Molecular insights into the powerful mucus-based adhesion of limpets (*Patella vulgata* L.)

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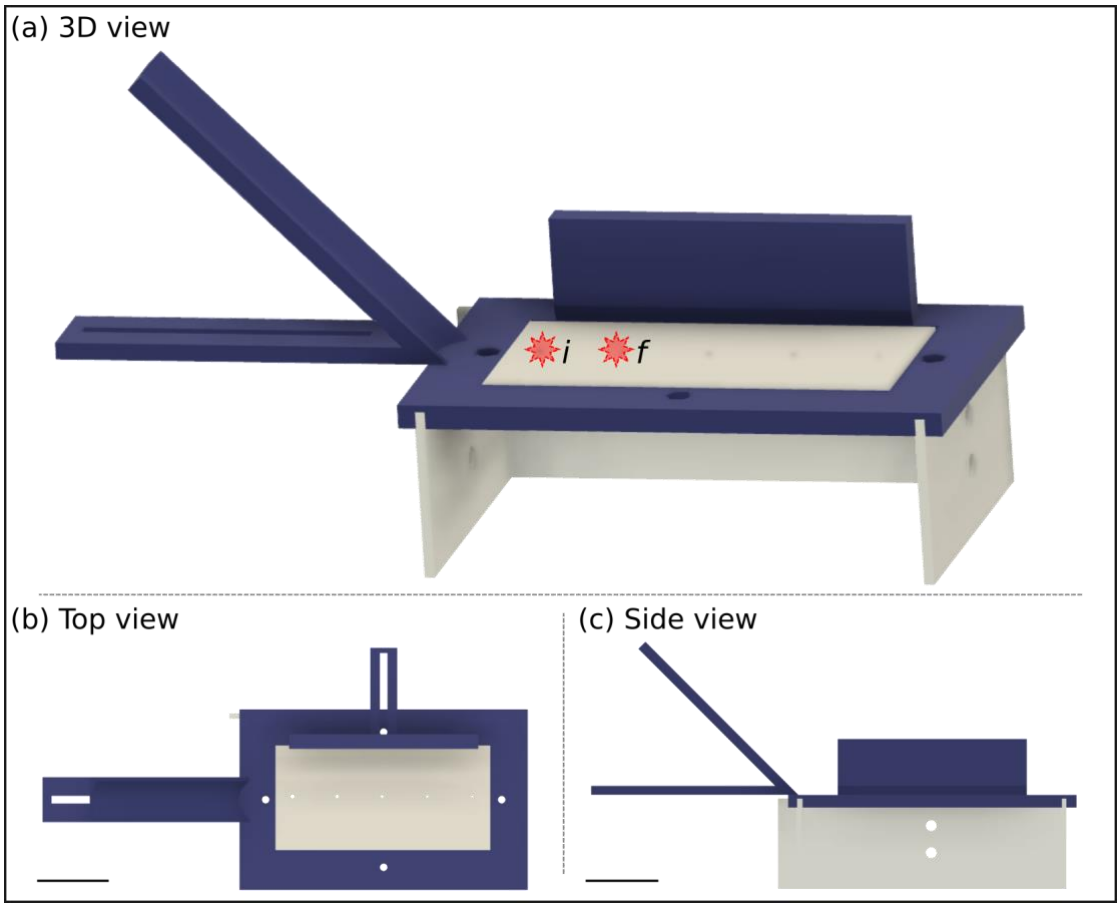
Supplementary Materials



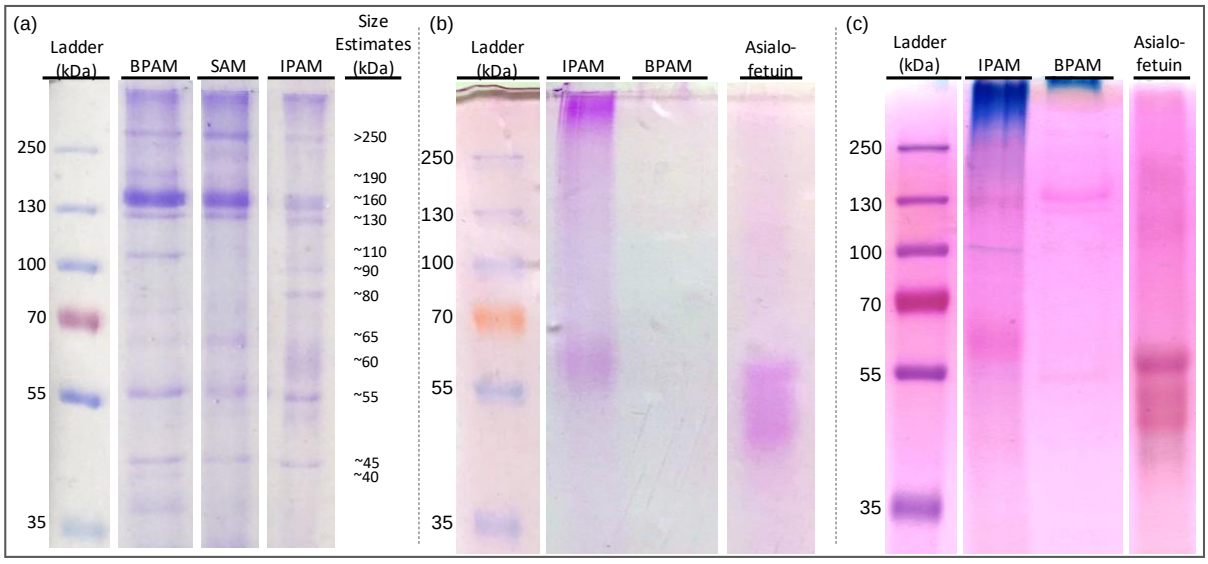
Supplementary Figure 1: Schematic of the *in vivo* pressure sensor rig made using 3D printed PLA components and laser-cut acrylic sheets. (a) 3D view, showing the 45° ramp used to accelerate the ball bearing. Red stars indicate initial *i* and final *f* position of limpets during free locomotion trials. (b) & (c) Top and side view of the set-up, respectively. Scale bar 40 mm.

Additional information on sequence quality check, trimming, and assembly
Quality check and trimming
Raw reads were filtered to remove reads with adaptors, reads with >5% unknown bases, and low quality reads (defined by BGI as reads in which the proportion of bases with a quality score < 10 was greater than 20%).

Assembly settings

The full software setting for read alignment using bowtie2, v2.2.5 was as follows: *Parameters: -q --phred64 --sensitive --dpad 0 --gbar 99999999 --mp 1,1 --np 1 --score-min L,0,-0.1 -I 1 -X 1000 --no-mixed --no-discordant -p 1 -k 200.*

Supplementary Figure 2: Example of a limpet that managed to crawl up and adhere to plastic mesh.



Supplementary Figure 3: SDS-PAGE gels of protein extracts from *P. vulgata* pedal sole mucus stained for additional information. BPAM: bulk primary adhesive mucus; SAM: secondary adhesive mucus; IPAM: interfacial primary adhesive mucus. (a) Coomassie Blue stain identified at least 11 prominent protein bands, ranging from ~40 to greater than 250 kDa. Note the approximate protein band sizes to the right of the gel image. (b) Smeared purple bands from PAS staining confirms that the presence of glycosylation for specific proteins within IPAM and not in BPAM. The strong smearing at the top of the gel indicates large complexes that failed to properly migrate into the gel. Asialo-fetuin from bovine serum used to as a reference glycoprotein to illustrate smearing pattern. (c) Multi-coloured bands from Stains-All highlight several differences between BPAM and IPAM (blue for highly acidic proteins and Ca^{2+}-binding proteins, purple for intact proteoglycans, and pink for weakly acidic proteins).

Supplementary Table 1: Transcriptome sequencing and assembly results of mRNA isolated from limpet pedal sole.

Sequencing output	
Total raw reads (M)	256.35
Total clean reads (M)	220.91
Total clean bases (Gbp)	33.14
High quality reads	
Q20 (%)	97.74%
Q30 (%)	94.30%
De novo assembly result	
Transcript number	86,396
Total size (bp)	81,369,009
Transcript N50 length (bp)	1,860
Transcript mean length (bp)	941
GC percentage (%)	35.59%

Supplementary Table 2: Functional annotation across seven databases.

Values	Total	Nr	Nt	SwissProt	KEGG	KOG	InterPro	GO	Intersection	Overall
Number	86,396	32,756	12,014	22,852	24,514	21,018	24,900	17,754	4,510	37,261
Percentage	100%	37.91%	13.91%	26.45%	28.37%	24.33%	28.82%	20.55%	5.22%	43.13%

Supplementary Table 3: List of limpet protein sequences tested with ISH.

Trinity assigned ID	Manuscript ID
Unigene47813	P-vulgata_1
Unigene46021	P-vulgata_2
CL3399.Contig1	P-vulgata_3
CL6799.Contig2	P-vulgata_4
CL6346.Contig2	P-vulgata_6
CL4559.Contig2	NA*
Unigene2524	NA
Unigene11734	P-vulgata_7
Unigene35700	NA
CL3409.Contig2	NA
Unigene36817	P-vulgata_8
CL489.Contig2	NA
CL5700.Contig1	P-vulgata_10
Unigene11944	P-vulgata_11
CL1231.Contig2	P-vulgata_12
Unigene15438	P-vulgata_14

*: Not applicable (NA), where a manuscript ID was not generated for a given Trinity-assigned protein ID as it was not included in downstream manual annotation and ISH was unsuccessful.

List of primers used to synthesise ISH probes

Manuscript ID	Primer Sequence
FW_P-vulgata_1_T7	GGATCCTAATACGACTCACTATAGGTTCCACATCAACCTTGTTCACC
FW_P-vulgata_2	TTTACGAATGTACGGCATCACC
FW_P-vulgata_3	TGTGATGGAGCAAACTGTACCC
FW_P-vulgata_4	GAAAACGACCATGTTCCAATCC
FW_P-vulgata_6_T7	GGATCCTAATACGACTCACTATAGGGCTGGGTTTCTCATTAGGTTCG
FW_P-vulgata_7_T7	GGATCCTAATACGACTCACTATAGGTCCTGGTGATTTCAATGGTAGC
FW_P-vulgata_8	ATTCTATCGGCGCATATTACGG
FW_P-vulgata_10	CAAAAAGAACGTGTTCCAGTCG
FW_P-vulgata_11	CTGTTGTGGTTTACAACCATGC
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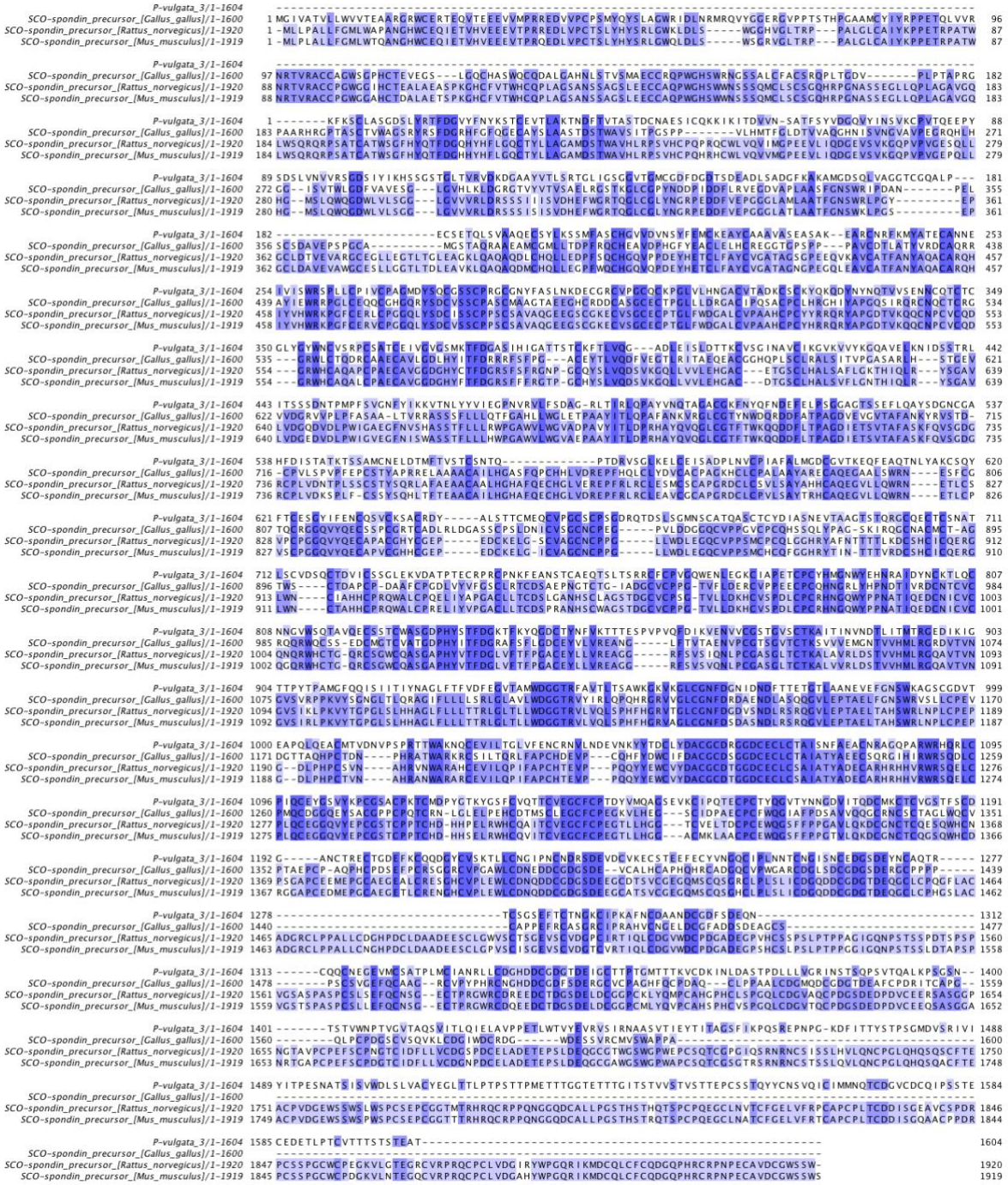
FW_P-vulgata_14_T7	GGATCCTAATACGACTCACTATAGGTTCCACTATGCGACACAAGAGC
FW_PV2524_T7	GGATCCTAATACGACTCACTATAGGTTTCGATCTAGGCAGAGAAATCC
FW_PV3409_T7	GGATCCTAATACGACTCACTATAGGTTCGAATTTTGCTCAACAGTGC
FW_PV35700_T7	GGATCCTAATACGACTCACTATAGGGGCCTCAAAACAGGTACATTGG
FW_PV4559	TTGTCTTGGAAGGAAACACAGC
FW_PV489	GGAGATATTGGTGCTGTTGACG
RV_P-vulgata_1	CAAAAGAAAAGGACGCAGATCC
RV_P-vulgata_2_T7	GGATCCTAATACGACTCACTATAGGCAATGCCACAGTTACAGTTCC
RV_P-vulgata_3_T7	GGATCCTAATACGACTCACTATAGGTGAAATCTTTTCCAGGGTTTGG
RV_P-vulgata_4_T7	GGATCCTAATACGACTCACTATAGGCAGGGTTTCCATCGAAGTTACC
RV_P-vulgata_6	TGCAAATGTGATATGCTCAACG
RV_P-vulgata_7	GATACGTGGTCCCAGAGAATCC
RV_P-vulgata_8_T7	GGATCCTAATACGACTCACTATAGGAAAGGTACAATGTGTGCGATCC
RV_P-vulgata_10_T7	GGATCCTAATACGACTCACTATAGGTAAACGTGTCTTGGATGGATGG
RV_P-vulgata_11_T7	GGATCCTAATACGACTCACTATAGGACTGGCCGTACTTGTCAAAAGG
RV_P-vulgata_12	AAATTACGTCGGAATGAATTAGCC
RV_P-vulgata_14	CCTTACGAAAAGGATGGATTCG
RV_PV2524	ACTAGCTCTGGTGGCTTACACG
RV_PV3409	GTAGAATGTACCGGCGAAGAGC
RV_PV35700	TACATCGAGTTGTCCCAACAGC
RV_PV4559_T7	GGATCCTAATACGACTCACTATAGGTTTCCAGGTCACCATCATAACG
RV_PV789_T7	GGATCCTAATACGACTCACTATAGGTCAATTTTATTCGGACGTGATCC

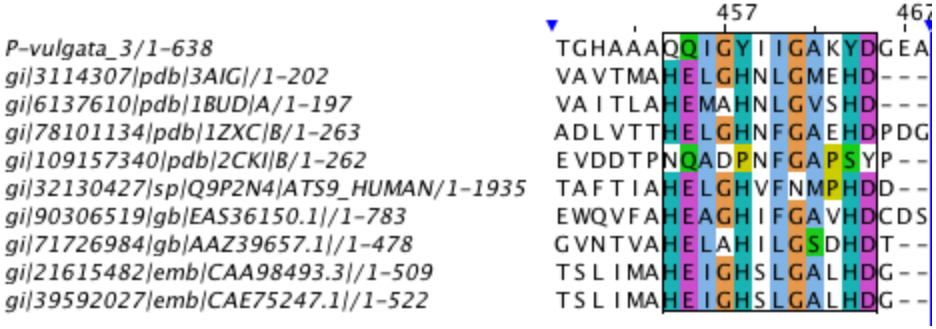
Supplementary Figure 4: Clustal alignments for SCO-spondin, Reprolysin, and Tc-MUC.

(a) Multiple SCO-spondin precursors and *P-vulgata_3* alignment



SCO-spondin *Gallus gallus* (truncated) and *P-vulgata_3* alignment showing SCOR regions

(b) Reprolysin CDD conserved sequences and *P-vulgata_8* alignment



(c) Tc-MUC and *P-vulgata_14* alignment

Detailed protocol of *in situ* hybridization (ISH)

RNA probe synthesis. Primers were designed with Primer3 (http://primer3.ut.ee, v4.1.0) and for antisense/sense probe production a T7/SP6 promoter region was added at the 5`end of the reverse/forward primers. cDNA was generated from isolated total RNA of the limpet tissue, (same limpet individual as the one sent for transcriptome sequencing) using Transcriptor First Strand cDNA Synthesis Kit (Roche). PCR reactions were performed with Q5® High-Fidelity DNA Polymerase (NEB) and their correct length verified on a 1 % agarose gel. The PCR products were purified with Wizard® SV Gel and PCR Clean-Up System (Promega). To synthesize single stranded digoxigenin-labelled RNA probes, T7/Sp6 polymerase (Promega) and DIG labelling mix (Roche) were used according to manufactures' protocols. DIG-labelled RNA probes were precipitated with isopropanol and bands length and intensity checked on a 1 % agarose gel. RNA probes were diluted in hybmix to an estimated concentration of 5ng/µl (based on gel images) and stored at -80°C until usage. Probes were used at a final concentration of approximately 0.2 ng/µl. We repeatedly observed unspecific background staining using sense probes, which should not be able to bind to any mRNA specifically (supplementary figure 4a). After extensive troubleshooting we realised that the precipitation with isopropanol was not sufficient to eliminate unbound DIG-labelled nucleotides, which were the source of this background staining. Please also see *background staining in the in situ hybridization experiments* addressing this issue.

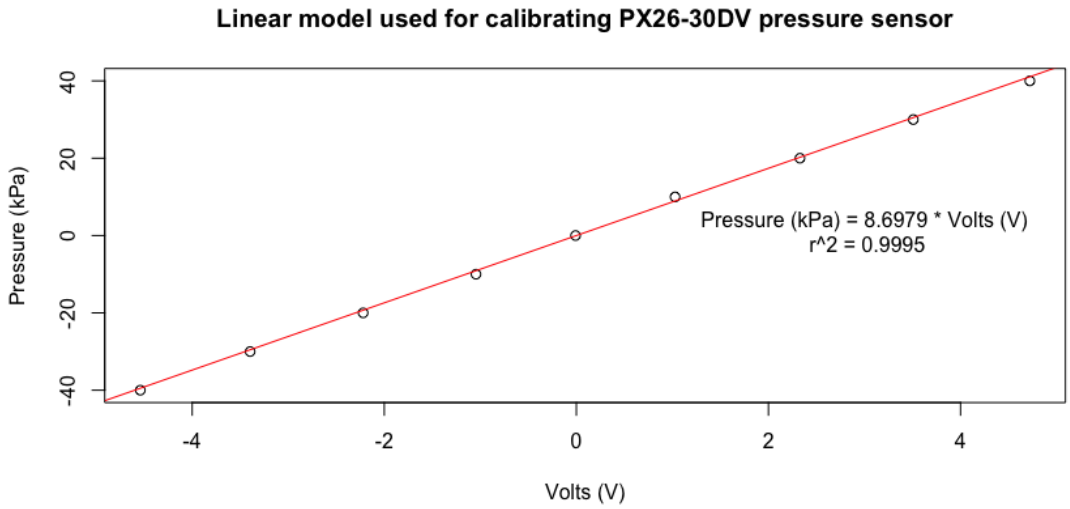
Paraffin section *in situ* hybridization. For section *in situ* hybridization, Patella tissue was dissected and fixed in 4% PFA in PBS overnight. After several washes in PBS, the tissue was dehydrated in an ethanol series until 100% ethanol. Tissue pieces were treated with xylene for 8 and 5 min and immediately placed in 60°C preheated liquid paraffin wax. The tissue was moved to fresh preheated paraffin wax every hour for three times and left at 60°C overnight. Afterwards, the samples were moved to room temperature for the paraffin blocks to harden. The paraffin-embedded tissue was then cut into 14 µm thick sections using a Microm HM 340 E microtome. The sections were placed on Superfrost ultra plus® slides (Thermo scientific) and heated in an oven at 60 °C for 1 h and allowed to cool down for 15 min. After dewaxing in xylene and rehydration, sections were postfixed in 4% PFA in PBS for 20 min, washed several times in PBS and treated with 0.2 M HCl for 10 min. Following washes in PBS, sections were treated with Proteinase K (20 µg/ml) for 20 min. Sections were postfixed in 4 % PFA in PBS for 5 min, washed in PBS and placed in 0.1 M triethanolamine (pH 8) containing 0.5 % acetic anhydride for 20 min (exchanging the solution after 10 min). Afterwards sections were washed and gradually dehydrated with ethanol. To dry the sections, Chloroform was dropped on the sections and allowed to evaporate. Slides were placed in a humidity chamber and incubated with hybmix at 55 °C for 30 min. Sections were incubated with RNA probes diluted in hybmix (0.2 ng/µl) overnight at 55 °C. To avoid evaporation of the liquid, sections were covered with fresh parafilm pieces during this step. The following day the sections were incubated in 50 % formamide in 2x SSC at 62 °C (3 x 20 min). After washes with 1x SSC and PBS, sections were blocked in blocking reagent (Roche) for 1 h at 4 °C. Sections were incubated with Anti-digoxigenin-AP Fab fragments (Roche) 1:2000 in blocking reagent overnight at 4 °C. Following several washes, the signal was developed using the NBT/BCIP system (Roche) at 37 °C. Colour reaction was stopped with ethanol and sections washed several times with PBS. Sections were mounted in Mowiol® 4–88 (Roth, Germany), prepared according to the manufacturer's protocol and images were taken with a Zeiss Axioscope A1 microscope.

Background staining in the *in situ* hybridization experiments. For ISH negative control samples, we used DIG-labelled RNA sense probes that should not bind to any mRNA sequences. However, we repeatedly observed unspecific background staining in the negative controls in glands ~100-150 µm from the epithelium, in the epithelium and secreted mucus (supplementary figure 4a). No staining was observed when no ISH probes were added to the samples (not shown). For interpretation of ISH results in this study, we only considered probes that consistently stained differently to the background in two to three repeated ISH experiments. Using *P-vulgata_3* as an example (supplementary figure 4c; modified version of Figure 3b), the positive stainings were distinct in intensity and locality compared to background stainings (supplementary figure 4a). Subsequent troubleshooting by B.L. in Mons, where all the ISH experiments were conducted, identified the probable cause as short DIG-labelled nucleotides that freely bound to unspecific regions of the tissue. When negative control probes were additionally purified through a Micro Bio-Spin® Chromatography Column (Biorad) and re-tested, the unspecific background staining issue was

resolved (supplementary figure 4b). Unfortunately, data collection for the project had concluded several months prior to this clarification, and the lead author had already returned to Cambridge, UK, hence ISH could not be repeated again. Nevertheless, by carefully considering the differences between background and signal staining patterns and by upholding a high threshold for inclusion, we are confident that we have reported valid results for the five candidate probes (shown in Figure 3 of the main text).

Supplementary Figure 5: Unspecific background staining due to DIG-labelled nucleotides present in the ISH probes. (a) Unspecific background stains in the negative control samples were diffuse and localised to ~100-150 µm from the epithelium. Scale bar 20 µm. (b) When negative control probes were additionally purified through microspin columns, background stains were eradicated. Scale bar 20 µm. (c) *P-vulgata_3* staining, showing the distinct staining pattern between the signal (green arrows) and the background (orange). Based on our prior experiences with ISH, the sharp intense stains indicated by the green arrows are indicative of positive stains. Additionally, the background and positive stains were segregated by location within the tissue. ISH probes that did not fit these criteria were not included in the analysis.

Additional information on PX26-30DV pressure sensor calibration: The pressure sensor was calibrated by connecting a syringe pump to an analogue pressure gauge (WIKA Instruments Ltd, UK), which in turn was connected to the sensor port. The syringe pump was used to increase the pressure (relative to ambient) from 0 to +40 kPa in 10 kPa bar steps, and the same to decrease the pressure from 0 to -40 kPa. Sensor output voltage was then plotted against pressure, and a linear regression (intercept set to 0) was fit to obtain the relationship Pressure (kPa) = 8.6979 * Volts (V), with a Pearson's correlation $r^2 = 0.9995$ (see below). This linear model was used to analyse the in vivo sub-pedal pressures from *P. vulgata*.



Additional information for manual vertical pull-off experiments: for the trial shown in Figure 3c, the limpet had been disturbed previously and had clamped down, corresponding to the slight negative pressure (around -1.4 kPa). The pull-off force was applied by gripping the limpet's shell with fingers and pulling upwards starting from ~20s until detachment at ~25s. Because of this method, we cannot state with confidence whether the applied detachment force was constant during the whole duration. For this particular trial, we observed an initial negative peak of around -2 kPa, followed by a second much larger peak of -5.7 kPa, and then the pressure returned to -2 kPa for ~3 s before full detachment. One possible explanation for this second dip is that as the limpet was being pulled, it attempted to clamp down, which would result in a decrease in sub-pedal pressure. Once it relaxed, the pressure returned to around -1.4 kPa, before being fully detached. We wrote in the Discussion that clamping can lead to a sub-pedal pressure reduction, although this still requires adhesion between the contact area and the surface, which we propose is provided by adhesive mucus.

Encoding cDNA sequences of the fourteen annotated protein (see Supplementary Table 3 for identifiers)

>Unigene47813_L1_2_1

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