



Table S1. Summary of mapping reads to mouse genome.

Sample Name	Total Reads	Total Mapped	Multiple Mapped	Uniquely Mapped
Germ-free Sample 1	98926086	96.20%	6.29%	89.91%
Germ-free Sample 2	94561652	96.58%	5.83%	90.75%
Germ-free Sample 3	88830420	96.68%	6.54%	90.15%
SPF Sample 1	103975920	96.11%	5.41%	90.70%
SPF sample 2	94930984	95.80%	5.47%	90.33%
SPF Sample 3	79055336	96.14%	7.26%	88.87%

Table S2. All differentially expressed genes.

Gene symbol	Gene name	GF vs. SPF Fold change	FDR p-value
Gm20388	ENSMUSG00000092329 predicted gene	-44.76	4.50E-05
Gm38947	ENSMUSG00000110365 predicted gene	-12.14	7.60E-06
Arntl	ENSMUSG00000055116 aryl hydrocarbon receptor nuclear translocator-like	-4.36	0.00E+00
Adamts4	ENSMUSG00000006403 a disintegrin-like and metallopeptidase	-3.87	1.80E-09
Cyp26b1	ENSMUSG00000063415 cytochrome P450, family 26, subfamily b, polypeptide 1	-3.5	2.00E-03
Diras2	ENSMUSG00000047842 DIRAS family, GTP-binding RAS-like 2	-3.14	3.90E-08
Egr1	ENSMUSG00000038418 early growth response 1	-3.06	2.10E-04
Fosb	ENSMUSG00000003545 FBJ osteosarcoma oncogene B	-2.81	8.30E-04
Spon2	ENSMUSG00000037379 spondin 2, extracellular matrix protein	-2.73	7.20E-13
Npas2	ENSMUSG00000026077 neuronal PAS domain protein 2	-2.61	8.10E-09
Nfil3	ENSMUSG00000056749 nuclear factor, interleukin 3, regulated	-2.2	3.00E-03
Rrad	ENSMUSG00000031880 Ras-related associated with diabetes	-2.1	1.20E-03
Spon1	ENSMUSG00000038156 spondin 1, (f-spondin) extracellular matrix protein	-2.04	5.20E-05
Stc1	ENSMUSG00000014813 stanniocalcin 1	2	1.10E-03
Atp1b1	ENSMUSG00000026576 ATPase, Na ⁺ /K ⁺ transporting, beta 1 polypeptide	2.01	3.20E-09
Mthfd11	ENSMUSG00000040675 methylenetetrahydrofolate dehydrogenase (NADP ⁺ dependent) 1-like	2.03	2.10E-04
Pfkfb3	ENSMUSG00000026773 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	2.12	6.10E-09
Gsdmc3	ENSMUSG00000055827 gasdermin C3	2.16	5.20E-05
Gsdmc2	ENSMUSG00000056293 gasdermin C2	2.21	2.70E-04
Per3	ENSMUSG00000028957 period circadian clock 3	2.35	3.50E-10
Mchr1	ENSMUSG00000050164 melanin-concentrating hormone receptor 1	2.36	3.70E-03
Tef	ENSMUSG00000022389 thyrotroph embryonic factor	2.42	0.00E+00
Tsc22d3	ENSMUSG00000031431 TSC22 domain family, member 3	2.43	0.00E+00
Usp2	ENSMUSG00000032010 ubiquitin specific peptidase 2	2.56	2.10E-06
Acsm3	ENSMUSG00000030935 acyl-CoA synthetase medium-chain family member 3	2.58	1.30E-03
Hlf	ENSMUSG00000003949 hepatic leukemia factor	2.63	5.70E-10
Per2	ENSMUSG00000055866 period circadian clock 2	2.87	0.00E+00
Tubb2b	ENSMUSG00000045136 tubulin, beta 2B class IIB	2.9	1.20E-06
Ttr	ENSMUSG00000061808 transthyretin	2.99	4.20E-07
Tcap	ENSMUSG00000007877 titin-cap	3.21	7.70E-04
Per1	ENSMUSG00000020893 period circadian clock 1	3.77	0.00E+00
Mapk10	ENSMUSG00000046709 mitogen-activated protein kinase 10	4.7	9.00E-03
Dbp	ENSMUSG00000059824 D site albumin promoter binding protein	4.71	0.00E+00
Elavl2	ENSMUSG00000008489 ELAV (embryonic lethal, abnormal vision, Drosophila)-like 2 (Hu antigen B)	5	5.30E-03
Ptprn	ENSMUSG00000026204 protein tyrosine phosphatase, receptor type, N	5.5	8.70E-03
Rab3c	ENSMUSG00000021700 RAB3C, member RAS oncogene family	5.58	2.10E-04
Ngfr	ENSMUSG00000000120 nerve growth factor receptor (TNFR superfamily, member 16)	5.66	1.10E-03
Prph	ENSMUSG00000023484 peripherin	5.67	1.30E-03
Gap43	ENSMUSG00000047261 growth associated protein 43	5.87	1.80E-03
Chgb	ENSMUSG00000027350 chromogranin B	6	7.20E-03
Orm1	ENSMUSG00000039196 orosomucoid 1	6.09	1.30E-03

<i>Mt3</i>	ENSMUSG00000031760	metallothionein 3	6.46	8.40E-04
<i>Cnr1</i>	ENSMUSG00000044288	cannabinoid receptor 1 (brain)	7.21	8.30E-04
<i>Nos1</i>	ENSMUSG00000029361	nitric oxide synthase 1, neuronal	7.73	7.10E-04
<i>Syt4</i>	ENSMUSG00000024261	synaptotagmin IV	7.95	5.10E-03
<i>Ptprn2</i>	ENSMUSG00000056553	protein tyrosine phosphatase, receptor type, N polypeptide 2	7.97	7.70E-04
<i>Eef1a2</i>	ENSMUSG00000016349	eukaryotic translation elongation factor 1 alpha 2	8.02	1.00E-04
<i>Mmp12</i>	ENSMUSG000000049723	matrix metalloproteinase 12	8.03	2.50E-03
<i>Pcsk1n</i>	ENSMUSG000000039278	proprotein convertase subtilisin/kexin type 1 inhibitor	8.33	3.20E-04
<i>Ciart</i>	ENSMUSG00000038550	circadian associated repressor of transcription	8.37	0.00E+00
<i>Slc5a7</i>	ENSMUSG00000023945	solute carrier family 5 (choline transporter), member 7	8.51	1.80E-03
<i>Mrap2</i>	ENSMUSG00000042761	melanocortin 2 receptor accessory protein 2	8.59	3.80E-03
<i>Stmn3</i>	ENSMUSG00000027581	stathmin-like 3	8.82	7.10E-04
<i>Nefl</i>	ENSMUSG00000022055	neurofilament, light polypeptide	8.91	2.10E-04
<i>Snap25</i>	ENSMUSG00000027273	synaptosomal-associated protein 25	8.93	9.20E-04
<i>Ppp2r2c</i>	ENSMUSG00000029120	protein phosphatase 2, regulatory subunit B, gamma	9.11	3.30E-03
<i>Vip</i>	ENSMUSG00000019772	vasoactive intestinal polypeptide	9.38	1.80E-03
<i>Slc18a3</i>	ENSMUSG00000100241	solute carrier family 18 (vesicular monoamine), member 3	9.44	7.10E-04
<i>Rit2</i>	ENSMUSG00000057455	Ras-like without CAAX 2	9.6	6.00E-03
<i>Cartpt</i>	ENSMUSG00000021647	CART prepropeptide	9.68	2.30E-03
<i>Vstm2l</i>	ENSMUSG00000037843	V-set and transmembrane domain containing 2-like	10.16	8.70E-03
<i>Cplx1</i>	ENSMUSG00000033615	complexin 1	10.18	5.10E-03
<i>Htr3a</i>	ENSMUSG00000032269	5-hydroxytryptamine (serotonin) receptor 3A	10.22	1.10E-04
<i>Slc10a4</i>	ENSMUSG00000029219	solute carrier family 10 (sodium/bile acid cotransporter family), member 4	10.32	1.80E-03
<i>Kcnq2</i>	ENSMUSG00000016346	potassium voltage-gated channel, subfamily Q, member 2	10.36	2.80E-03
<i>Pirt</i>	ENSMUSG00000048070	phosphoinositide-interacting regulator of transient receptor potential channels	10.55	1.70E-03
<i>Htr3b</i>	ENSMUSG00000008590	5-hydroxytryptamine (serotonin) receptor 3B	10.69	1.50E-03
<i>Vat1l</i>	ENSMUSG00000046844	vesicle amine transport protein 1 like	11.2	2.20E-05
<i>Npy</i>	ENSMUSG00000029819	neuropeptide Y	11.83	7.40E-04
<i>A730017C20Rik</i>	ENSMUSG00000050875	RIKEN cDNA A730017C20 gene	11.85	3.60E-04
<i>Zcchc12</i>	ENSMUSG00000036699	zinc finger, CCHC domain containing 12	11.94	7.30E-04
<i>Tubb3</i>	ENSMUSG00000062380	tubulin, beta 3 class III	11.99	3.10E-06
<i>Jph3</i>	ENSMUSG00000025318	junctophilin 3	12.34	1.30E-03
<i>Alb</i>	ENSMUSG00000029368	albumin	12.42	8.40E-04
<i>Chrna3</i>	ENSMUSG00000032303	cholinergic receptor, nicotinic, alpha polypeptide 3	12.58	7.50E-04
<i>Pagr1a</i>	ENSMUSG00000030680	PAXIP1 associated glutamate rich protein 1A	13.03	5.50E-10
<i>Chrn4</i>	ENSMUSG00000035200	cholinergic receptor, nicotinic, beta polypeptide 4	13.09	3.30E-03
<i>Hpcal4</i>	ENSMUSG00000046093	hippocalcin-like 4	13.22	3.60E-04
<i>Th</i>	ENSMUSG00000000214	tyrosine hydroxylase	13.4	4.40E-03
<i>Fstl5</i>	ENSMUSG00000034098	folliculin-like 5	13.81	2.50E-03
<i>Dbh</i>	ENSMUSG00000000889	dopamine beta hydroxylase	14.24	2.20E-04
<i>Ctnna2</i>	ENSMUSG00000063063	catenin (cadherin associated protein), alpha 2	14.3	1.10E-03
<i>Iglc2</i>	ENSMUSG00000076937	immunoglobulin lambda constant 2	14.35	6.00E-03
<i>Unc80</i>	ENSMUSG00000055567	unc-80, NALCN activator	14.89	1.30E-03
<i>Tlx2</i>	ENSMUSG00000068327	T cell leukemia, homeobox 2	15.25	6.20E-03
<i>Slc7a14</i>	ENSMUSG00000069072	solute carrier family 7 (cationic amino acid transporter, y+ system), member 14	15.77	1.80E-04
<i>Cacng5</i>	ENSMUSG00000040373	calcium channel, voltage-dependent, gamma subunit 5	16.27	6.60E-03
<i>Serpina3k</i>	ENSMUSG00000058207	serine (or cysteine) peptidase inhibitor, clade A, member 3K	19.95	1.90E-05
<i>Igkv15-103</i>	ENSMUSG00000076523	immunoglobulin kappa chain variable 15-103	20.65	2.40E-03
<i>Gm21320</i>	ENSMUSG00000110439	predicted gene, 21320	23.4	6.70E-06
<i>Gria2</i>	ENSMUSG00000033981	glutamate receptor, ionotropic, AMPA2 (alpha 2)	25.48	1.40E-03
<i>Svop</i>	ENSMUSG00000042078	SV2 related protein	32.84	6.90E-04
<i>Iglv2</i>	ENSMUSG00000076940	immunoglobulin lambda variable 2	60.24	1.90E-03
<i>Ighv1-36</i>	ENSMUSG00000094051	immunoglobulin heavy variable 1-36	159.03	6.30E-03
<i>Igkv4-68</i>	ENSMUSG00000076549	immunoglobulin kappa variable 4-68	159.55	3.80E-03
<i>Mup7</i>	ENSMUSG00000073842	major urinary protein 7	208.29	2.90E-03
<i>Igkv1-122</i>	ENSMUSG00000095497	immunoglobulin kappa chain variable 1-122	240.32	1.20E-03

Table S3. All KEGG pathways with significant changes in expression.

KEGG number	Pathway
Upregulated Pathways	
mmu00020	Citrate cycle (TCA cycle)
mmu00100	Steroid biosynthesis
mmu00190	Oxidative phosphorylation
mmu00240	Pyrimidine metabolism
mmu00270	Cysteine and methionine metabolism
mmu00280	Valine, leucine and isoleucine degradation
mmu00480	Glutathione metabolism
mmu00620	Pyruvate metabolism
mmu00770	Pantothenate and CoA biosynthesis
mmu00900	Terpenoid backbone biosynthesis
mmu01200	Carbon metabolism
mmu01212	Fatty acid metabolism
mmu03008	Ribosome biogenesis in eukaryotes
mmu03010	Ribosome
mmu03013	RNA transport
mmu03040	Spliceosome
mmu03050	Proteasome
mmu04144	Endocytosis
mmu04146	Peroxisome
mmu04216	Ferroptosis
mmu04530	Tight junction
mmu04721	Synaptic vesicle cycle
mmu04722	Neurotrophin signaling pathway
mmu04964	Proximal tubule bicarbonate reclamation
mmu05014	Amyotrophic lateral sclerosis (ALS)
Downregulated Pathways	
mmu04060	Cytokine-cytokine receptor interaction
mmu04512	ECM-receptor interaction
mmu04640	Hematopoietic cell lineage
mmu04974	Protein digestion and absorption
mmu05150	Staphylococcus aureus infection
Pathways with up and downregulation	
mmu00100	Steroid biosynthesis
mmu00270	Cysteine and methionine metabolism
mmu00280	Valine, leucine and isoleucine degradation
mmu00900	Terpenoid backbone biosynthesis
mmu01200	Carbon metabolism
mmu01212	Fatty acid metabolism
mmu03010	Ribosome
mmu04020	Calcium signaling pathway
mmu04144	Endocytosis
mmu04145	Phagosome
mmu04146	Peroxisome
mmu04151	PI3K-Akt signaling pathway
mmu04216	Ferroptosis
mmu04360	Axon guidance
mmu04371	Apelin signaling pathway
mmu04514	Cell adhesion molecules (CAMs)
mmu04520	Adherens junction

mmu04662	B cell receptor signaling pathway
mmu04666	Fc gamma R-mediated phagocytosis
mmu04710	Circadian rhythm
mmu04974	Protein digestion and absorption
mmu05012	Parkinson's disease
mmu05014	Amyotrophic lateral sclerosis (ALS)
mmu05202	Transcriptional misregulation in cancer
mmu05230	Central carbon metabolism in cancer

Table S4. Enriched Molecular Functions in GF bladders.

GO Molecular Function	# (total number in reference genome)	# (total number of genes in our dataset with that annotation)	Fold Enrichment	P value	FDR p-value
acetylcholine receptor activity	6	2	77.3	5.02E-04	1.38E-02
neuropeptide hormone activity	12	2	38.65	1.61E-03	3.85E-02
binding	5268	40	1.76	9.69E-05	4.65E-03
ligand-gated ion channel activity	131	7	12.39	2.21E-06	4.24E-04
ion channel activity	330	8	5.62	1.05E-04	4.03E-03
transmembrane transporter activity	827	13	3.65	6.14E-05	5.89E-03
transporter activity	957	13	3.15	2.56E-04	8.18E-03
cation channel activity	116	4	8	1.79E-03	3.82E-02
sequence-specific DNA binding					
RNA polymerase II transcription factor activity	229	7	7.09	7.17E-05	4.59E-03
acetylcholine receptor activity	6	2	77.3	5.02E-04	1.38E-02
neuropeptide hormone activity	12	2	38.65	1.61E-03	3.85E-02
binding	5268	40	1.76	9.69E-05	4.65E-03
ligand-gated ion channel activity	131	7	12.39	2.21E-06	4.24E-04
ion channel activity	330	8	5.62	1.05E-04	4.03E-03

Table S5. Cellular components, reactome pathways and protein class enriched in GF tissue.

GO Cellular Component	# (total number in reference genome)	# (total number of genes in our dataset with that annotation)	Fold Enrichment	P value	FDR p-value
neuron projection	251	9	8.31	1.82E-06	1.16E-04
cell projection	387	9	5.39	5.22E-05	1.67E-03
synapse	130	4	7.14	2.68E-03	3.43E-02
plasma membrane	1731	19	2.55	1.42E-04	3.03E-03
membrane	2384	21	2.04	1.37E-03	

Reactome pathways	# (total number in reference genome)	# (total number of genes in our dataset with that annotation)	Fold Enrichment	P value	FDR p-value
PPARA activates gene expression	4	2	> 100	2.71E-04	4.55E-02
Catecholamine biosynthesis	4	2	> 100	2.71E-04	4.09E-02
BMAL1:CLOCK,NPAS2 activates circadian gene expression	20	5	57.97	6.52E-08	9.87E-05
Circadian Clock	28	5	41.41	2.83E-07	1.43E-04
Acetylcholine	17	4	54.56	1.81E-06	5.47E-04
Neurotransmitter Release Cycle					
Neurotransmitter Release Cycle	47	4	19.74	6.83E-05	1.29E-02
Transmission across Chemical Synapses	194	8	9.56	2.60E-06	6.55E-04
Neuronal System	307	9	6.8	8.87E-06	1.92E-03

Protein Class	# (total number in reference genome)	# (total number of genes in our dataset with that annotation)	Fold Enrichment	P value	FDR p-value
acetylcholine receptor	42	4	22.09	4.54E-05	9.75E-03
GABA receptor	42	4	22.09	4.54E-05	4.88E-03

Table S6. Shapiro-Wilk test for normal distribution.

	SPF Male	GF Male	SPF Female	GF Female	SPF Male	GF Male	SPF male	Antibiotics male
W	0.9712	0.9526	0.9251	0.7017	0.9675	0.8613	0.9382	0.8499
P value	0.9001	0.7615	0.5427	0.0064	0.8258	0.2331	0.5327	0.0284
Passed normality test (alpha=0.05)?	Yes	Yes	Yes	No	Yes	Yes	Yes	No
P value summary	ns	ns	ns	**	ns	ns	ns	*

Table S7. Expression of genes for pattern recognition receptors.

Gene symbol	Identifier	Gene name	Mean Total Counts ^a	Fold change	FDR p-value
Bcl10	ENSMUSG00000028191	B cell leukemia/lymphoma 10 caspase recruitment domain family, member 9	1272	1.04	1E+00
Card9	ENSMUSG00000026928	CD209a antigen	53	-1.42	1E+00
Cd209a	ENSMUSG00000031494	C-type lectin domain family 7, member a	498	-1.36	1E+00
Clec7a	ENSMUSG00000079293	hepatitis A virus cellular receptor 2	326	-1.34	1E+00
Havcr2	ENSMUSG00000020399	lectin, galactose binding, soluble 1	41	1.00	1E+00
Lgals1	ENSMUSG00000068220	lymphocyte antigen 75	14902	-1.00	1E+00
Ly75	ENSMUSG00000026980	MALT1 paracaspase	131	1.16	1E+00
Malt1	ENSMUSG00000032688	mannose receptor, C type 1	938	-1.16	1E+00
Mrc1	ENSMUSG00000026712	mannose receptor, C type 2	3268	-1.43	1E-01
Mrc2	ENSMUSG00000020695	NLR family, pyrin domain containing 3 nucleotide-binding oligomerization domain containing 1	2458	-1.28	1E+00
Nlrp3	ENSMUSG00000032691	NLR family, pyrin domain containing 3 nucleotide-binding oligomerization domain containing 1	53	-1.34	1E+00
Nod1	ENSMUSG00000038058	oligomerization domain containing 1	1133	-1.16	1E+00
Nod2	ENSMUSG00000055994	nucleotide-binding oligomerization domain containing 2	63	1.23	1E+00
Src	ENSMUSG00000027646	Rous sarcoma oncogene	6555	1.01	1E+00
Syk	ENSMUSG00000021457	spleen tyrosine kinase	638	-1.14	1E+00
Tlr1	ENSMUSG00000044827	toll-like receptor 1	85	-1.55	1E+00
Tlr2	ENSMUSG00000027995	toll-like receptor 2	588	-1.17	1E+00
Tlr3	ENSMUSG00000031639	toll-like receptor 3	1187	-1.02	1E+00
Tlr4	ENSMUSG00000039005	toll-like receptor 4	955	-1.19	1E+00
Tlr5	ENSMUSG00000079164	toll-like receptor 5	226	-1.25	1E+00
Tlr6	ENSMUSG00000051498	toll-like receptor 6	65	-1.11	1E+00
Tlr7	ENSMUSG00000044583	toll-like receptor 7	238	-1.03	1E+00
Tlr8	ENSMUSG00000040522	toll-like receptor 8	108	-1.21	1E+00
Tlr9	ENSMUSG00000045322	toll-like receptor 9 triggering receptor	112	-1.22	1E+00
Trem1	ENSMUSG00000042265	expressed on myeloid cells 1	1	-1.87	1E+00
Tyrobp	ENSMUSG00000030579	TYRO protein tyrosine kinase binding protein	1129	-1.22	1E+00

^aMean Total Counts is the mean of a normalized number of reads for each gene in the three SPF samples.

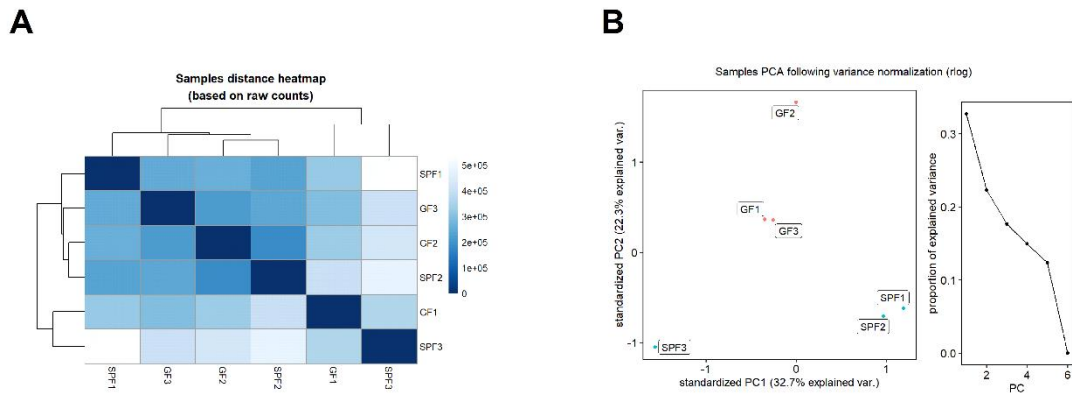


Figure S1. RNA-Seq sample comparison of GF and SPF groups. A. Complete clustering of samples using Euclidean distance calculated on raw counts B.PCA on rlog variance normalized counts.

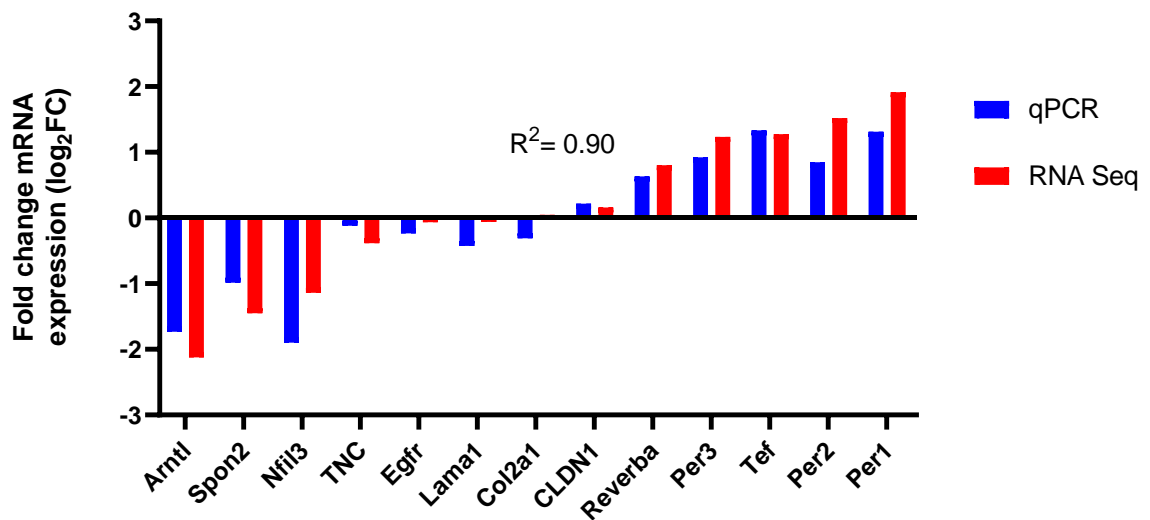


Figure S2. Concurrence of log2(fold change) from qPCR and RNA-seq analysis for 13 exemplar genes, correlation expressed with R2 correlation coefficient; Bars represent mean value.

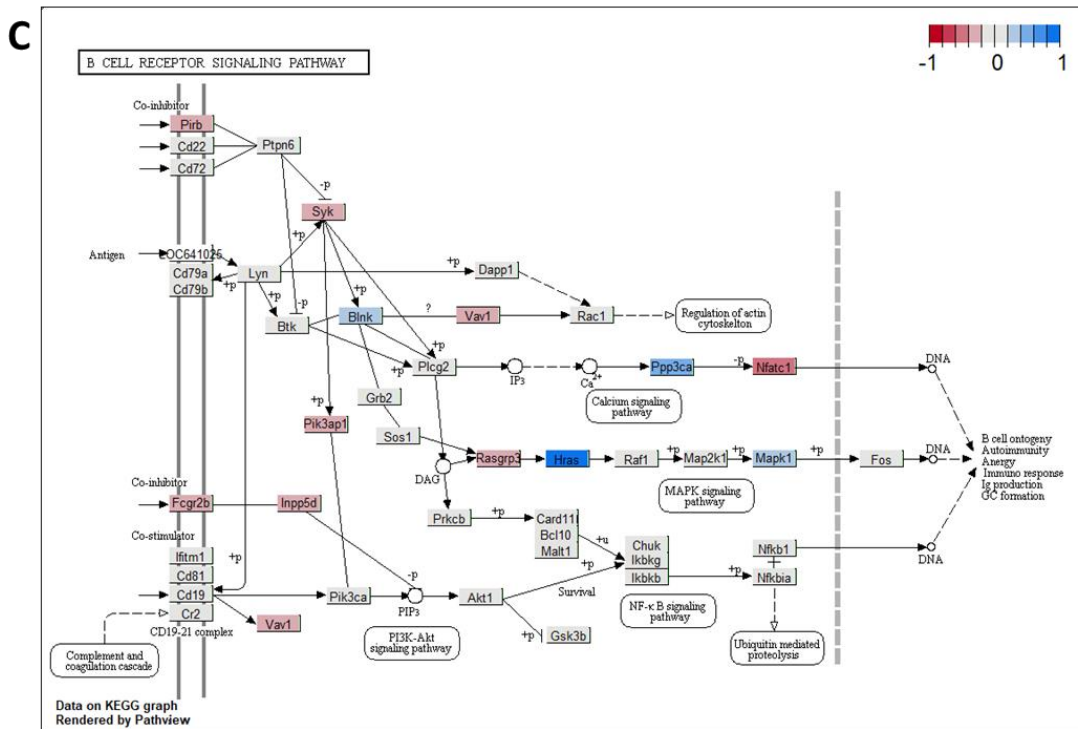


Figure S3. KEGG pathways with significant change in gene expression. The colouring is rendered from the summary $\log_2(\text{fold change})$ of all genes in one gene node, representing multiple genes with a redundant functional role. **A.** Circadian rhythm **B.** Calcium signalling pathway **C.** B cell receptor signalling pathway.

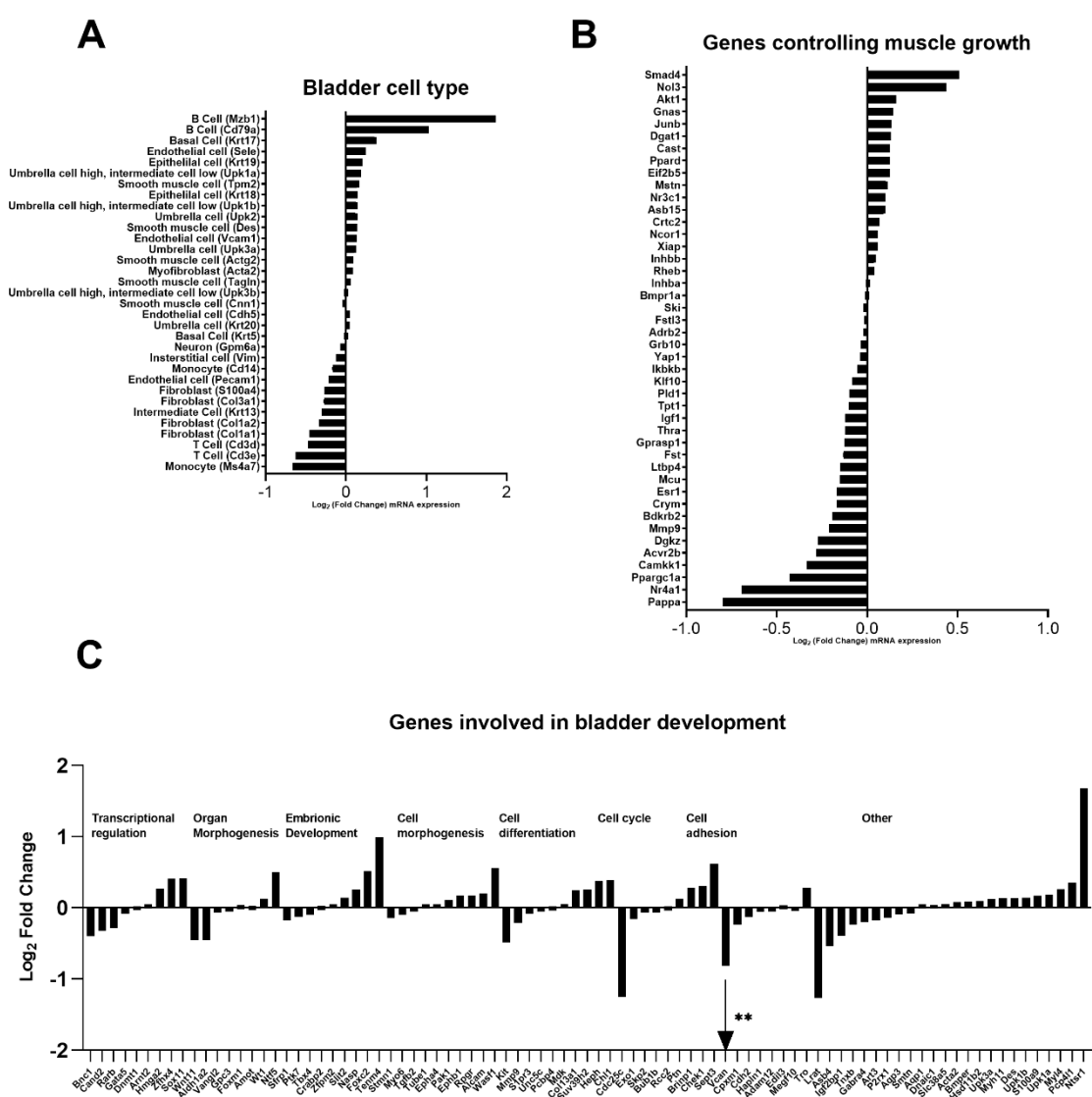


Figure S4. Log2 Fold change (GF vs SPF) of gene expression for **A.** gene markers for bladder cell types **B.** genes controlling muscle growth **C.** genes involved in bladder development; arrow with asterisks shows significant change (FDR p<0.01)