band (depicting from Tartar, 1961). (c) Schematic diagram showing the cilium and surrounding subcellular structures.

Figure 4

Overview of regeneration induction and sample collection. (a) Morphological photograph of *Stentor coeruleus* WHEL. (b) Shedding oral apparatus with 4% urea treatment for 1 minutes to induce regeneration. (c) Collection of a large number of shedding oral apparatus for proteome analysis. (d) Selecting of single regenerating somatic cells at the different time points for transcriptome analysis.

Supplementary Files

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