Supporting Information

Franzenburg et al. 10.1073/pnas.1213110109

SI Materials and Methods

Animal Culture. Experiments were carried out using *Hydra vulgaris* (AEP). All animals were cultured under constant, identical environmental conditions including culture medium, food, and temperature according to standard procedures. All animal experiments were conducted in accordance with the German law concerning animal experimentation.

Generation of Transgenic *H. vulgaris* (AEP) Expressing an EGFP–MyD88–Hairpin Fusion Construct in Endodermal and Ectodermal Cells. For generation of *H. vulgaris* (AEP) egfp:myd88–hairpin transgenics, a 430-bp antisense (as) fragment of *myd88* was amplified from *H. vulgaris* (AEP) cDNA using Platinum High-Fidelity polymerase (Invitrogen). The fragment was cloned into a modified hotG EGFP-expression vector using the AsisI and BsiWI restriction sites (Fig. 1*A*) adjacent to the *egfp*. A stop codon terminating the EGFP was inserted with the forward primer. Next, a 750-bp sense (s) fragment of *myd88* was amplified with the first 320 bp as the linker sequence and the last 430 bp as the reverse complement to the antisense fragment. This fragment was cloned into the expression vector using the BsiWI and EcoRI restriction sites at the 3′ end of the egfp:myd88_as construct (Fig. 1*A*). The resulting transfection construct was sequenced and plasmid DNA was purified using the Qiagen MidiPrep kit and injected into *H. vulgaris* (AEP) embryos as previously described (1). Embryos began to express the reporter gene 2–3 d after injection. Founder transgenic animals bearing the egfp:myd88–hairpin construct started to hatch 14 d after microinjection. One of them showed stable EGFP expression in a group of ectodermal as well as endodermal cells. The initial founder transgenic polyp was expanded further by clonal propagation. By selecting for EGFP expression using an Olympus SZX16 stereomicroscope, mass cultures of both, polyps with no transgenic cells (MyD88 control) and polyps with full endodermal and ectodermal expression of EGFP (MyD88 knockdown), were generated (Fig. 1 *C* and *D*). Successful down-regulation of *myd88* was verified by RT-PCR using primers targeting regions of the *myd88* gene that were not used in generating the hairpin construct (Fig. 1*E*).

Generation of Germ-Free *Hydra*. Polyps were incubated for 1 wk in an antibiotic solution containing 50 μg/mL each of ampicillin, rifampicin, streptomycin, and neomycin with daily exchange of the solution. After 1 wk of treatment, the polyps were transferred into sterile-filtered and autoclaved culture medium and fed with germ-free *Artemia salina* larvae (hatched in 30‰ artificial sea water containing the same antibiotics). Following 1 wk of recovery, the absence of bacteria was verified by plating homogenized polyps on R2A agar plates. After incubation at 18 °C for 3 d the cfu were counted. Absence of cfu indicated successful antibiotic treatment.

For culture independent analysis, total DNA was extracted from single polyps using the DNeasy Blood and Tissue kit (Qiagen). 16S rRNA genes were amplified using the universal primers Eub-27F and Eub-1492R (2) in a 30-cycle PCR. Sterility was verified by the absence of a PCR product (Fig. 1*G*).

Custom-Made *H. vulgaris* (AEP) Microarray. The microarray is based on a full transcriptome of *H. vulgaris* (AEP) sequenced by 454 technology (3). The final assembly contained 49,070 contigs resulting in 31,192 peptide predictions. For the microarray design (Agilent Technologies) we used all contigs with a peptide prediction and additionally, we integrated contigs without a peptide prediction that were larger than 260 bp. This results in a microarray platform having 45,220 oligos of 60 nucleotides in length, resembling 37,063 unique contigs.

RNA Isolation and Microarray Gene Expression Experiments. Total RNA was isolated from 15 polyps using the TRIzol Plus protocol (Invitrogen). Three MyD88-control and four biological replicates each of MyD88-knockdown and MyD88-control germ-free animals were conducted. Quality was checked by 260/280 and 260/230 ratios and visualization of rRNA bands by agarose gel electrophoresis. Four-hundred nanograms of total RNA per sample was labeled with Cy3 using the one-color Quick Amp Labeling kit protocol (Agilent). Labeled cDNAs were hybridized to custom-made Agilent *Hydra* (AEP) Gene Expression Microarray slides (4 × 44K) for 17 h at 65 °C and washed according to the Agilent protocol. Hybridized microarray slides were scanned using an Agilent High-Resolution Microarray scanner.

Microarray Data Extraction, Filtering, and Analysis. Raw microarray image files were processed and quality checked by Agilent's Feature Extraction 10.7 Image Analysis software. Background-subtracted signal-intensity values that contain correction for multiplicative surface trends (gProcessedSignal) generated by the Feature Extraction software were used for further data analysis. Using GeneSpring microarray data analysis software we filtered probes that were flagged as nonuniform or as population outlier. For all samples we calculated average signal-intensity values over the three (MyD88 control) or four (MyD88 knockdown and MyD88 control germ-free) biological replicates. Statistical analysis was conducted by ANOVA with Student–Newman–Keuls (SNK) post hoc test and false discovery rate (FDR) correction for multiple comparisons. After setting a threshold of ≥1.5-fold change compared with the control, resulting significant ($P \leq 0.05$) differentially expressed contigs were analyzed using the blast2go batchblast (4) with BLASTX ($\leq E^{-6}$) and domain prediction by InterProScan (5) and grouped into self-chosen categories. Microarray data are deposited at Gene Expression Omnibus (GEO) with the accession number GSE32383.

qRT-PCR. qRT-PCR was conducted in biological triplicates (n = 3), using the GoTaq qPCR Master mix (Promega) and a 7300 Real-Time PCR system (ABI). Template amounts were equilibrated for the *Hydra* EF1α gene (EF1α_F 5′-GCAGTACTGGTGAGTTTGAAG-3′ and EF1α_R 5′-CTTCGCTGTATGGTGGTTCAG-3′).

SP600125 c-Jun N-Terminal Kinase (JNK) Inhibitor Treatment. For the treatment with SP600125 (A. G. Scientific), polyps (25 each) were incubated at a density of 1 polyp per milliliter in SP600125 diluted in 5% (wt/vol) DMSO/*Hydra* medium for 30 min on ice. Following three short washes in 0.1% DMSO/*Hydra* medium, animals were transferred to SP600125 diluted in 0.1% DMSO/*Hydra* medium in the dark for 24 h at 18 °C (6). RNA was extracted using the TRIzol Plus protocol (Invitrogen).

DNA Extraction and Sequencing of 16S rRNA Genes. For total DNA extraction, single polyps were subjected to the DNeasy Blood and Tissue kit (Qiagen) after being washed three times with sterile filtered culture medium. Extraction was performed following the manufacturer's protocol, except that DNA was eluted in 50 μL. For sequencing of the bacterial 16s rRNA genes, the variable region 2 (V2) was amplified using the universal forward primer V2_B_Pyro_27F (5′-CTATGCGCCTTGCCAGCCCGCTCAG-

*TC***AGAGTTTGATCCTGGCTCAG**-3′), which consists of the 454 FLX amplicon primer B (underlined), a two-base linker (italics), and the universal 16S primer 27F (bold) and the barcoded reverse primer V2_A_338R (5′-CGTATCGCCTCCCTCGCGCCATCAGNNNNNNNNNN*CA***TGCTGCCTCCCGTAGG**AGT-3′) which contains the 454 FLX amplicon primer A (underlined), a sample specific 10-mer barcode (N's), a two-base linker (italics), and the universal 16S primer 338R (bold). Twenty-five–microliter PCR reactions were performed using the Phusion Hot-Start II DNA polymerase (Finnzymes) following the manufacturer's instructions. PCR conditions consisted of an initial denaturation step (98 °C, 30 s) followed by 30 cycles of denaturation (98 °C, 9 s), annealing (55 °C, 30 s), and elongation (72 °C, 20 s). PCR was terminated by a final elongation of 72 °C for 10 min. All reactions were performed in duplicates, which were combined after PCR. PCR products were extracted from agarose gels with the Qiagen MinElute Gel Extraction kit and quantified with the Quant-iT dsDNA BR Assay kit on a NanoDrop 3300 Fluorometer. Equimolar amounts of purified PCR product were pooled and further purified using Ampure Beads (Agencourt). A sample of each library was run on an Agilent Bioanalyzer before emulsion PCR and sequencing as recommended by Roche. Amplicon libraries were subsequently sequenced on a 454 GS-FLX using titanium sequencing chemistry.

16S rRNA 454 Analysis. 16S rRNA amplicon sequence analysis was conducted using the Qiime 1.3 package (7). Using the sequence fasta-file, a quality file, and a mapping file that assigned the 10-nt barcodes to the corresponding sample as input, the sequences were analyzed using the following parameters: length between 300 and 400 bp, no ambiguous bases, and a maximum of a single mismatch to the primer sequence. Chimeric sequences were identified and removed using Chimera Slayer (8). Resulting high-quality reads ranged from 2,739 to 7,394 per sample. Sequences were normalized to 2,700 sequences per sample, grouped into operational taxonomic units (OTUs) at a ≥99% sequence identity threshold, and classified by Ribosomal Database Project (RDP) classifier. Beta diversity was assessed using the weighted UniFrac metric (1,000 replicates) and visualized by principal coordinate analysis (PCoA). Alpha diversity measurements were conducted using the chao1 metric and observed species (Fig. S4). 454 data are deposited at MG-Rast with Project ID 1719.

***Pseudomonas aeruginosa* Infection.** *P. aeruginosa* strain PA14 was cultured in LB medium for 16 h at 37 °C. Fifty milliliters of culture was centrifuged for 10 min at 1,380 × *g*. The resulting pellet was resuspended in 50 mL sterile *Hydra* medium. This resulted in an optical density (OD_{600}) of 0.93, which was diluted to an OD_{600} of 0.1 with sterile *Hydra* medium. Single MyD88-knockdown and -control polyps (n = 25 each) were transferred in single wells of 24-well plates and incubated in 1 mL of the PA14 solution. Colony-forming units per milliliter were counted by plating 100 μL of a 1/10,000 dilution of OD_{600}= 0.1. Mean resulting cell count was 1.8×10^8 cells per milliliter. Plates were sealed and stored at 20 °C. Polyps were screened daily and scored by following the criteria shown in Fig. 5.

1. Wittlieb J, Khalturin K, Lohmann JU, Anton-Erxleben F, Bosch TC (2006) Transgenic Hydra allow in vivo tracking of individual stem cells during morphogenesis. *Proc Natl Acad Sci USA* 103(16):6208–6211.
2. Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* 173(2):697–703.
3. Hemmrich G, et al. (2012) Molecular signatures of the three stem cell lineages in Hydra and the emergence of stem cell function at the base of multicellularity. *Mol Biol Evol*, 10.1093/molbev/mss13.
4. Conesa A, et al. (2005) Blast2GO: A universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* 21(18):3674–3676.
5. Zdobnov EM, Apweiler R (2001) InterProScan—an integration platform for the signature-recognition methods in InterPro. *Bioinformatics* 17(9):847–848.
6. Philipp I, et al. (2009) Wnt/beta-catenin and noncanonical Wnt signaling interact in tissue evagination in the simple eumetazoan Hydra. *Proc Natl Acad Sci USA* 106(11): 4290–4295.
7. Caporaso JG, et al. (2010) QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 7(5):335–336.
8. Haas BJ, et al.; Human Microbiome Consortium (2011) Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. *Genome Res* 21(3):494–504.

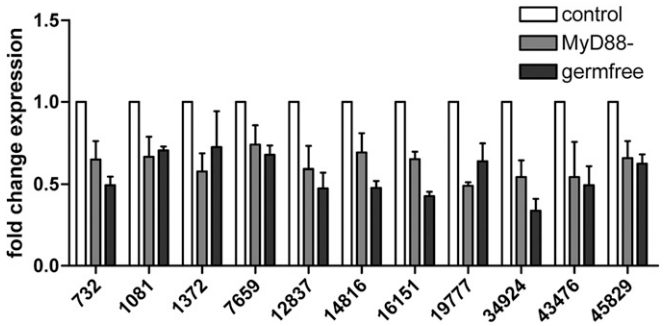

Fig. S1. Differential gene expression between control, MyD88-knockdown, and germ-free polyps. Expression changes of MyD88 candidates were validated by qRT-PCR. The cDNA amounts were equilibrated by elongation factor 1 alpha (EF1α); the graphic shows means + SD (n = 3). Genes are named according to their contig number in the *Hydra vulgaris* AEP transcriptome; an annotation is shown in Table 1.

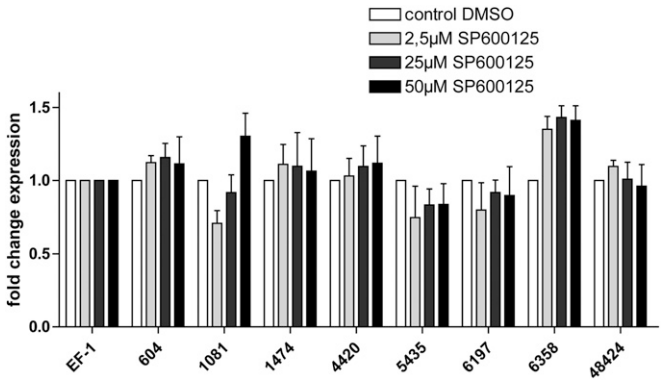

Fig. S2. JNK inhibitor SP600125 does not cause drastic unspecific changes in the *Hydra* transcriptome. Relative expression level of randomly chosen genes upon administration of the JNK inhibitor SP600125, determined by qRT-PCR. None of the tested genes showed a concentration-dependent down-regulation upon SP600125 treatment. cDNA amounts were equilibrated by elongation factor 1 alpha; the graphic shows means + SD (n = 3).

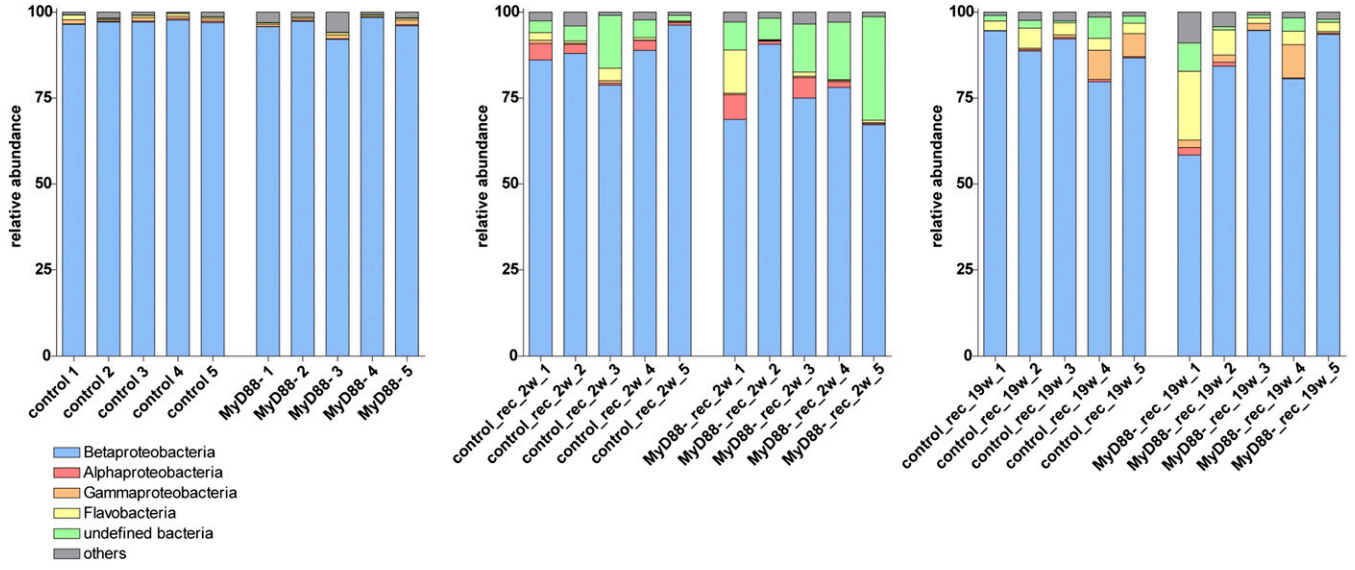

Fig. S3. Abundance of the major bacterial classes in the *Hydra vulgaris* (AEP) microbiota for all sequenced samples. The fraction of "others" includes all rare fractions with a relative abundance of <1%.

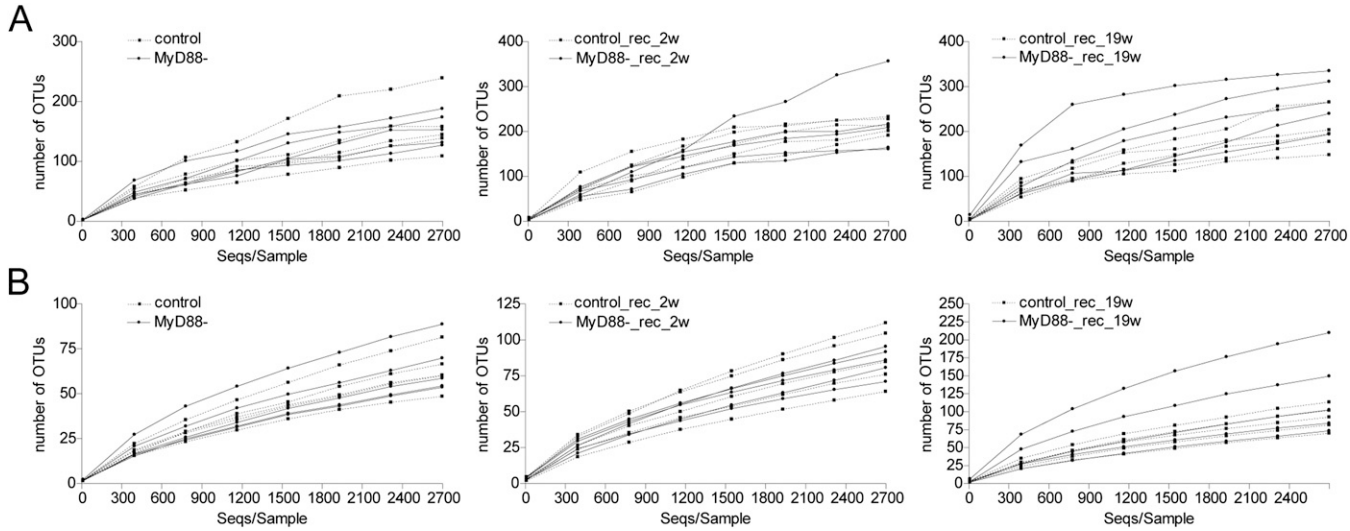

Fig. S4. Alpha-diversity curves for all sequenced samples. (*A*) Chao1-bacterial diversity estimation. (*B*) Observed species. Number of reads was capped in silico to the smallest sample (2,700 reads).

Table S1. Putative TLR-cascade members show differential expression in germ-free and/or MyD88-deficient conditions in *Hydra*

			MyD88$^-$		Germ-free	
Annotation	Contig*	Blastp†	Fold change	*P* value	Fold change	*P* value
LBP	9861	7.00*E*-40	1.01	0.723	0.74	0.026
LRR1	47938		1.15	0.032	1.13	0.022
LRR2	46369		1.08	0.049	1.17	0.033
TRR1	708		1.06	0.268	0.82	0.045
TRR2	10818		0.97	0.532	0.88	0.032
MyD88	11552	3.00*E*-34	0.23	0.000	0.91	0.115
IRAK	7139	1.00*E*-20	0.88	0.261	0.88	0.261
Tollip	46616	1.00*E*-65	0.98	0.590	0.87	0.017
TRAF4	12390	2.00*E*-26	1.01	0.963	0.64	0.010
	13328	2.00*E*-40	1.02	0.946	0.61	0.010
	44978	1.00*E*-13	0.89	0.100	0.65	0.004
TRAF3	**45514**	**7.00*E*-31**	**0.85**	**0.021**	**0.68**	**0.003**
	45513		**0.80**	**0.020**	**0.57**	**0.003**
	12389		**0.78**	**0.006**	**0.56**	**0.002**
TRAF6	46773	3.00*E*-92	0.95	0.479	1.19	0.069
TAK1	**474**	**1.00*E*-95**	**0.84**	**0.020**	**0.79**	**0.000**
IKKβ	47555	3.00*E*-88	0.92	0.623	1.05	0.689
ankyrin_rp	**7685**		**1.34**	**0.003**	**1.77**	**0.000**
IkappaB	8355	3.00*E*-17	1.01	0.857	1.09	0.080
NfkappaB	9522	3.00*E*-46	0.99	0.714	0.92	0.005
p38 alpha	**10926**	**2.00*E*-151**	**0.82**	**0.003**	**0.74**	**0.001**
p38 beta	**35834**	**3.00*E*-18**	**0.74**	**0.018**	**0.60**	**0.001**
JNK	13307	0.0	0.95	0.559	0.90	0.084
JSP-1	**12280**	**3.00*E*-44**	**0.79**	**0.003**	**0.63**	**0.005**
cJun	43498	2.00*E*-26	0.89	0.378	1.05	0.038

Boldface indicates genes that are significantly differentially regulated in both MyD88$^-$ and germ-free polyps.
*Contigs are available at http://compagen.zoologie.uni-kiel.de/.
†*Hydra vulgaris* (AEP) predicted peptides were blasted (blastp) against human proteins (National Center for Biotechnology Information). *E* values for the human ortholog are depicted.