Supporting Information

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SI Materials and Methods

Molecular Biology. For each total RNA extraction, tube feet (approximately 200–500 mg) were rapidly dissected from one sea star and immediately frozen in liquid nitrogen. They were then homogenized in Tri Reagent solution (Applied Biosystems) using a Polytron homogenizer (Kinematica), and total RNA was extracted according to Applied Biosystems' instructions. A 1-µg aliquot of total RNA was reverse-transcribed by using the FirstChoice RLM-RACE kit (Ambion). Inosine-containing degenerate sense oligonucleotide primers were designed on the basis of the peptide sequence HEASGEYY obtained by tandem MS (MS/MS) (1). They were used in combination with the 3′RACE outer primer (Ambion) to amplify DNA fragments from the cDNA obtained with the 3′RACE technique of the RLM-RACE kit. PCRs were carried out with the PuReTaq Ready-To-Go PCR beads kit (GE Healthcare) in a Thermal iCycler (Bio-Rad). An initial denaturation step of 3 min at 94 °C was performed, followed by 35 cycles of 30 s at 94 °C, 30 s at 47 °C and 2 min at 72 °C. A final elongation step was carried out for 10 min at 72 °C. The PCR products were subjected to 1% (wt/vol) agarose gel electrophoresis, and the DNA fragments amplified were excised and purified with Freeze 'N Squeeze DNA Gel Extraction Spin Columns (Bio-Rad). They were then cloned into a pCR2.1-TOPO vector by using the TOPO TA Cloning kit (Invitrogen) and transformed into Top10 chemically competent *Escherichia coli* cells. Several clones were selected for plasmid isolation with the GeneJET Plasmid Miniprep Kit (Fermentas), and their insert size was checked by PCR by using the vector-encoded universal M13F and M13R primers. Clones of the expected size were sequenced with the GenomeLab DTCS Quick Start Kit (Beckman-Coulter) in a Beckman CEQ 2000 XL DNA analysis system (Beckman-Coulter). The sequences obtained were checked manually by using BioEdit (2) and aligned using Clustal X (3), and the consensus sequence was created by using BioEdit. New sense and antisense primers were designed on the basis of this sequence and were used in nested PCR in combination with the 3′ and 5′RACE inner and outer primers. PCRs were performed as described earlier, except that, in this case, we used the DreamTaq DNA Polymerase (Fermentas) and/or the Pfu DNA Polymerase (Promega) and we adapted the annealing temperature according to the primer used. The PCR products were purified, cloned as described earlier, and sequenced with an ABI3730 sequencer by a commercial service (StarSeq). CodonCode Aligner (CodonCode) was used to align the sequences together with the two complete sequences resulting from a tBLASTn search using the peptide sequence HEASGEYYR in the tube foot transcriptome (purchased from GIGA-Genomics). The consensus sequence was translated into protein sequence by using the Translate tool of the ExPASy Proteomics Server (4). The presence of specific domains were predicted by the Conserved Domain Database (5), the presence of a signal peptide was predicted with the SignalP 4.1 Server (6), and molecular weight values were calculated by using the Compute pI/Mw tool of the ExPASy Proteomics Server (4). The sequence of Sea star footprint protein-1 (Sfp1) has been deposited in the GenBank database under accession number KJ472215.

MS and Data Analysis. Tube feet (approximately 300 mg) were rapidly dissected from one sea star and immediately frozen in liquid nitrogen. They were then homogenized in a 1.5 M Tris·HCl buffer (pH 7.8) containing 0.5 M DTT, 6 M urea, 2% (wt/vol) SDS, and protease inhibitors (Complete Protease Inhibitor Mixture Tablets; Roche) by using a Polytron homogenizer (Kinematica). The solution was incubated for 1 h at room temperature and centrifuged for 10 min at 19,000 × *g*. Proteins in the supernatant were resolved on a 4–20% (wt/vol) Mini-PROTEAN TGX precast polyacrylamide gel (Bio-Rad). The gel lane was entirely cut up into 27 serial slices by using a GridCutter (Gel Company). Gel slices were washed twice in 25 mM ammonium bicarbonate (ABC) buffer and then twice in ABC buffer containing 50% (vol/vol) acetonitrile, each time for 15 min under agitation. They were then incubated in 50 mM DTT in ABC buffer at 56 °C for 45 min to reduce disulfide bridges and subsequently in 50 mM iodoacetamide in ABC buffer in the dark. Again, the gel pieces were washed twice in ABC buffer and then twice in acetonitrile buffer. They were then incubated in 15 µL of a digestion solution made up of 10 ng/µL of trypsin (modified porcine trypsin, sequencing grade; Promega) in ABC buffer. This incubation was first performed for 15 min at 4 °C and then overnight at 37 °C. The reaction was stopped by adding 1.5 µL of 1% (vol/vol) formic acid. Tryptic peptides were analyzed by reverse-phase HPLC–electrospray ionization MS/MS using an Eksigent Ultra Plus nano-LC 2D HPLC system connected to a quadrupole time-of-flight TripleTOF 5600 mass spectrometer (AB SCIEX). Briefly, after injection, peptide mixtures were transferred onto the analytical C18-nanocapillary HPLC column (C18 Acclaim PepMap100, 75 µm i.d. × 25 cm, 3-µm particle size, 100-Å pore size; Dionex) and eluted at a flow rate of 300 nL/min by using the following gradient: 2–35% solvent B in A (from 0 to 12 min), 35–90% solvent B in A (from 12 to 14 min), 90% solvent B in A (from 14 to 19 min), 90–2% solvent B in A (from 19 to 20 min) and 2% solvent B in A (from 20 to 50 min), with a total run time of 50 min including mobile phase equilibration. Solvents were prepared as follows: mobile phase A, 2% acetonitrile/98% of 0.1% formic acid (vol/vol) in water; mobile phase B, 98% acetonitrile/2% of 0.1% formic acid (vol/vol) in water. Data acquisition was performed with a TripleTOF 5600 System (AB SCIEX) fitted with a Nanospray III source (AB SCIEX) and a pulled quartz tip as the emitter (New Objectives). Data were acquired by using an ion spray voltage of 2.6 kV, curtain gas of 15 psi, and ion source gas of 4 psi. For data-dependent acquisition, survey scans were acquired in 250 ms and as many as 50 product ion scans were collected if exceeding a threshold of 100 counts per second and with a 2+ to 4+ charge state. Total cycle time was fixed to 1.85 s. Four time bins were summed for each scan at a pulser frequency value of 15.42 kHz through monitoring of the 40 GHz multichannel TDC detector with four-anode/channel detection. A sweeping collision energy setting of 35 ± 15 eV was applied to all precursor ions for collision-induced dissociation.

Protein searches were performed against a translated version [performed using CAMERA 2.0 Portal (7)] of the tube foot transcriptome with the database search engine ProteinPilot (AB SCIEX) using the Paragon algorithm (4.0.0.0, 459). The following sample parameters were used: trypsin digestion, cysteine alkylation set to iodoacetamide. Processing parameters were set to "Biological modification" and a thorough ID search effort was used. The peptide and fragment mass tolerances were <10 ppm. During the search, Protein Pilot performs an automatic mass recalibration of the data sets based on highly confident peptide spectra. Specifically, a first search iteration is done to select high-confidence peptide identifications that are used to recalibrate the MS and MS/MS data, which is automatically researched. The

false discovery rate (FDR) analysis was done by using the integrated tools in ProteinPilot software. This tool automatically creates a concatenated forward and reverse decoy database, and provides an Excel output of the experimentally determined FDR at the spectral, peptide, and protein levels with standard statistical errors (8). The discriminating variable for the Paragon Algorithm is the peptide confidence value, which is a 0–99 scaled real number (8). For database searches, a cutoff peptide confidence value of 95 was chosen. The Protein Pilot FDR analysis tool (PSPEP) algorithm (8) provided, at a minimum, a global FDR of 1% and a local FDR at 5% in all cases. In addition, all peptides were manually inspected.

To evaluate the relative abundance of Sfp1 in footprints, we proceeded as follows. Proteins were extracted from footprints in a 1.5 M Tris·HCl buffer (pH 8.5) containing 7 M GuHCl, 20 mM EDTA, and 0.5 M DTT and incubated for 1 h at 60 °C under agitation. The sulfhydryl groups of the proteins were then carbamidomethylated with iodoacetamide used in a 2.5-fold excess (wt/wt) to DTT in the dark at room temperature for 20 min. The reaction was stopped by adding mercaptoethanol in the same quantity as iodoacetamide. The suspension was centrifuged at $16,000 \times g$ for 15 min at 4 °C and the supernatant was collected. Protein concentration was measured by using the Non-Interfering Protein Assay Kit (Calbiochem) with BSA as a protein standard. A total of 50 μg of proteins were precipitated in acetone overnight at −20 °C with an acetone/protein ratio of 4/1. After 15 min of centrifugation at $16,000 \times g$, and acetone evaporation, the resulting pellet was submitted to overnight enzymatic digestion by using modified porcine trypsin at an enzyme/substrate ratio of 1:50 at 37 °C. Tryptic peptides were analyzed by MS as detailed earlier. Resulting MS/MS data were searched for protein candidates against the tube foot transcriptome using MASCOT Daemon software (Matrix Sciences). The peptide mass tolerance was set to ±20 ppm, and fragment mass tolerance was set to ±0.05 Da. Carbamidomethyl cysteine was set as fixed modification, and oxidized methionine and deamidated asparagine were set as variable modifications. MASCOT Daemon was used to calculate an abundance index, the exponentially modified protein abundance index, which gives an evaluation of absolute protein contents in complex mixtures (9).

Western Blotting. Adhesive footprints were collected as previously described (1). Freeze-dried footprint material samples (approximately 2 mg) were suspended and incubated for 45 min at 4 °C in a 1.5 M Tris·HCl buffer (pH 7.8) containing 0.5 M DTT, 5 M urea, 2% (wt/vol) SDS, and protease inhibitors. For tube foot extraction, tube feet (approximately 400 mg) were rapidly dissected from one sea star and immediately frozen in liquid nitrogen. They were homogenized in one of the following buffers using a Heidolph Silent Crusher-M homogenizer (Labotal Scientific Equipment): (*i*) 1.5 M Tris·HCl buffer (pH 7.8) containing 0.5 M DTT, 5 M urea, 2% (wt/vol) SDS, and protease inhibitors; (*ii*) 1.5 M Tris·HCl buffer (pH 7.8) containing 5 M urea, 2% (wt/vol) SDS, and protease inhibitors; and (*iii*) 1.5 M Tris·HCl buffer (pH 7.8) containing protease inhibitors. The extracts were stored at 4 °C for 15 min to 1 h until use. Footprint and tube foot extracts were centrifuged for 10 min at $19,000 \times g$ and loaded on 8% (wt/vol) SDS/PAGE gels. After electrophoresis, the proteins were blotted onto a PVDF membrane using 25 mM Tris, 192 mM glycine, and 20% (vol/vol) methanol as transfer buffer. The blots were then immunodetected as previously described (1). In brief, they were probed with the four sets of purified polyclonal antibodies diluted as follows: anti-THDGMYEIDPDGAGGE (Sfp1α), 1:1,000; anti-VDGNDFEYITDEDGRD (Sfp1β), 1:500; anti-YGTYPTADCPETLEGA (Sfp1γ), 1:2,500; and anti-SGEYYRQGESVITKDC (Sfp1δ), 1:10,000, followed by HRP-conjugated anti-rabbit antibodies (Amersham) and chemiluminescence detection (Roche).

Histology and Immunohistochemistry. Tube feet were fixed in 4% (wt/vol) paraformaldehyde (PAF) in sodium phosphate buffer (PBS solution, pH 7.4), rinsed in PBS solution, dehydrated through an ethanol series, embedded in paraffin, and sectioned longitudinally as previously described (1). One section was stained with Heidenhain azan trichrome (10); the others were subjected to an indirect immunohistochemical staining method according to the following protocol. Antigen retrieval was achieved by incubation in a solution containing 0.05% (wt/vol) trypsin (Sigma) and 0.1% (wt/vol) $CaCl_2$ for 15 min at 37 °C. After one wash of 3 min in water, sections were blocked for 30 min in Tris-buffered saline solution containing 0.05% (vol/vol) Tween 20 and 3% (wt/vol) BSA (TBS-T-BSA). The four sets of purified antibodies diluted 1:100 in TBS-T-BSA were applied to the sections for 1 h 45 min at room temperature. After three washes of 5 min in TBS-T, the sections were incubated for 1 h in Alexa Fluor 488-conjugated goat anti-rabbit immunoglobulins (Invitrogen) diluted 1:100 in TBS-T-BSA. Following three final washes of 10 min in TBS-T, the sections were mounted with Vectashield (Vector Laboratories). Sections were observed by using a Zeiss Axioscope A1 microscope [excitation filter, bandpass (BP) 475/40 nm; beam splitter filter, farb teiler (FT) 500 nm; emission filter, BP 530/50 nm].

Footprint Immunolabeling. Footprints were fixed in 4% (wt/vol) PAF in PBS solution, rinsed in PBS solution, dehydrated in ethanol, and stocked in 70° ethanol. Upon use, they were rehydrated in distilled water followed by TBS-T and submitted to the immunolabeling method detailed earlier, without the antigen retrieval step. Footprints were observed by using a Zeiss Axioscope A1 microscope and a Olympus FluoView FV1000 confocal microscope.

Transmission EM and Immunocytochemistry. Tube feet were fixed for 3 h at 4 °C in 3% (vol/vol) glutaraldehyde in cacodylate buffer (0.1 M, pH 7.8; adjusted to 1,030 mOsm with NaCl). They were then rinsed in cacodylate buffer and postfixed for 1 h in 1% (wt/vol) OsO_4 in the same buffer. Another set of tube feet were also fixed on ice with 4% (wt/vol) PAF, 0.25% (vol/vol) glutaraldehyde in PBS solution (pH 8.0), and rinsed in PBS solution. The first set of tube feet were processed as previously described (11). Tube feet from the second set were dehydrated through an ethanol series, embedded in LR White Resin (Fluka), and processed as follows. Ultrathin (~70 nm) longitudinal sections were mounted on gold grids. They were incubated for 30 min in 0.5 M NH_4Cl to block free aldehyde groups, rinsed in TBS, and blocked for 1 h in 10% (vol/vol) goat serum in TBS-T-BSA. The four sets of purified antibodies diluted 1:100 in TBS-T-BSA were applied to the sections overnight at 4 °C. After five washes of 5 min in TBS, the sections were incubated for 90 min in goat anti-rabbit immunoglobulins conjugated to 15 nm gold particles (BB International) diluted 1:50 in TBS-T-BSA. Following several washes in TBS, and then ultrapure water, they were further stained with aqueous uranyl acetate and lead citrate and observed with a Zeiss LEO 906E transmission electron microscope.

Scanning EM. To collect footprints, tube feet were allowed to attach to and then detach from clean glass coverslips. Some tube feet were also cut off while still attached to these substrata. Both types of samples (footprints and attached tube feet) were then chemically fixed in Bouin fluid for 24 h, dehydrated in graded ethanol, and dried by the critical-point method. They were mounted on aluminum stubs, coated with gold in a sputter-coater, and observed with a JEOL JSM-6100 scanning electron microscope.

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MKAALLVVLLVACSANLVTSHDATPASCQDVLAQGNTHDGMYEIDPDGAGGEPQFQVYCELAGGKGVSIIHHDAECADREACKVDGFEEPGSFVKKVNYKGNI
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LVDTSITVSSTYVHEGDSHSFGPRSARLRHVGMHENLGGAWVADQKDESPWLQVDFRVEGTVTGLITQGRHAKVNWWVTRYAVYYSVNCEDWMPVEDANGDEM
VFEGNVDSDTEVERYFPNAVKAQCIRVYPRGFFGRAAMRLEVLGDFEIAANEYGMTVKTRDNLDNKNFILLAAGIPFESDGYITEWIFWPKTSAAFKAGVWRV
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DNEKIRSPYEKDGVTIIDGCNSRVILMTDFGLRVVWDGRSKVEVYLTEDYKHEVEGLCGNYDGQQGPDYFMRSGEPTTDINKFGESWGVDELCIGCQDCIEKD
ECRENELIEEAQKACHVLINPHGPFSMCHGIISVDAYYKECTFDFCKLYPNMENLCEDIQVYADDCNDKKIQIGIWRTPGFCNYPCPVGMVTTEIGTECPNTC
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EIASYPVRADTELISCEGNTLDLDCGFLKISVIRASYGRGQGDFVCTETGDDTSTTPLTEDCIFKSAFRLISEACHGKQTCTITSDTGLFGTDSCPTVSKYAK
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VRDLCHTKQSCLVQADTGTFGNPWAEGDEYMEITYKCSPQKQ

Fig. S1. Predicted amino acid sequence of Sfp1. The signal peptide is underlined (solid line) as well as the peptide sequences used for polyclonal antibody production (dotted line). Peptide sequences highlighted by colored residues denote peptides retrieved by MS analyses. Each color corresponds to one Sfp1 subunit (blue for Sfp1α, yellow for Sfp1β, green for Sfp1γ, red for Sfp1δ). The arrows indicate the cleavage sites between the different subunits.

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  1                 10                  20                  30                  40                  50                  60                  70
1 RFTNDVCENNDCNPLGGQCLPLRGSD-YQCSCKANFFGDKCEREYAMCRAWGDPHYTGFNGRNFDYQGMCKYTLATPC
2                     KAQNSDGYTCNCPAEWGGKNCEVKTNKCFGWGDPHYGQFDGAKYNFMGACTYVLTRTV
3 VLEDDKCDPNPC--INGECESVSIEPKYNCKCLPGFTGTHCEIGKATCKALGDPHYITFDDVR

  80                  90                  100       105
1 VDNKDFLFR
2 AGSTKDAFEVTAKNEKSLRQPSVSSTR
3
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Fig. S2. Identification of the cleavage sites between the four Sfp1 subunits. Three regions from the amino acid sequence of Sfp1, each overlapping two successive subunits, have been aligned with Geneious (version 7.0; Biomatters) to highlight a putative conserved cleavage site. These regions were identified by selecting sequence between the last MS-derived peptide that mapped to one subunit and the first to map to the next subunit (Fig. S1). Amino acids highlighted in colors are conserved between the three sequences. The box indicates the conserved GDPHY sequence.

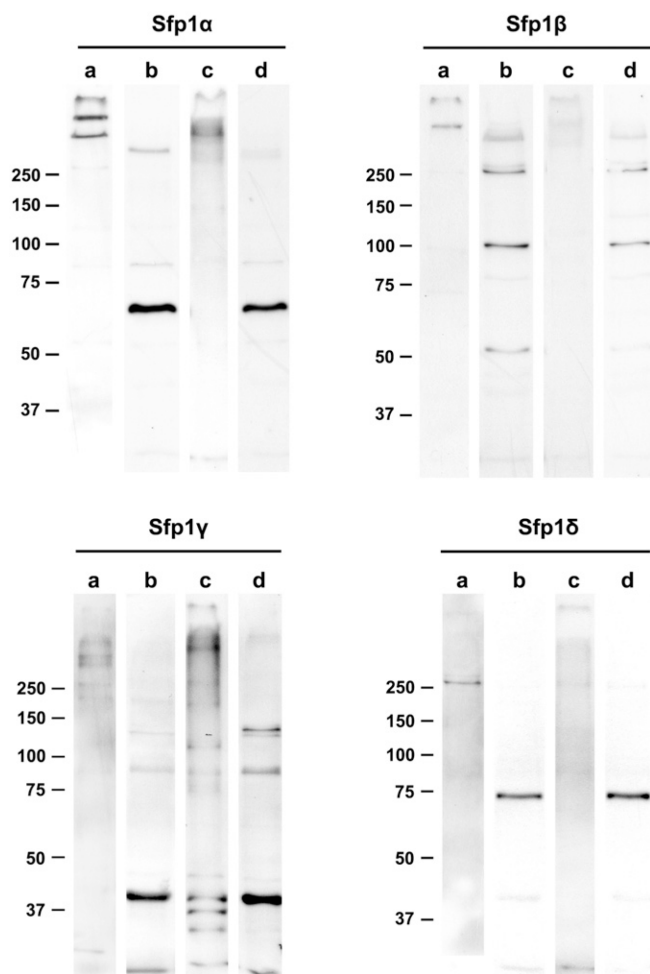

Fig. S3. Influence of DTT on the protein Sfp1 shows that its four subunits are linked by disulfide bonds. Proteins were extracted from tube feet in 1.5 M Tris·HCl buffer (pH 7.8) containing protease inhibitors (lanes a and b), or in 1.5 M Tris·HCl buffer (pH 7.8) containing 5 M urea, 2% (wt/vol) SDS, and protease inhibitors (lanes c and d), and were separated by SDS/PAGE on 8% (wt/vol) polyacrylamide gels. A fraction of each extract (lanes b and d) was supplemented with 0.5 M DTT before loading on the gels. Proteins were then transferred onto PVDF membranes, and the four Sfp1 subunits were detected in Western blot using specific polyclonal antibodies.

Table S1. Amino acid composition of Sfp1 and of the footprint material from *Asterias rubens* expressed as residues of amino acid per 100 residues

Amino acid	Sfp1	Footprints*
Ala	6.1	6.2
Arg	4.6	4.1
Asn	5.1	11.8†
Asp	7.3	
Cys	5	0
Cys/2	—	3.2‡
Gln	3.9	10.2†
Glu	6.6	
Gly	8.2	9.7
His	1.8	2.1
Ile	5.5	4.5
Leu	5.4	6.1
Lys	5.1	5.6
Met	2	1.7
Phe	4.9	3.8
Pro	4.4	6.1
Ser	6.2	7.6
Thr	5.7	7.8
Trp	1.4	—
Tyr	3.4	2.7
Val	7.5	6.7

*Amino acid analysis from Flammang et al (11).
†Deamidation during acid hydrolysis eliminates the distinction between Asn and Asp and between Glu and Gln; therefore, these two amino acid pairs are presented as Asx and Glx, respectively.
‡Reported values are for half-cystine, cysteine residues being destroyed during acid hydrolysis.

Other Supporting Information Files

Dataset S1 (PDF)